ANTIBODY CARRIERS OF CpG OLIGODEOXYNUCLEOTIDES FOR SOLID TUMOR IMMUNOTHERAPY

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ABSTRACT

ROLAND CHEUNG: Antibody Carriers of CpG Oligodeoxynucleotides for Solid Tumor Immunotherapy
(Under the direction of Moo J. Cho, Ph.D.)

The use of CpG oligodeoxynucleotide (ODN) TLR9 agonists for oncology therapy has been primarily limited to its use as an adjuvant in combination therapies. Successful anti-cancer therapy has been limited to the intratumoral and peritumoral routes of administration. Subcutaneous (SC) administration has showed promise, but results have been modest at best. For cancer monotherapy with CpG, we developed a delivery system utilizing an endogenous antibody as a carrier for CpG ODNs. Our system first involves conjugation of a small molecule 2,4-dinitrophenyl (DNP) hapten to CpG. Next, we immunize mice against the hapten such that a high titer of anti-DNP antibodies is maintained in the systemic circulation. Upon injection of DNP-CpG, an immune complex will be formed. Subsequently, the immune complex will be taken up by dendritic cells for CpG processing by TLR9 endosomal receptors.

This system was shown to be effective in tumor growth inhibition in our animal model upon intravenous (IV) delivery of CpG ODNs, an administration route that has never shown to be effective. In the following investigations, we found that the SC route of administration exhibited great efficacy. Anti-tumor response was as good as IV delivery and showed an improvement over SC administration of underivatized CpG. Pharmacokinetic analysis suggests that increased half-life of CpG may play a role toward the therapeutic

effect. In vitro studies suggest that our immune complex effectively activates dendritic cells and that this effect is possibly due to facilitated processing of the immune complexes via Fc receptor-mediated endocytosis and not due to the extent of CpG uptake. Based on collective results, we proposed mechanisms by which we were able to garner such a positive immunological response. Additional opportunities stemming from this work will certainly be of great value. The establishment of SC and IV formulations that exhibit desirable properties carries tremendous implications for the deliver of other therapeutic agents.

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TABLE OF CONTENTS

APPENDIX

LIST OF FIGURES

LIST OF TABLES

LIST OF DATA FILES ON CD

(CD stored with master copy of dissertation by Dr. Moo J. Cho)

File 1 Pharmacokinetics and Biodistribution

File 2 PD Tumor Measurements Series 1

File 3 Antibody Titer Series 1

File 4 PD Tumor Measurements Series 2

File 5 Antibody Titer Series 2

File 6 Dendritic Cell Uptake

File 7 Dendritic Cell Activation

File 8 Tumor Cytokine Assay Single-dose

File 9 Tumor Cytokine Assay Multiple-dose

LIST OF ABBREVIATIONS AND SYMBOLS

Ab	Antibody
APC	Antigen-presenting cell, allophycoerythrin
CD	Cluster of differentiation
CpG	Cytosine-phosphate-guanine
CFA	Complete Freund's adjuvant
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenyl
DNP-BSA	Dinitrophenylated bovine serum albumin
DNP-CpG	2,4-Dinitrophenyl derivative of CpG oligonucleotide
DNP-KLH	Dinitrophenylated keyhole limpet hemocyanin
DNP-ss	Dinitrophenylated oligonucleotide scramble sequence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPR	Enhanced permeability and retention
Fab	Fragment of antigen binding
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcR	Fc receptor

FcRn	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage-colony stimulating factor
HBSS	Hank’s Balanced Salt Solution
IC	Immune Complex
ID	Injected dose
IFA	Incomplete Freund’s adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IT	Intratumoral
IV	Intravenous
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mIgG	Monoclonal immunoglobulin G
NK	Natural killer
ODN	Oligodeoxynucleotide, oligonucleotide
OPD	*o*-Phenylenediamine dihydrochloride
pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PD	Pharmacodynamic

pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PK	Pharmacokinetic
PS	Phosphorothioate
PT	Peritumoral
RES	Reticuloendothelial system
rm	Recombinant mouse
RT	Room temperature
SA	Specific activity
SC	Subcutaneous
SEM	Standard error of the mean
$t_{1/2}$	Half-life
T_H	T helper
TNF	Tumor necrosis factor
TNP	2,4,6-Trinitrophenyl
TLR	Toll-like receptor

CHAPTER I

INTRODUCTION

Historical Perspective

It has been observed for hundreds, and perhaps even thousands of years, that cancer patients who developed concurrent pathogenic infections would experience remissions of their malignancies.[1] Many surgeons in the 1800s noted the link between erysipelas infections (a superficial infection of the skin caused by *Streptcoccus pyogenes*) and cancer remissions, but years would pass before a breakthrough in active cancer immunotherapy. In 1891, Dr. William B. Coley, then a young surgeon at New York Memorial Hospital, experienced great distress from the loss of a female cancer patient. Frustrated with the failure of surgery, he spent numerous hours examining all records of sarcoma cases at New York Hospital hoping to find alternative treatments. Coley eventually discovered a seven-year-old record describing a man with sarcoma of the neck whose case was deemed hopeless after multiple failed surgeries. The patient's last surgery resulted in a severe infection of erysipelas and high fever, but the man survived the infection and the tumor healed completely. Coley spent weeks eagerly searching for this patient in lower Manhattan's east side slums and found him in good health and free from cancer.

Coley's interest in erysipelas led him to discover historical literature and other publications that recorded similar findings linking acute infections with tumor regression. He then decided to treat cancer patients by infecting them with erysipelas. Some cases met with great success, even after metastasis, but limitations to the treatments soon became apparent: some patients never developed an infection while others developed an infection so severe that they proved to be fatal. Coley also observed that inducing a febrile response was vital in producing anti-cancer effect.

To address these difficulties, Coley eventually developed a vaccine comprised of two heat-killed bacteria: the Gram-positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens*. This vaccine became known as "Coley's toxins". Although thousands of patients were treated with Coley's toxins, responses to the vaccine were still highly variable and depended on many factors including cancer type, route of administration, and precision of the vaccine formulation. Eventually, after Coley's death in 1936, use of his vaccine diminished as radiation and chemotherapy became the preferred methods of treatment. As a result of the Kefauver Harris Amendment in 1963, the Food and Drug Administration gave Coley's toxins "new drug" status, making it illegal to sell them in the United States.

Thanks to the efforts of Coley's daughter, Helen Coley Nauts, and Wayne Martin, a chemical engineer who had a great interest in medicine, doctors and researchers developed a renewed interest in Coley's toxins. In line with contemporary regulations and advances in medical research, the need and desire for a more refined and better understood method of cancer treatment has led to the eventual development of CpG oligodeoxynucleotides (ODNs).

CpG Oligodeoxynucleotides and Toll-like Receptor 9

Until the 1980s, the active bacterial component in Coley's toxin was thought to be lipopolysaccharide. A Japanese research group working with Bacillus Calmette-Guerin (BCG), a vaccine against tuberculosis that is prepared from a strain of the attenuated live *Mycobacterium bovis,* found that the anti-tumor activity came from bacterial DNA.[2-4] Arthur Krieg's work in the 1990s contributed greatly to the understanding of the

immunotherapeutic effects of bacterial DNA and the design of short, synthetic DNA sequences meant to mimic bacterial DNA for the induction of selective immune responses.

The vertebrate immune system recognizes invading pathogens using pattern recognition receptors. The Toll-like receptors are among the most well-characterized receptor family with Toll-like receptor (TLR) 9 established as the receptor that recognizes and processes bacterial DNA.[5,6] The ability of this receptor to distinguish between prokaryotic and eukaryotic DNA comes from a fundamental evolutionary differences. TLR9 recognizes and is activated by cytosine-phosphate-guanine (CpG) dinucleotides which are unmethylated and common in bacterial DNA, but are found methylated and with suppressed frequency in the vertebrate genome.[7]

Knowledge of this has fueled the development of immunostimulatory CpG ODNs, also known as TLR9 agonists. These short, synthetic DNA oligodeoxynucleotide sequences, ranging from eight to 30 nucleotides long, contain unmethylated CpG dinucleotides in specific base contexts called CpG motifs. Specific modifications such as those to the sugar, base, backbone, and number of CpG, can be made to selectively elicit a type and the degree of an immune response.[8] For example, CpG ODNs usually contain phosphorothioate (PS) modifications to the backbone to hinder nuclease degradation. CpG ODNs are currently classified into one of four classes: A, B, C, and P. To date, most of the vaccine work has utilized B-class CpG ODNs. Class B is potent in producing IFN-α and B-cell activation and most well characterized in terms of anti-tumor activity and mechanism.[7,9] For many murine-based *in vivo* and cellular experiments, the B-class CpG ODNs used is CpG 1826. It is a well-established ODN specifically developed for mouse studies and has the following 20-mer

sequence with two CpG motifs: 5'-TCCATGACGTTCCTGACGTT-3'. A commonly used control is the non-CpG-containing ODN, ODN 1982, which has identical base composition as 1826, but ordered in a non-stimulatory sequence: 5'-TCCAGGAGTTCTCTCAGGTT-3'.

TLR9 activation results in a cascade of innate and adaptive immune responses.[10] Since dendritic cells (DCs) act in the initiation of primary responses and as the interface between the two arms of the immune system,[11] they are logical targets for CpG ODN delivery.

Dendritic Cells and the Immune System

Dendritic cells are the most potent of antigen presenting cells (APCs) and specialize in antigen presentation.[11,12] Immature DCs originate from bone marrow and migrate throughout the body. DCs exist in all lymphoid and most non-lymphoid tissues and, with high endocytic capacity, continuously sample their environment for foreign antigens. There are multiple means by which DCs capture antigens: phagocytosis, macropinocytosis, pinocytosis, and receptor-mediated endocytosis.[13]

When activated, DCs follow the unidirectional flow of the lymph through the lymphatic vessels until they reach the secondary lymphoid organs such as the lymph nodes where they will interact with T cells.[14] DCs are so effective at stimulating the immune system because of their ability to interact with natural killer (NK) cells of the innate arm and with $CD4^+$ (T helper cells) and $CD8^+$ (Cytotoxic T cells, CTL) T cells of the adaptive arm.[15] The major histocompatibility complex (MHC) molecules, known in humans as the human leukocyte antigen (HLA), are the molecules that present the antigen to T cells. The two

types, MHC class I and MHC class II, are distinct in their involvement in antigen processing. MHC I presents antigen fragments derived from cytosolic proteins to CD8+ T cells. MHC II presents antigen fragments derived from exogenous proteins to CD4+ T cells. MHC II antigens come from extracellular pathogens which have been endocytosed. DCs are unique because they can effectively use MHC I to also present exogenous antigen fragments to CD8+ T cells through a specialized cross-presentation mechanism.[16]

Plasmacytoid dendritic cells (pDCs), a subset of immature DCs, and B cells are the only human immune cells known to constitutively express TLR9 (Figure 1.1).[17] When activated by CpG ODNs, pDCs excrete cytokines and chemokines which activate cells of the innate immune system which can confer direct anti-tumor effects.[18-20] DC stimulation also results in increased expression of co-stimulatory molecules such as CD80 and CD86 to signal proliferation of T-cells, particularly type 1 helper T cells (T_H1). In the presence of tumor-associated antigens, DCs can process these antigens for MHC presentation to T cells. Both the co-stimulatory and MHC signals are required for successful activation of T cells.[21] Suggestive of DC involvement in CpG ODNs therapy, intralymphatic administration considerably improved therapeutic response to CpG ODNs[22] while another study was able to treat large murine tumors using combined DC and CpG ODNs therapy.[23] Still others have attempted to use receptor-mediated delivery of antigens to DCs for anticancer applications.[24] These findings warrant the use of CpG ODNs for dendritic cell activation, ultimately resulting in T cell effector function,[25] in cancer immunotherapy. As such, delivery of CpG ODNs to DCs would benefit greatly using a drug delivery system.

Endogenous Antibodies as Drug Carriers

A major concern with current synthetic delivery systems is the inherent immunogenicity.[26] Even some protein therapeutics can elicit immune responses and attempts have been made to minimize their immunogenicity.[27] The ideal drug delivery system must not only carry intact cargo to the target location, but avoid activating the immune system or undesired neutralization that leads to elimination from the body. Herein lies the enormous potential of endogenous antibodies in CpG ODNs and general delivery. For example, DCs, in addition to TLR9, express cell-surface Fc receptors such as FcγR which bind the Fc domain of immunoglobulin G (IgG) antibodies. This implicates the potential for IgG to be used for delivery to DCs. Because of this and other desirable properties, natural and synthetic antibodies have been developed for many applications in medicine and research. In fact, more than 20 antibody products are approved by the U.S. Food and Drug Administration (FDA). Typically, the antibody itself is the therapeutic entity. CpG ODNs have been used to boost the therapeutic effect of an antibody treatment,[28] but only recently has the opposite been done: using antibodies to support the activity of CpG ODNs.[29]

Antibodies, also called immunoglobulins (Igs), are serum glycoproteins which all have the same basic structure of two identical heavy chains and two identical light chains held together by disulfide bonds in a 'Y' shape (Figure 1.2). They are naturally-occurring proteins produced and secreted by B cells of immune system to neutralize or destroy ligands to which they bind. Human Igs have two types of light chains, κ and λ, but are classified into five families, or isotypes, based on their heavy chain: immunoglobulin α (IgA), δ (IgD), ε

(IgE), γ (IgG), and μ (IgM). The isotypes differ in size, overall structure, and antigen binding sites.

Of all Igs, IgG makes up approximately 80% of the total at an average concentration of about 10 mg/ml and a maximum concentration as high as 17 mg/ml in the blood, making it the second most abundant blood protein after albumin. Most, if not all, therapeutic antibodies to date are of the IgG class. An example of IgG's role in protective immunity is its natural function in providing passive humoral immunity to a fetus from the mother. Based on structural differences of the heavy chains, which allow for differences in receptor binding, IgGs can be further divided into four subclasses: IgG_1, IgG_2, IgG_3, and IgG_4. Each intact IgG has a high molecular weight of approximately 150 kDa. Each light chain is around 25 kDa in size and each heavy chain is about 50 kDa. A single IgG molecule has two identical antigen-binding domains, or Fab (fragment of antigen binding), and an Fc (fragment crystallizable) domain. The Fc domain is involved in the binding to a variety of cell-surface receptors.

Not only is the neonatal Fc receptor (FcRn), specific for the transport of IgG, important in conferring passive immunity from mother to fetus, it plays an important role in IgG homeostasis. Also known as the Brambell receptor, FcRn protects viable IgG from catabolism and allows IgG to be recycled via endocytic pathways thereby giving IgG a long circulatory half-life ($t_{1/2}$) of greater than 21 days in humans.

The FcRn protection pathway initially involves the uptake of IgG into various cells via non-specific fluid phase pinocytosis. As the endosome matures, a characteristic decrease in pH follows which provides a favorable condition for viable IgG binding to FcRn. Eventually, the endosome fuses with the lysosome and unbound IgG will be digested by

proteases. The IgG-FcRn complex is trafficked back to the cell surface when the endosome fuses with the plasma membrane. Exposure to the physiological pH causes the complex to disassociate, releasing IgG back into the plasma or interstitial fluid.[30-32]

Upon IgG binding to an antigen, however, different processing pathways can occur. A polymeric antigen-IgG immune complex (IC) is subject to degradation in the lyosomes after FcRn-mediated uptake.[33] However, a 1:1 antigen-IgG immune complex (IC), being structurally similar to free IgG in that it cannot crosslink antigen, is subject to uptake via FcγR-mediated endocytosis into APCs such as DCs. There are three types FcγR on various immune cells that recognize the Fc domain of IgG molecules, each showing a different affinity for IgG or IgG-containing ICs. Of the three, only FcγRI (CD64) processes monomeric ICs[34,35] and exhibits a high affinity (10^{-9} M) for IgG.[36] As a result of their ability to recognize their specific antigen, they have been exploited for their effector function or targeted drug delivery. A highly unique approach is to use them as a drug carrier for therapeutic compounds that otherwise would exhibit undesirable pharmacokinetic (PK) profiles, such as CpG ODNs.

Constitutive fluid-phase endocytosis including macropinocytosis is energy-consuming and non-specific in solute uptake. The process diminishes significantly as the DC matures.[37] The rationale behind the use of FcγR in delivering CpG in a monovalent IC is supported by the discovery that the FcγRs expressed on DCs can effectively take up and process ICs[38] and are involved in the efficient priming of T_H cell responses *in vivo*.[39] Indeed, a DNA vaccine comprised of an antigen fused to an IgG Fc fragment[40] and ovalbumin/anti-ovalbumin ICs successfully demonstrated both major histocompatibility complex (MHC) I-

and II-derived responses when targeted to DCs in mice.[41] Several *in vitro* studies have shown that DCs present IgG-complexed antigens more effectively to CD8+ T and CD4+ cells than soluble antigens.[42,43] In a murine tumor model, treatment has been shown to be more effective using DCs which have been pre-loaded *ex vivo* with ICs compared with direct injection of soluble ICs.[44]

Unlike the often intensive and difficult synthetic process required to develop nanoparticulate carriers, antibodies can be produced in high quantities through both *in vivo* and *in vitro* methods. Monoclonal antibodies (mAbs) are extensively used in basic research, diagnostics, and as therapeutic agents for cancer, inflammation, and infectious disease. The monoclonal antibody production process permits large quantities of antibodies targeted against a particular antigen to be produced. Initially, a mouse is immunized by injection of an antigen to stimulate the production of antibodies targeted against the particular antigen. The antibody-secreting B cells are isolated from the spleen. Monoclonal antibodies are produced by fusing a single B cell to a tumor cell and this generated hybrid cell, hence called a hybridoma, is then grown in culture. By allowing the hybridoma to multiply in culture, a uniform population of cells is produced, each of which produces identical antibody molecules. These antibodies are called monoclonal antibodies because they are produced by the identical offspring of a single, cloned antibody-secreting cell. Hybridoma cells can be grown using two methods. A major benefit using hybridomas is that they can be cultured indefinitely in cell culture media. Alternatively, the cells can be injected into the peritoneal cavity of mice where they produce tumors containing an antibody-rich fluid called ascites fluid. Although mAb production is constant, offers high concentration, purity, and homogeneity, monospecificity sometimes limits their use and production often requires more

time, skill, and money relative to polyclonal antibody (pAb) production. Monoclonal antibody usage as drug carriers in humans may not be ideal since murine mAbs in humans have been shown to only have serum half-lives of one to two days[45,46] and can be immunogenic. Even humanized, or chimeric, mAbs do not have the same ideal PK properties as fully human IgGs. However, there is promise in utilizing mAbs as drug carriers since fully human mAbs have recently been produced through advanced genetic manipulations.[47]

Polyclonal antibodies are produced by immunizing an animal against a particular antigen and, if desired, are then isolated from collected blood. The concentration of antigen-specific antibodies ranges from 50 to 200 μg/ml although absolute quantity is dependent on the size and age of the animal.[48] Due to aforementioned issues with mAbs, pAbs are a promising alternative for drug delivery. Artificial active immunization involves injection of an antigen into the host to elicit production of antigen-specific IgGs in a large excess of antigen. The resulting stoichiometric excess of antibody to antigen is desirable for sustained immune complex formation.

Passive Tumor Targeting and the EPR Effect

Antibody usage in drug delivery has enormous potential. Not only can antibodies bind cargo and deliver it to DCs, they can be used in passive tumor targeting. The enhanced permeability and retention (EPR) effect is a phenomenon described over 20 years ago for passive targeting of macromolecules to solid tumors.[49] Tumors trigger angiogenesis and maintain a high density of vessels to obtain the vast amount of oxygen and nutrients

necessary to sustain their growth. Unlike in normal tissues, the neovasculature exhibits abnormal vascular architecture. These defective vessels are leaky due to large openings called fenestrae which can range from 100 to 600 nm.[50] The lack of a smooth muscle layer results in irregular blood flow. Impaired lymphatic clearance[51] and reduced venous blood flow in the tumor leads to retention and accumulation of macromolecules and nanoparticles.[52]

Tumor targeting strategies based on the EPR effect have been reported with soluble polymeric drugs[53,54] as well as particulates such as liposomes.[55] A standard explanation is that macromolecules or particulates are passively trapped in partially denuded endothelium. A major weakness in this argument is the failure to recognize potential competition with a large excess of endogenous macromolecules such as immunoglobulins and albumin and particulates such as platelets and lipoprotein particles. Furthermore, synthetic nanocarriers require meticulous design for size control since they must avoid the major clearance mechanisms. The kidneys are known to filter compounds with molecular weighs less than about 70,000 daltons (Da) and particles less than 10 nm in size whereas those greater than 100 nm are subject to clearance by the liver.[56] Nanoparticles must also be shielded from the reticuloendothelial system (RES), a system of phagocytic cells found in the circulation and tissue, primarily comprised of monocytes and macrophages, which engulf and destroy foreign or abnormal cells and substances.

Interstitial transport of macromolecules from the site of retention in the endothelium to tumor interior can be unfavorable. This is because the interstitial transport of macromolecules and particulates is mainly via solvent drag (i.e. convection) and because

high interstitial fluid pressure and elevated oncotic pressure, a result of impaired lymphatics in the tumor, would work against the convective transport.[57,58] The dilemma of the large size needed in exploiting EPR effect and the small size desirable for interstitial transport could well be the reason for the poor *in vivo* outcome of many tumor-targeted macromolecular drug delivery strategies that otherwise have shown tremendous potential in cellular systems.

In the proposed use of IgG as a carrier, the dilemma is largely avoided as our target is not tumor cells per se but DCs in the tumor vicinity.[10] Therefore, it may not be required for our CpG-containing ICs to penetrate deep into the tumor tissue. All in all, the multitude of benefits that antibodies can provide may offer an enhanced synergistic effect for anti-tumor therapy.

Routes of Administration

To date, CpG ODNs use in monotherapy continues to be limited. Although intra- and peri-tumoral injections have been effective in murine models,[10] these routes limit the use of CpG ODNs to only specific types of cancer. Intravenous (IV) administration is often used because the drug has 100% bioavailability such that maximum absorption and distribution is ensured. However, IV delivery of any ODN results in rapid clearance from the circulation to the clearing organs, particularly the liver and kidneys. IV dosing also subjects ODN to dilution and non-specific binding to serum proteins. Only recently has it been shown that it can be effective in anti-cancer therapy after intravenous administration.[29]

Due to the failed attempts of intravenous CpG ODNs delivery, the subcutaneous (SC) route of administration has been studied although not established for monotherapy.[7,59,60] SC

administration offers many practical benefits. IV and intramuscular (IM) injections require skilled personnel whereas SC injections can be self-administered by a patient and can be painless. In fact, many drugs such as insulin and heparin have been highly successful through SC administration. The SC route is very promising especially since it allows for slow release from the injection site and for accumulation in the lymph nodes where many DCs are located.

Development of tumor vaccines using CpG ODNs monotherapy has not been successful although use of CpG ODNs as adjuvants[61-63] or in multi-modal treatments show clinical promise.[64,65] The importance of delivery route was recently manifested by Pfizer's decision not to develop CpG 7907 (also known as PF-3512676) any further in treating non-small cell lung cancer, after the initial human clinical studies show no clear-cut efficacy with a systemic route of administration (http://www.forbes.com/healthcare/2007/06/20/pfizer-coley-drug-biz-bizhealth-cx_mh_0620pfizer.html). The following drug delivery strategies were devised to resolve issues with CpG delivery regardless of administration route.

Development of a Drug Delivery System

In order for IgG to be used as a CpG ODNs carrier, major considerations must be taken into account. IgG must recognize and bind specifically to CpG ODNs with a high enough affinity such that the cargo is not subject to clearance mechanisms before it reaches the DC targets, but also must bind in such a way that does not obstruct its uptake by DCs nor obstruct binding of CpG ODNs to TLR9. To maximize binding, IgG would ideally exist in large excess such that 100% of administered CpG ODNs is bound. A 1:1 or 1:2

stoichiometric ratio of IgG to ligand, such that either one or both Fab domains are occupied, would confer an apparent IC $t_{1/2}$ similar to that of the antibody itself.[66-70] Initially, it would appear that ICs containing CpG as an antigen would eliminate the CpG ODN rather than extending its circulation time. In reality, IC formation can have a wide range of PK effects on the bound antigen depending upon the size of the antigen, the stoichiometry of the IC, and the titer of the antibody.[70] Antigen crosslinking must be avoided since polymeric ICs not only neutralize the cargo, but are themselves subject to enhanced clearance. Monovalent ICs, however, do not induce antigen crosslinking and enjoy a prolonged life time. This PK property has been utilized in the systemic delivery of small molecules[69] as well as proteins,[68] and may also be applied to ODN delivery.

Binding of IgG to CpG ODNs can readily accomplished by simple chemical modification (Figure 1.3). The most straightforward method is to add a hapten to CpG. 2,4-Dinitrophenyl (DNP) moiety can easily be conjugated to CpG ODNs through a linker during automated solid-phase ODN synthesis. This small molecular-weight hapten lacks any inherent immunogenicity and mono-derivatization on CpG ODNs avoids crosslinking. Since any chemical modification at the 5'-end of CpG ODNs reduces their immune-stimulating activity,[8] DNP is introduced only at the 3'-end. Logically, an anti-DNP antibody would need to be used. For *in vitro* and mechanistic studies, anti-trinitrophenyl (TNP) $mIgG_1$ from 1B7.11 and IGEL a2 hybridoma cells and anti-DNP IgG_1 from ANO6 hybridomas are available. For *in vivo* polyclonal antibody production against a specific antigen, standard immunization protocols have long been established. Antigens are usually proteins or polysaccharides. In order for smaller molecules such as DNP compounds to become antigenic, multiple molecules are extensively conjugated to large carrier proteins, such as

albumin or keyhole limpet hemocyanin (KLH). A primary injection of this highly derivatized carrier protein along with an adjuvant via a relevant administration route into the host will trigger antibody production against the antigen. Additional injections, called boosters, can be administered at regular time intervals to maintain a high antibody titer.

The strength of association is one variable that can easily be controlled to improve serum $t_{1/2}$ *in vivo* and has been optimized for the effects of several variables on the total interactive energy, or avidity, between anti-DNP monoclonal antibody and DNP-modified ODNs.[67] Monovalent 1:1 ICs indeed exhibit a long circulation $t_{1/2}$, providing a sufficient time for tumor accumulation in DNP-immunized mice whereas ICs with CpG haptenized with two DNP (DNP_2-CpG ODNs) showed increased clearance possibly due to polymeric IC formation.[29] Perhaps more importantly, the Fc domain of the ICs appears to promote their entry to DCs thereby facilitating DC activation.

Since IgG is found ubiquitously throughout blood and tissue alike, CpG can be injected via multiple routes of administration to form ICs after active immunization. A substantial amount of endogenous IgG is found in the interstitial tissue in dynamic equilibrium with plasma, approximately at 25% of the plasma concentration.[71] Therefore, regardless of most administration routes, CpG ODNs is expected to remain as ICs which will eventually find their DC targets (Figure 1.4).

As a proof on concept, we first employed our system for intravenous delivery.[29] In the first pharmacokinetic study using this endogenous immune complex system, underivatized CpG administered intravenously to non-immunized, tumor-bearing mice exhibited rapid clearance with almost complete elimination after only two hours. In contrast,

DNP-derivatized CpG administered to DNP-immunized, tumor-bearing mice showed a long, sustained systemic circulation with high serum levels even after 72 hours (Figure 1.5). The PK properties of DNP-CpG were highly favorable. Compared to free CpG, DNP-CpG had a 266-fold increase in elimination half-life, a 1650-fold increase in systemic exposure, a 13-fold decrease in the tissue volume of distribution, and a 1530-fold decrease in systemic clearance. DNP_2-CpG, a CpG derivative which contains two DNP haptens was used to determine whether a greater binding affinity to the anti-DNP antibody could confer additional improvements in PK properties. Although DNP_2-CpG showed better PK properties than free CpG, DNP-CpG improvements were much greater (Table 1). Since DNP_2-CpG has two DNP haptens, antibody crosslinking could have occurred which subsequently led to the rapid elimination of such polymeric immune complexes. Based on this finding, all further studies utilized DNP-CpG.

Analysis of the biodistribution of free CpG shows that the ODN is found primarily in the clearance organs while only up to 2% of the injected dose is found in the tumor tissue at any given time. In contrast, tumor accumulation of DNP-CpG was up to 10-fold higher with over 20% of the injected dose found in the tumor tissue. A high percentage of the dose was sustained in the tumor tissue even after 72 hours (Figure 1.6). This finding supports the involvement of the EPR effect as we had proposed.

In a preliminary tumor growth inhibition study, PBS, free CpG, and DNP-CpG were intravenously administered to DNP-immunized, tumor-bearing mice. Administration of free CpG did not result in any anti-tumor response and the rapid tumor growth pattern mirrored that of the PBS negative control. DNP-CpG treatments, however, were able to inhibit the

rate of tumor growth, especially when the anti-DNP antibody titer was high (Figure 1.7). Around dose 3, the time when antibody titers dropped to low levels, treatments were no longer effective which attests to the need for immune complex formation.

In the following chapters, we further employ this intricately developed drug delivery strategy in the treatment of solid tumors and explore the possible mechanistic effects involved. Our approach was already shown to be effective when treatments were administered intravenously. Further studies confirm these previous results. Furthermore, contralateral subcutaneous (SC) injections were also found to be effective in the same mouse model. One possible explanation of these successful outcomes was that we were able to provide: (1) sustained circulation of the CpG ODNs through spontaneous formation of monovalent ICs *in vivo*; (2) targeting of the CpG ODNs to DCs in the tumor margin via the EPR effect; and (3) facilitated uptake of ICs by DCs through the Fc receptor expressed on pDCs.

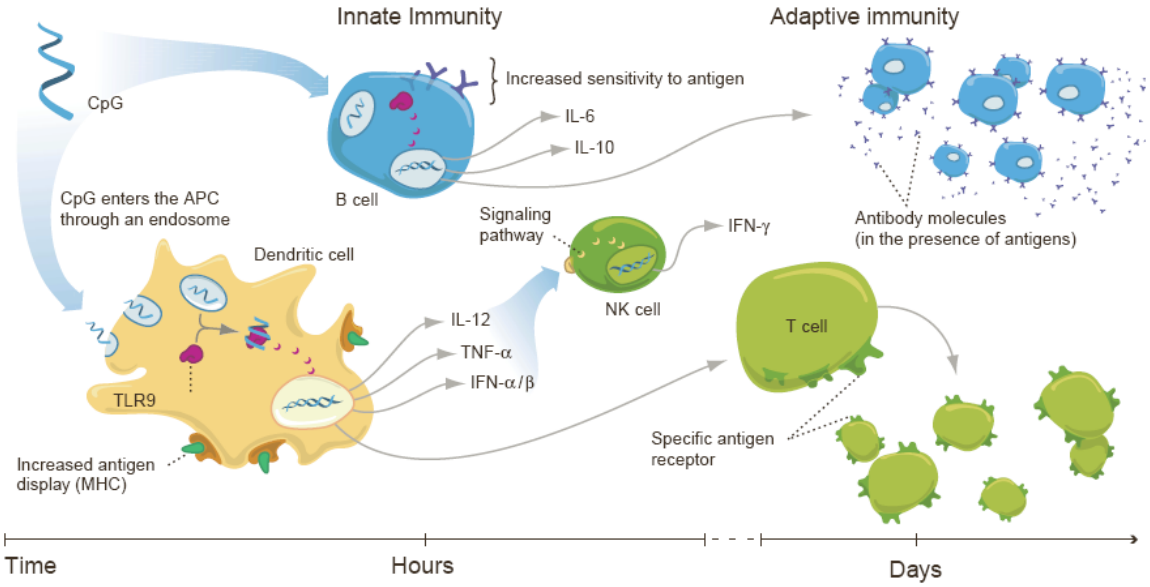

Figure 1.1 Activation of the immune system by CpG oligodeoxynucleotides. This figure was borrowed from Krieg, AM. CpG motifs: the active ingredient in bacterial extracts? *Nat. Med.* **2003** *9*:831-835.

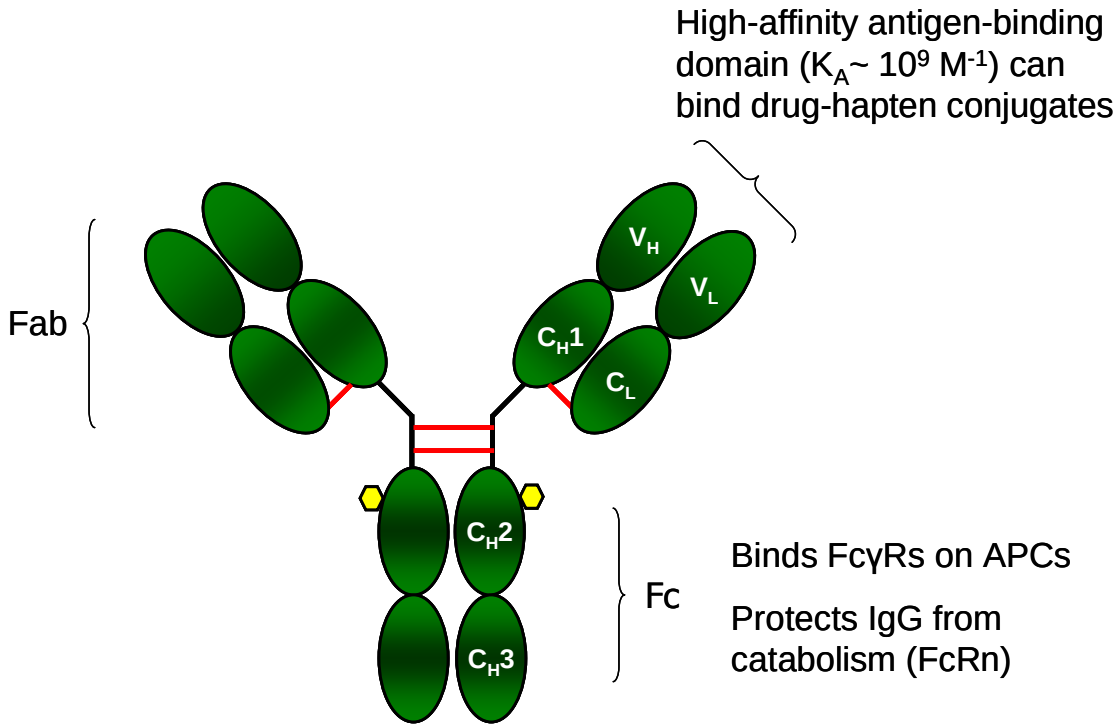

Figure 1.2. Structure and function of immunoglobulin G. IgG can be used as an endogenous protein carrier because it can be found in high levels *in vivo*, can bind drugs with high affinity, exhibits a long circulatory half-life and lack of antigenicity, and can target drugs to antigen-presenting cells via Fc receptor-mediated endocytosis.

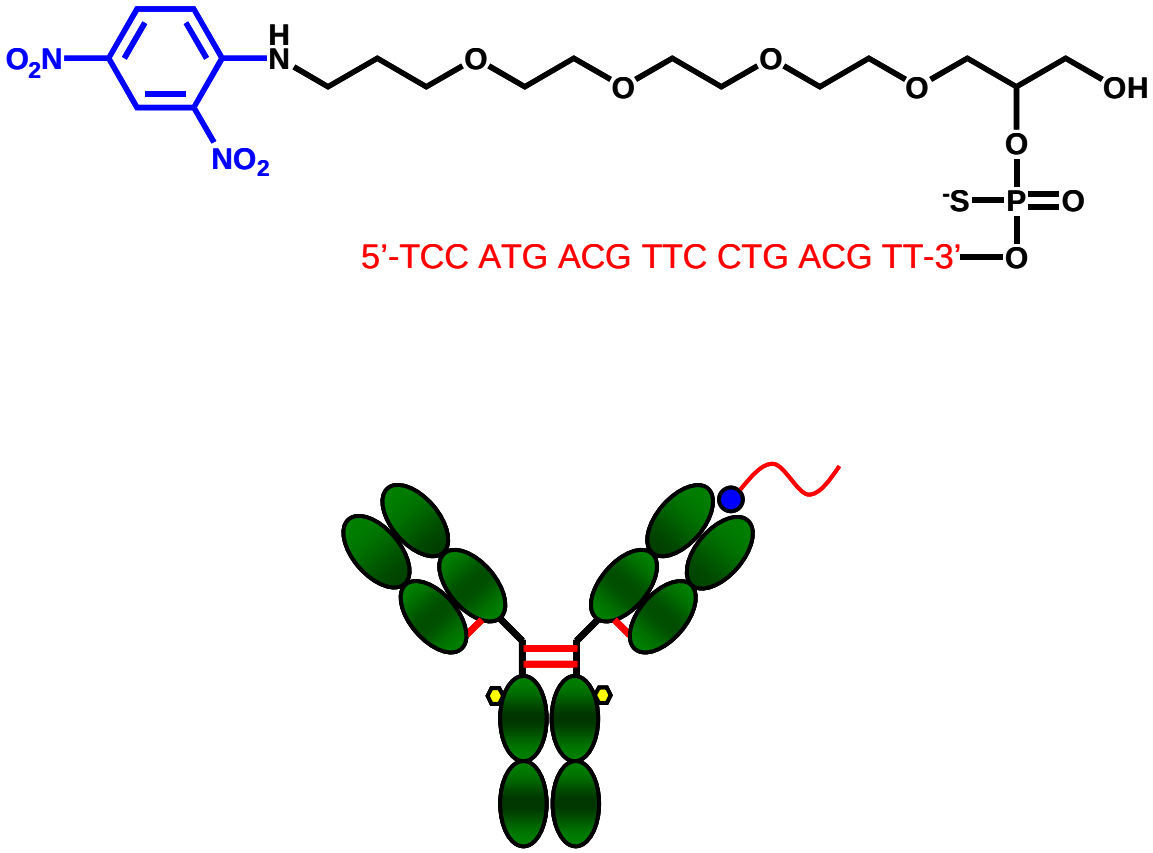

Figure 1.3. Haptenization of CpG for immune complexation. DNP-CpG forms a non-crosslinking complex with an anti-DNP antibody. The CpG oligonucleotide contains a phosphorothioate backbone. The complex exhibits characteristics similar to those of the antibody alone.

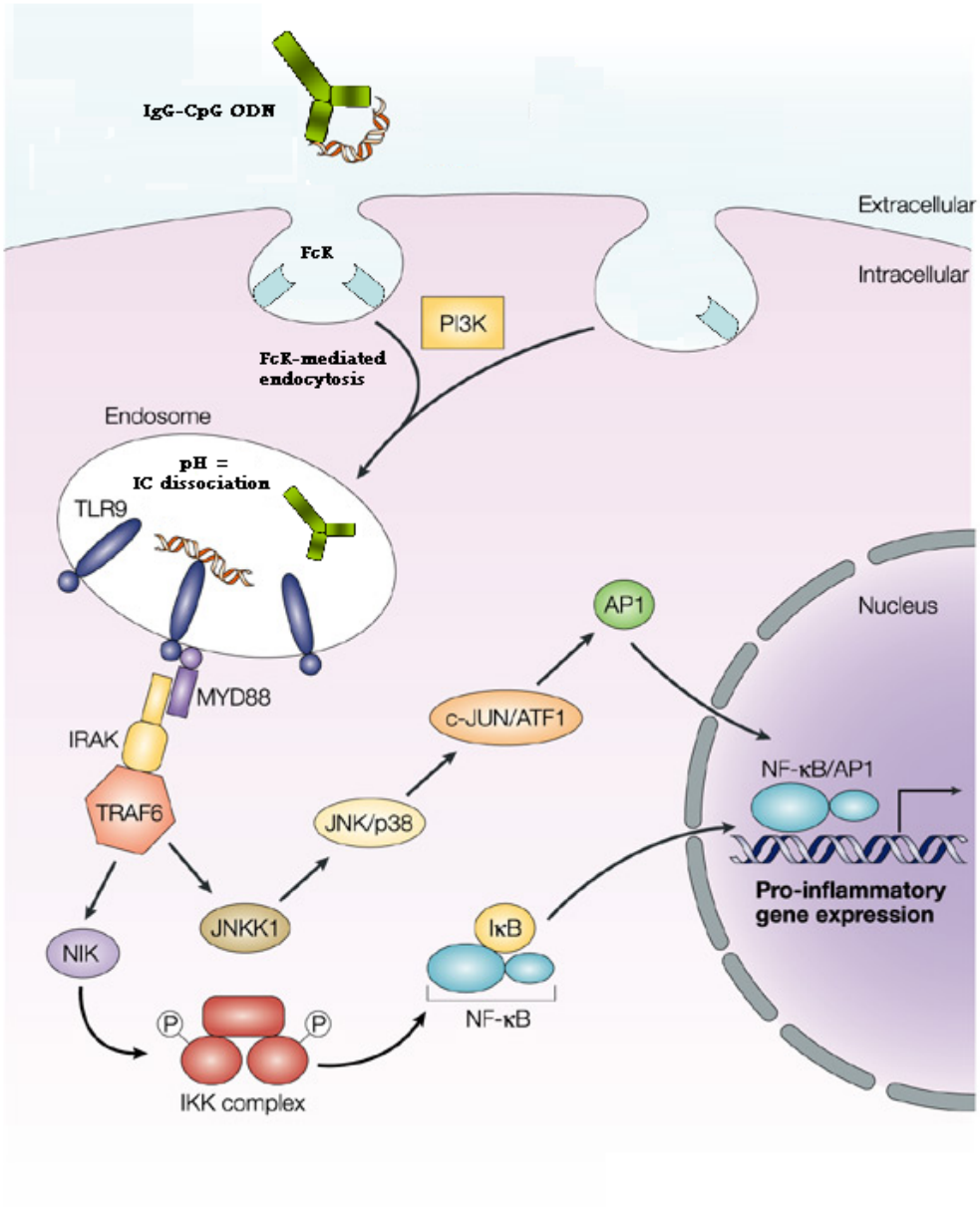

Figure 1.4. Proposed mechanism by which dendritic cells uptake and process a monovalent, non-crosslinking immune complexes. Dendritic cells uptake the immune complex via FcγRI-mediated endocytosis. The complex dissociates in the acidic pH of the endosome. The liberated CpG oligonucleotide binds to the endosomal receptor TLR9 to initiate an immune response. (Figure modified from Bell, E. *Nat. Rev. Immunol.* **2004**, *4*, 3-3)

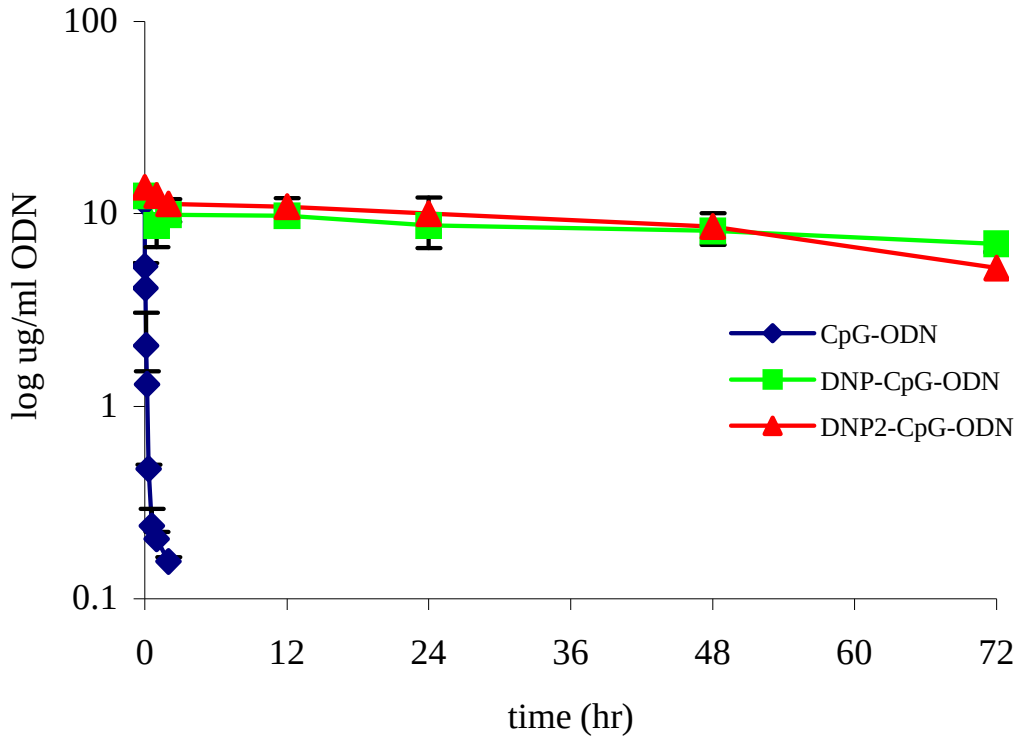

Figure 1.5. Serum pharmacokinetic profile of free CpG, DNP-CpG, and DNP_2-CpG after a single intravenous dose. Serum concentrations of [^{3}H]-CpG-ODN in CT26 tumor-bearing mice and of DNP-[^{3}H]-DNP-CpG and DNP_2-[^{3}H]-CpG-ODN in DNP-immunized, CT26 tumor-bearing mice. The above oligodeoxynucleotides (2.8 nmole, 18-22 μg) were injected as an intravenous bolus via the tail vein. At specific time points, mice were exsanguinated by decapitation or cardiac puncture; collected blood was processed for serum and counted via liquid scintillation counting. n = 3 mice per time point. Figure is from reference 29.

Table 1. Serum pharmacokinetic parameters following a single intravenous bolus injection of [^{3}H]-CpG-ODN, DNP-[^{3}H]-CpG-ODN, and DNP_2-[^{3}H]-CpG-ODN into DNP-immunized, tumor-bearing mice.			
	[^{3}H]-CpG-ODN*	DNP-[^{3}H]-CpG-ODN	DNP_2-[^{3}H]-CpG-ODN
$t_{1/2,\alpha}$ (hr)	0.0215 ± 0.00427	0.121 ± 0.200 (5.63 ↑) †	0.259 ± 0.519 (12.0 ↑)
$t_{1/2,\beta}$ (hr)	0.694 ± 0.745	185 ± 95.8 (266 ↑)	74.6 ± 15.0 (108 ↑)
AUC (hr·μg / ml)	1.46 ± 0.903	2410 ± 1120 (1650 ↑)	1290 ± 214 (884 ↑)
V_1 (ml)	1.75 ± 0.131	1.54 ± 0.172	1.60 ± 0.167
V_2 (ml)	8.46 ± 6.24	0.657 ± 0.234 (12.9↓)	0.228 ± 0.194 (37.0↓)
V_{ss} (ml)	10.2 ± 6.26	2.20 ± 0.159 (4.63↓)	1.83 ± 0.103 (5.59↓)
CL (ml/hr)	12.6 ± 7.85	0.00825 ± 0.00385 (1530↓)	0.0170 ± 0.00281 (746↓)
MRT (hr)	0.807 ± 0.949	267 ± 138 (331 ↑)	108 ± 21.6 (134 ↑)
* In non-immunized, tumor-bearing mice; control ODN is shaded gray † Fold-change relative to control ODN. Arrows indicate direction of change			

Table 1. Serum pharmacokinetic parameters of free CpG, DNP-CpG, and DNP_2-CpG after a single intravenous dose. Table from E. Palma, "Antibody-Mediated Systemic Delivery of CpG Oligonucleotides for Solid Tumor Immunotherapy," PhD Dissertation submitted to the University of North Carolina, 2006

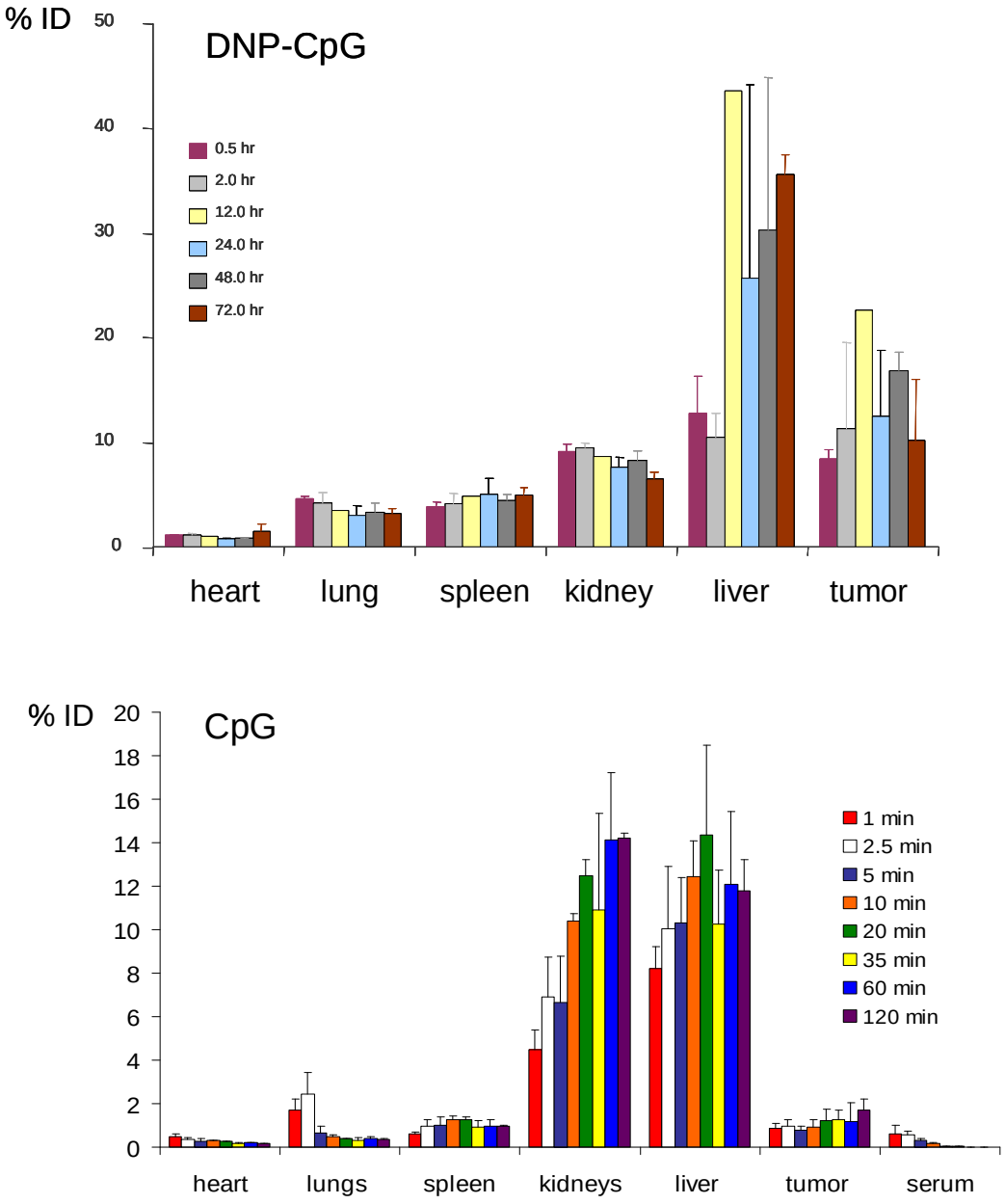

Figure 1.6. Biodistribution of DNP-CpG and free CpG after a single intravenous dose. Tumor-bearing mice were pre-immunized against DNP. The colored bars represent the average percent of the total injected dose at given time points after the given dose. n = 3 mice per time point. A large amount was found in the clearance organs. However, a very high percentage was found in the tumor tissue which supports the involvement of the EPR effect. See reference 29.

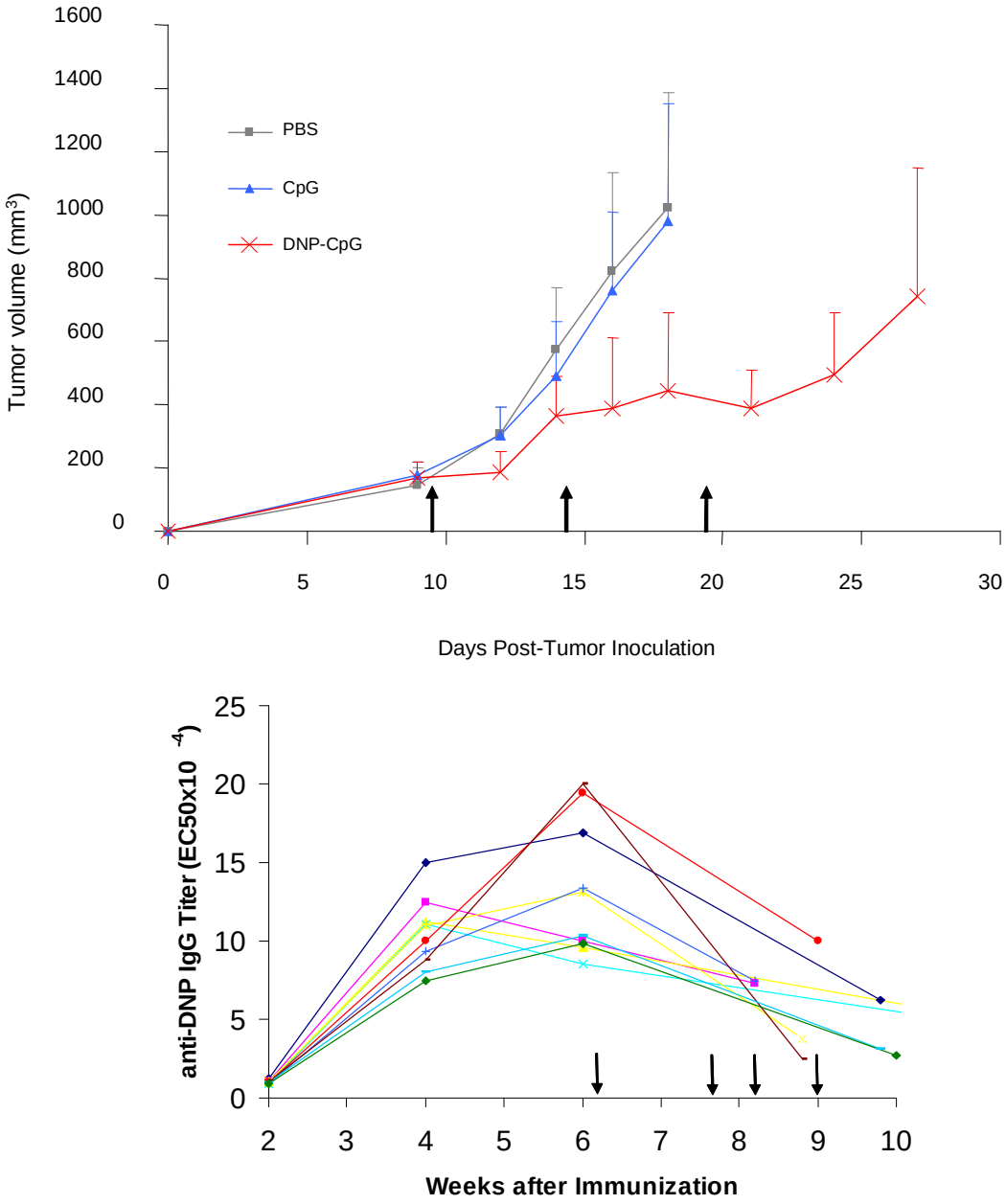

Figure 1.7. Comparison of tumor growth inhibition between intravenous dosing of free CpG and DNP-CpG in immunized mice. Tumor growth inhibition by PBS (negative control, n=7), free CpG (n=10), and DNP-CpG (n=10) administered intravenously in DNP-immunized, CT26 tumor-bearing mice. Tumor volumes were plotted against time in days after tumor inoculation. Data are expressed as means ± SEM. In the lower graph, the anti-DNP titer after immunization with DNP-KLH along with Freund's adjuvants is shown. n = 10 mice. CT26 cell inoculation took place immediately after 6 weeks after immunization, marked by the first arrow. Mice were subsequently treated three times with DNP-CpG as indicated by subsequent arrows. Note that the titer did not increase with the DNP-CpG treatments, supporting that B cells were not activated with DNP-CpG treatment. See reference 29.

REFERENCES

(1) Hoption Cann, S. A.; van Netten, J. P.; van Netten, C. Dr William Coley and tumour regression: a place in history or in the future. *Postgrad. Med. J.* **2003**, *79*, 672-680.

(2) Tokunaga, T.; Yamamoto, T.; Yamamoto, S. How BCG led to the discovery of immunostimulatory DNA. *Jpn. J. Infect. Dis.* **1999**, *52*, 1-11.

(3) Tokunaga, T.; Yamamoto, H.; Shimada, S.; Abe, H.; Fukuda, T.; Fujisawa, Y.; Furutani, Y.; Yano, O.; Kataoka, T.; Sudo, T. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **1984**, *72*, 955-962.

(4) Shimada, S.; Yano, O.; Inoue, H.; Kuramoto, E.; Fukuda, T.; Yamamoto, H.; Kataoka, T.; Tokunaga, T. Antitumor activity of the DNA fraction from Mycobacterium bovis BCG. II. Effects on various syngeneic mouse tumors. *J. Natl. Cancer Inst.* **1985**, *74*, 681-688.

(5) Akira, S.; Uematsu, S.; Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **2006**, *124*, 783-801.

(6) Iwasaki, A.; Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol* **2004**, *5*, 987-995.

(7) Krieg, A. M.; Efler, S. M.; Wittpoth, M.; Al Adhami, M. J.; Davis, H. L. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J. Immunother.* **2004**, *27*, 460-471.

(8) Agrawal, S.; Kandimalla, E. R. Medicinal chemistry and therapeutic potential of CpG DNA. *Trends Mol. Med.* **2002**, *8*, 114-121.

(9) Krieg, A. M. Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug Discov.* **2006**, *5*, 471-484.

(10) Heckelsmiller, K.; Rall, K.; Beck, S.; Schlamp, A.; Seiderer, J.; Jahrsdörfer, B.; Krug, A.; Rothenfusser, S.; Endres, S.; Hartmann, G. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.* **2002**, *169*, 3892-3899.

(11) Banchereau, J.; Briere, F.; Caux, C.; Davoust, J.; Lebecque, S.; Liu, Y. J.; Pulendran, B.; Palucka, K. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **2000**, *18*, 767-811.

(12) Reis e Sousa, C. Dendritic cells in a mature age. *Nat. Rev. Immunol.* **2006**, *6*, 476-483.

(13) Wilson, N. S.; Villadangos, J. A. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. *Adv. Immunol.* **2005**, *86*, 241-305.

(14) Randolph, G. J.; Angeli, V.; Swartz, M. A. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.* **2005**, *5*, 617-628.

(15) Raulet, D. H. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* **2004**, *5*, 996-1002.

(16) Watts, C. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu. Rev. Immunol.* **1997**, *15*, 821-850.

(17) Iwasaki, A.; Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **2004**, *5*, 987-995.

(18) Ikeda, H.; Chamoto, K.; Tsuji, T.; Suzuki, Y.; Wakita, D.; Takeshima, T.; Nishimura, T. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. *Cancer Sci.* **2004**, *95*, 697-703.

(19) Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **2003**, *3*, 133-146.

(20) Dredge, K.; Marriott, J. B.; Todryk, S. M.; Dalgleish, A. G. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. *Cancer Immunol. Immunother.* **2002**, *51*, 521-531.

(21) Bretscher, P. The two-signal model of lymphocyte activation twenty-one years later. *Immunol. Today* **1992**, *13*, 74-76.

(22) von Beust, B. R.; Johansen, P.; Smith, K. A.; Bot, A.; Storni, T.; Kündig, T. M. Improving the therapeutic index of CpG oligodeoxynucleotides by intralymphatic administration. *Eur. J. Immunol.* **2005**, *35*, 1869-1876.

(23) Heckelsmiller, K.; Beck, S.; Rall, K.; Sipos, B.; Schlamp, A.; Tuma, E.; Rothenfusser, S.; Endres, S.; Hartmann, G. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. *Eur. J. Immunol.* **2002**, *32*, 3235-3245.

(24) Proudfoot, O.; Apostolopoulos, V.; Pietersz, G. A. Receptor-mediated delivery of antigens to dendritic cells: anticancer applications. *Mol. Pharm.* **2007**, *4*, 58-72.

(25) Brunner, C.; Seiderer, J.; Schlamp, A.; Bidlingmaier, M.; Eigler, A.; Haimerl, W.; Lehr, H. A.; Krieg, A. M.; Hartmann, G.; Endres, S. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell

activation in vitro and therapeutic anti-tumor immune responses in vivo. *J. Immunol.* **2000**, *165*, 6278-6286.

(26) Jiskoot, W.; van Schie, R. M. F.; Carstens, M. G.; Schellekens, H. Immunological risk of injectable drug delivery systems. *Pharm. Res.* **2009**, *26*, 1303-1314.

(27) Onda, M. Reducing the immunogenicity of protein therapeutics. *Curr. Drug Targets* **2009**, *10*, 131-139.

(28) Jahrsdörfer, B.; Weiner, G. J. CpG oligodeoxynucleotides as immunotherapy in cancer. *Update Cancer Ther.* **2008**, *3*, 27-32.

(29) Palma, E.; Cho, M. J. Improved systemic pharmacokinetics, biodistribution, and antitumor activity of CpG oligodeoxynucleotides complexed to endogenous antibodies in vivo. *J. Control. Release* **2007**, *120*, 95-103.

(30) Lencer, W. I.; Blumberg, R. S. A passionate kiss, then run: exocytosis and recycling of IgG by FcRn. *Trends Cell Biol.* **2005**, *15*, 5-9.

(31) Lobo, E. D.; Hansen, R. J.; Balthasar, J. P. Antibody pharmacokinetics and pharmacodynamics. *J Pharm Sci* **2004**, *93*, 2645-2668.

(32) Junghans, R. P. Finally! The Brambell receptor (FcRB). Mediator of transmission of immunity and protection from catabolism for IgG. *Immunol. Res.* **1997**, *16*, 29-57.

(33) Qiao, S.; Kobayashi, K.; Johansen, F.; Sollid, L. M.; Andersen, J. T.; Milford, E.; Roopenian, D. C.; Lencer, W. I.; Blumberg, R. S. Dependence of antibody-mediated presentation of antigen on FcRn. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 9337-9342.

(34) Fossati, G.; Bucknall, R. C.; Edwards, S. W. Fcgamma receptors in autoimmune diseases. *Eur. J. Clin. Invest.* **2001**, *31*, 821-831.

(35) Gessner, J. E.; Heiken, H.; Tamm, A.; Schmidt, R. E. The IgG Fc receptor family. *Ann. Hematol.* **1998**, *76*, 231-248.

(36) Ravetch, J. V.; Bolland, S. IgG Fc receptors. *Annu. Rev. Immunol.* **2001**, *19*, 275-290.

(37) Norbury, C. C. Drinking a lot is good for dendritic cells. *Immunology* **2006**, *117*, 443-451.

(38) Regnault, A.; Lankar, D.; Lacabanne, V.; Rodriguez, A.; Théry, C.; Rescigno, M.; Saito, T.; Verbeek, S.; Bonnerot, C.; Ricciardi-Castagnoli, P.; Amigorena, S. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med.* **1999**, *189*, 371-380.

(39) Hamano, Y.; Arase, H.; Saisho, H.; Saito, T. Immune complex and Fc receptor-mediated augmentation of antigen presentation for in vivo Th cell responses. *J. Immunol.* **2000**, *164*, 6113-6119.

(40) You, Z.; Huang, X.; Hester, J.; Toh, H. C.; Chen, S. Y. Targeting dendritic cells to enhance DNA vaccine potency. *Cancer Res.* **2001**, *61*, 3704-3711.

(41) Rafiq, K.; Bergtold, A.; Clynes, R. Immune complex-mediated antigen presentation induces tumor immunity. *J. Clin. Invest.* **2002**, *110*, 71-79.

(42) de Jong, J. M. H.; Schuurhuis, D. H.; Ioan-Facsinay, A.; Welling, M. M.; Camps, M. G. M.; van der Voort, E. I. H.; Huizinga, T. W. J.; Ossendorp, F.; Verbeek, J. S.; Toes, R. E. M. Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4+ T cells upon immune-complex uptake in vivo. *Immunology* **2006**, *119*, 499-506.

(43) Amigorena, S.; Bonnerot, C. Fc receptor signaling and trafficking: a connection for antigen processing. *Immunol. Rev.* **1999**, *172*, 279-284.

(44) Schuurhuis, D. H.; van Montfoort, N.; Ioan-Facsinay, A.; Jiawan, R.; Camps, M.; Nouta, J.; Melief, C. J. M.; Verbeek, J. S.; Ossendorp, F. Immune complex-loaded dendritic cells are superior to soluble immune complexes as antitumor vaccine. *J. Immunol.* **2006**, *176*, 4573-4580.

(45) Ober, R. J.; Radu, C. G.; Ghetie, V.; Ward, E. S. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *Int. Immunol.* **2001**, *13*, 1551-1559.

(46) Goodman, G. E.; Beaumier, P.; Hellström, I.; Fernyhough, B.; Hellström, K. E. Pilot trial of murine monoclonal antibodies in patients with advanced melanoma. *J. Clin. Oncol.* **1985**, *3*, 340-352.

(47) Hudson, P. J.; Souriau, C. Engineered antibodies. *Nat. Med.* **2003**, *9*, 129-134.

(48) Lipman, N. S.; Jackson, L. R.; Trudel, L. J.; Weis-Garcia, F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J.* **2005**, *46*, 258-268.

(49) Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* **1986**, *46*, 6387-6392.

(50) Gullotti, E.; Yeo, Y. Extracellularly Activated Nanocarriers: A New Paradigm of Tumor Targeted Drug Delivery. *Mol. Pharm.* **2009**.

(51) Padera, T. P.; Kadambi, A.; di Tomaso, E.; Carreira, C. M.; Brown, E. B.; Boucher, Y.; Choi, N. C.; Mathisen, D.; Wain, J.; Mark, E. J.; Munn, L. L.; Jain, R. K. Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* **2002**, *296*, 1883-1886.

(52) Maeda, H.; Bharate, G. Y.; Daruwalla, J. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. *Eur. J. Pharm. Biopharm.* **2009**, *71*, 409-419.

(53) Fox, M. E.; Szoka, F. C.; Fréchet, J. M. J. Soluble Polymer Carriers for the Treatment of Cancer: The Importance of Molecular Architecture. *Acc. Chem. Res.* **2009.**

(54) Peterson, C. M.; Shiah, J.; Sun, Y.; Kopecková, P.; Minko, T.; Straight, R. C.; Kopecek, J. HPMA copolymer delivery of chemotherapy and photodynamic therapy in ovarian cancer. *Adv. Exp. Med. Biol.* **2003,** *519*, 101-123.

(55) Ishida, O.; Maruyama, K.; Tanahashi, H.; Iwatsuru, M.; Sasaki, K.; Eriguchi, M.; Yanagie, H. Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo. *Pharm. Res.* **2001,** *18*, 1042-1048.

(56) Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C. Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Mol. Pharm.* **2008,** *5*, 505-515.

(57) Stohrer, M.; Boucher, Y.; Stangassinger, M.; Jain, R. K. Oncotic pressure in solid tumors is elevated. *Cancer Res.* **2000,** *60*, 4251-4255.

(58) Jain, R. K. The Eugene M. Landis Award Lecture 1996. Delivery of molecular and cellular medicine to solid tumors. *Microcirculation* **1997,** *4*, 1-23.

(59) Murad, Y. M.; Clay, T. M.; Lyerly, H. K.; Morse, M. A. CPG-7909 (PF-3512676, ProMune): toll-like receptor-9 agonist in cancer therapy. *Expert Opin. Biol. Ther.* **2007,** *7*, 1257-1266.

(60) Wilson, K. D.; Raney, S. G.; Sekirov, L.; Chikh, G.; deJong, S. D.; Cullis, P. R.; Tam, Y. K. Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles. *Int. Immunopharmacol.* **2007,** *7*, 1064-1075.

(61) Chikh, G.; de Jong, S. D.; Sekirov, L.; Raney, S. G.; Kazem, M.; Wilson, K. D.; Cullis, P. R.; Dutz, J. P.; Tam, Y. K. Synthetic methylated CpG ODNs are potent in vivo adjuvants when delivered in liposomal nanoparticles. *Int. Immunol.* **2009,** *21*, 757-767.

(62) Salem, A. K.; Weiner, G. J. CpG oligonucleotides as immunotherapeutic adjuvants: innovative applications and delivery strategies. *Adv. Drug Deliv. Rev.* **2009,** *61,* 193-194.

(63) Chaung, H. CpG oligodeoxynucleotides as DNA adjuvants in vertebrates and their applications in immunotherapy. *Int. Immunopharmacol.* **2006,** *6,* 1586-1596.

(64) Kim, H.; Ko, H.; Ju, H.; Kim, K.; Roh, S.; Lee, H.; Im, S. CpG-ODN-based immunotherapy is effective in controlling the growth of metastasized tumor cells. *Cancer Lett.* **2009,** *274,* 160-164.

(65) Vollmer, J.; Krieg, A. M. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv. Drug Deliv. Rev.* **2009,** *61,* 195-204.

(66) O'Hear, C. E.; Foote, J. Antibody buffering of a ligand in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **2005,** *102,* 40-44.

(67) Palma, E.; Klapper, D. G.; Cho, M. J. Antibodies as drug carriers III: design of oligonucleotides with enhanced binding affinity for immunoglobulin G. *Pharm. Res.* **2005,** *22,* 122-127.

(68) Rehlaender, B. N.; Cho, M. J. Antibodies as drug carriers. II. For proteins. *Pharm. Res.* **2001,** *18,* 753-760.

(69) Rehlaender, B. N.; Cho, M. J. Anti-drug antibodies as drug carriers. I. For small molecules. *Pharm. Res.* **2001,** *18,* 745-752.

(70) Rehlaender, B. N.; Cho, M. J. Antibodies as carrier proteins. *Pharm. Res.* **1998,** *15,* 1652-1656.

(71) Bazin-Redureau, M. I.; Renard, C. B.; Scherrmann, J. M. Pharmacokinetics of heterologous and homologous immunoglobulin G, F(ab')2 and Fab after intravenous administration in the rat. *J. Pharm. Pharmacol.* **1997,** *49,* 277-281.

CHAPTER II

PHARMACOKINETICS OF CpG OLIGODEOXYNUCLEOTIDES UPON SUBCUTANEOUS ADMINISTRATION IN MICE

Abstract

A prolonged circulatory half-life that will allow for a sustained supply to (intra)cellular targets are important in oligonucleotide delivery in its pharmacological intervention in a given disease state. To confer favorable pharmacokinetic (PK) properties to our model CpG oligodeoxynucleotide (ODN), we have devised a 2,4-dinitrophenyl derivative of CpG ODN (DNP-CpG) which exhibits a favorable systemic half-life in the presence of endogenously produced anti-DNP antibodies. We have previously shown that intravenously (IV) administered DNP-CpG exhibits prolonged circulation *in vivo* whereas underivatized CpG (free CpG) is rapidly eliminated.

In this study, we characterized and compared the pharmacokinetics and biodistribution of underivatized [^{3}H]-CpG and DNP-[^{3}H]-CpG in DNP-immunized, CT26 tumor-bearing mice upon subcutaneous (SC) administration. Serum and tissue samples for PK and biodistribution analysis were collected at set post-dose time points for analysis by liquid scintillation counting. In both cases, an absorption and elimination phases are apparent although DNP-CpG exhibits slightly faster peak serum levels and gradual elimination. A high percentage of the injected dose remains in the injection site, but there is little accumulation in the tumor for both treatments. These results characterize the differences between the subcutaneous administration of DNP-CpG in an immune complex (IC) forming system and free CpG.

Introduction

Small oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs can activate immune responses, providing potential applications in cancer and other diseases.[1,2] However, for CpG monotherapy to be effective in tumor models, these ODNs must be injected intra- or peri-tumorally.[3] Recently, we were able to dramatically improve the pharmacokinetics (PK) of intravenously administered 20-mer CpG, known as CpG 1826 in the literature, by injecting its 2,4-dinitrophenyl (DNP) derivative to tumor-bearing mice that had been immunized against the DNP hapten. More significantly, the intravenous administration inhibited tumor growth.[4]

The PK behavior of the systemically administered ODNs with a phosphorothioate backbone is largely nucleotide sequence-independent, equivalent across non-human and human species on a normalized-dose basis. It is thus accepted that animal PK study results can be extrapolated to humans.[5] Upon bolus IV administration, these ODNs generally show a serum profile consisting of a rapid tissue distribution phase with a $t_{1/2}$ of a few minutes followed by an elimination phase with a $t_{1/2}$ of 15 to 45 min. The poor anti-tumor effect of intravenously administered CpG ODNs in mice[3] and humans[6] is possibly due to their poor uptake by target cells; i.e., the amount of the CpG delivered to target cells, such as dendritic cells, was insufficient. This is in turn caused by the rapid renal clearance of this class of ODNs.[7-9] One solution is to use an antibody carrier to exploit the enhanced permeability and retention (EPR) for passive targeting of CpG ODNs to dendritic cells. Leaky vasculature and impaired lymphatics in the tumor vicinity will allow accumulation of antibodies and subsequent presentation of CpG ODNs to surrounding dendritic cells.

In our preliminary studies, BALB/c mice were first immunized with DNP. They were subsequently inoculated with BALB/c-derived colon carcinoma CT26 cells. When the solid tumor was clearly palpable, [^{3}H]-labeled DNP derivative of CpG 1826 ([^{3}H]-DNP-CpG) was injected via tail vein. At a given time interval, CpG concentrations in blood, organs, and tumor tissue were determined. These PK studies lasted for three days. In a separate series of experiments but in a similar protocol, anti-tumor effect of DNP-CpG was continuously monitored over a 30-day period with weekly IV dosing. The PK/pharmacodynamic (PD) results are highlighted as follows.[4] The serum elimination phase $t_{1/2}$ increased from 0.69 hr for CpG to 185 hr for DNP-CpG, which is an increase of nearly 270-fold. The $t_{1/2}$ observed is in a reasonable agreement with the value for free IgG_1 in mice.[10,11] Accordingly, the mean residence time increased 330-fold. The area under curve in serum concentration increased 1,650-fold. Clearance decreased 1,260-fold. In accordance of these extraordinary PK findings and the expected EPR effect, the fraction of injected dose (% ID) found in tumor tissue ranged from 10 to as high as 23% and persisted for 3 days. These numbers correspond to approximately 40 and 90 %ID/gm tumor, respectively. In contrast, CpG accumulated less than 1% and not detectable after 2 hrs. Weekly IV dosing of 0.1 mg/mouse inhibited tumor growth when the initial tumor was on the average 175 mm^3. To our best knowledge, this is the first time anti-tumor effect was successfully observed subsequent to the IV route of administration. Tumor shrinkage was confirmed macroscopically as well as histologically. Cell infiltration, presumably lymphocytes, was clearly noticed.

Since a substantial amount of endogenous IgG is found in the interstitial tissue in dynamic equilibrium with plasma, approximately at 25% of the plasma concentration[12], we

reasoned that a significant amount of SC dose of DNP-CpG would exist as an IC at the injection site in the tissue. These complexes will be drained to lymphatics and eventually discharged to general circulation where a high anti-DNP titer is available for further IC formation. In the present study, we investigated the PK and organ distribution including tumor accumulation subsequent to SC administration of DNP-CpG relative to unconjugated, free CpG in DNP-immunized, CT26 tumor-bearing mice.

Experimental Procedure

General Procedures and Materials

All reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Forty female BALB/c mice, five weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the UNC Eshelman School of Pharmacy Kerr Hall Animal Facility according to NIH guidelines. All procedures were approved by the UNC Institutional Animal Care and Use Committee. Molecular biology-grade water, phosphate buffered saline (PBS), sodium carbonate, thimerosal, Tween 20, ethylenediaminetetraacetic acid (EDTA), 30% H_2O_2 solution, β-mercaptoethanol, Tris base, complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA) were purchased from Sigma-Aldrich (St. Louis, MO). Hammersten-grade casein was purchased from Research Organics (Cleveland, OH). Dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) was purchased from Calbiochem (San Diego, CA). BALB/c-derived colon carcinoma CT26 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) with low (1 g/L) glucose supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin. All cell culture reagents, including trypsin-EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Cell culture vented flasks and conical tubes were purchased from Corning, Inc. (Corning, NY).

CpG 1826 (free CpG) and dinitrophenylated CpG 1826 (DNP-CpG) were synthesized with a phosphorothioate backbone and provided in lyophilized form by Dr. Rowshon Alam of the Nucleic Acid Core Facility of the Program Project Grant in Pharmacodynamics of Genes and Oligonucleotides at the University of North Carolina at Chapel Hill. Solvable and Ultima Gold were purchased from PerkinElmer (Waltham, MA). Ketamine HCl (Ketaved, 100 mg/ml) used during PK experiments was provided by Dr. Philip Smith at the UNC Eshelman School of Pharmacy.

Oligonucleotide Radiolabeling

Oligonucleotides were tritium labeled with [^{3}H]-water (5 Ci/g; Sigma-Aldrich, St. Louis, MO) according to similar methods previously desccribeed.[13] Five milligrams of DNP-CpG in water was placed in a 1.0 ml Reacti-Vial (Pierce, Rockford, IL) and lyophilized overnight. Two hundred microliters of 50 mM sodium phosphate containing 0.1 mM EDTA at pH 7.8 was added to DNP-CpG and lyophilized overnight. Subsequently 200 μl of [^{3}H]-water was added to the dry DNP-CpG with 84 μl β-mercaptoethanol. The reaction mixture was placed in an oil bath at 90^{o}C for 6 hours. The sample was then removed from the oil bath and lyophilized overnight. Free CpG was radiolabeled similarly. Specific activity (SA) and purity were determined to be in the range of 6.5-7.6 x 10^4 dpm/μg. All ODNs were dissolved in sterile PBS prior to use *in vivo*.

Vaccine Preparation, Active Immunization, and Tumor Inoculation

To prepare the primary vaccine, 20 mg of DNP-KLH was dissolved in 10 ml of endotoxin-free water to give a 2 mg/ml solution. Prior to vaccination, an equal volume of CFA was added. The mixture was emulsified by vortexing at maximum rpm for 15 minutes using a multi-tube vortexer (VWR, West Chester, PA). Secondary vaccines were prepared similarly, but replacing CFA with IFA.

Forty BALB/c mice were immunized intraperitoneally with 200 μl of freshly prepared primary vaccine. A booster injection was given two weeks later using 200 μl of the secondary vaccine. Anti-DNP antibody titer was monitored by ELISA. Four to five weeks after primary vaccination, DNP-immunized mice were inoculated with $2.5x10^5$ CT26 cells suspended in 100 μl HBSS into the right flank. Tumor growth was monitored daily.

Pharmacokinetics and Biodistribution

On the ninth day after tumor inoculation, 18 mice were dosed subcutaneously in the left flank with 100 μg of [^{3}H]-CpG (SA = $7.6x10^4$ dpm/μg) in 100 μl of PBS. At 5, 10, 20, 60, 120, and 240 minutes post-dose, three mice per time point were anesthetized intraperitoneally with 100 μl of 100 mg/ml ketamine·HCl. Additionally, 21 mice were dosed subcutaneously with 108 μg of DNP-[^{3}H]-CpG (SA = $6.5x10^4$ dpm/μg) in 100 μl of PBS. At 0.5, 2, 6, 12, 24, 48 and 72 hours post-dose, mice were anesthetized as described above. Mice were exsanguined by cardiac puncture and each blood sample was allowed to clot in a 1.6 ml eppendorf tube for two hours at room temperature (RT). Serum was collected after centrifugation of coagulated blood at 500 x g for 15 minutes and stored at -20^{o}C for further analysis. The injection site, heart, lungs, spleen, liver, kidneys, and tumor were subsequently

harvested, rinsed briefly with water, blotted dry, placed in pre-weighed 20 ml scintillation vials, flash frozen in liquid nitrogen, and stored at -80^{o}C until analyzed.

Organ and tissue samples were processed as previously described[14] with slight modifications. Samples were thawed at RT and homogenized in 3 ml of cold homogenizing buffer comprised of 10 mM EDTA, 1 mM β-mercaptoethanol, and 100 mM Tris buffer at pH 7.5 using a PowerGen 700 tissue homogenizer (Thermo Fisher Scientific, Waltham, MA) followed by addition of 2 ml of Solvable® tissue solubilizer. Vials were then heated overnight in a 60^{o}C shaking water bath. Solubilized organs were decolorized using 0.2 ml of 30% H_2O_2 and heating once more at 60^{o}C for 2 hours. Vials were cooled to RT and 10 ml of Ultima Gold scintillation cocktail was added. Serum samples were processed only by the addition of 25 μl of sample to 10 ml of scintillation cocktail. The radioactivity in dpm of all samples was measured using a Packard Tri-Carb 2900TR Liquid Scintillation Analyzer and corrected for background radioactivity. No additional correction was performed for organ-associated quenching nor organ-associated radioactivity from resident blood volume.

Results

Serum concentration-versus-time data was derived from radioactivity measurements. As seen in Figure 2.1, biphasic pharmacokinetic profiles were observed for both [^{3}H]-CpG and DNP-[^{3}H]-CpG. At points prior to two hours, [^{3}H]-CpG levels are higher than DNP-[^{3}H]-CpG levels. However the opposite was seen after two hours with DNP-[^{3}H]-CpG levels sustained above [^{3}H]-CpG levels. [^{3}H]-CpG showed a large drop in serum levels between 1 to 2 hours. Serum levels persisted past the measured time points for both groups. A noncompartmental analysis was performed using WinNonlin 5.2.1 (Pharsight Corporation,

Cary, NC) to elucidate the elimination half-life (Figure 2.2). Analysis using the method of residuals gives an elimination half-life estimate of 1.08 hours for [^{3}H]-CpG. This estimate is comparable to that obtained from intravenous data.[4] This analysis indicates the ascending portion of the serum data actually represents the elimination phase while the descending portion represents the rate-limiting absorption. In contrast, the descending portion of the DNP-[^{3}H]-CpG plot represents rapid absorption whereas the terminal portion of the plot represents slow elimination. Data for DNP-[^{3}H]-CpG results in an estimate of 715 hours which is consistent with the substantial increase in elimination half-life as seen with intravenous data for DNP-[^{3}H]-CpG compared to [^{3}H]-CpG.

Figures 2.3 and 2.4 present the tissue distribution profile of [^{3}H]-CpG and DNP-[^{3}H]-CpG, respectively. In both cases, injection site radioactivity levels were initially high, but diminished over time, a finding consistent with SC administration. Almost no radioactivity was detected in the heart and lungs. At all time points, both ODNs were found in the liver and kidneys. [^{3}H]-CpG was found in the spleen at low levels while DNP-[^{3}H]-CpG levels were almost zero. As expected, levels of [^{3}H]-CpG in the tumor were low. However, a key observation was seen with DNP-[^{3}H]-CpG. Even at later time points, negligible levels of DNP-[^{3}H]-CpG were detected in the tumor tissue. This finding is in stark contrast to the results seen with IV data in which accumulation of DNP-[^{3}H]-CpG in the tumor was as high as 30% of the injected dose. This suggests that the two routes of administration may utilize two different mechanisms for presentation of CpG to target cells.

Discussion

During this experiment, anti-DNP antibody titer levels were confirmed to be high by ELISA. Based on the available results, the time to peak serum concentration for DNP-[^{3}H]-CpG nor the maximum serum level could not be concretely established since we do not have data at the 1 hour time point. However, noncompartmental analysis using the method of residuals was employed to determine whether flip-flop kinetics was present. Based on the results, [^{3}H]-CpG was shown to exhibit flip-flop kinetics in which the absorption phase,, represented by the declining portion of the concentration-time profile, is much slower than the elimination rate constant, represented by the ascending portion of the curve. This result is typical of extravascular administration, especially in cases involving depot injections such as SC administration. In contrast, the initial declining portion of the DNP-[^{3}H]-CpG data represents a rapid absorption while the flattened portion indicates slow elimination. As shown in the DNP-[^{3}H]-CpG profile, serum levels of DNP-[^{3}H]-CpG after two hours are maintained higher than [^{3}H]-CpG. This additionally supports the argument that antibodies can be used as systemic carriers to mediate ODN half-life in the circulation.[4] Analysis of the DNP-[^{3}H]-CpG data is also supportive of the above conclusion since the elimination half-life was over 700 hours which is much higher than that of [^{3}H]-CpG.

Detection of [^{3}H]-CpG in the kidney and liver agrees with the typical renal and hepatic clearance observed for many synthetic ODNs.[15] Since DNP-[^{3}H]-CpG is assumed to exist as an IC, it is unlikely that the levels found in the kidney and liver are due to the same clearance mechanisms but rather Fc receptor-mediated association with glomerular epithelial cells[16,17] or Kupffer cells.[18-20] Accumulation in the spleen is likely due to ODN uptake by plasmacytoid DCs.[21] Negligible accumulation of [^{3}H]-CpG in the tumor tissue was expected

and coincided with the high radioactivity levels in the liver and kidneys. This is consistent with results from IV administration.[4] However, the remarkable observation that DNP-[^{3}H]-CpG did not accumulate in the tumor greatly differs from IV data. This may be a result of multiple reasons. Intravenously administered DNP-CpG is 100% bioavailable to circulation and a higher concentration has immediate access to the tumor periphery. DNP-CpG administered subcutaneously exhibits lower serum concentrations due to a depot effect at the injection site thereby limiting serum concentration and the extent of tumor accumulation. Tumor accumulation is also restricted since DNP-CpG and its IC with anti-DNP will be first subject to lymphatic drainage and transport before reaching the circulation[22]. As a result, it could well be immediately processed by DCs found in the lymph nodes.[23] Ultimately, the amount of DNP-CpG that does reach the circulation may be low such that a high percentage will be processed by blood DCs. In fact, these outcomes may be beneficial to CpG delivery since a clinical regimen can potentially be established to use a lower dose and lower dosing frequency. Based on the accumulation results, it can be proposed that, depending on the route of administration, the EPR effect may or may not be vital in the processing of CpG-containing immune complexes. In fact, the EPR effect may play a minimal, if any, role in anti-cancer effects upon SC administration. More discussion on this subject can be found in Chapter III.

Conclusions

The similarities in the pharmacokinetic and biodistribution profiles of [^{3}H]-CpG and DNP-[^{3}H]-CpG suggest that both may also demonstrate similar anti-tumor efficacy. However, pharmacokinetic analysis of serum data indicates that DNP-CpG would have

greater anti-tumor efficacy. Based on these findings, subcutaneous delivery of DNP-CpG in pre-immunized hosts exhibits potential in anti-cancer therapy. In one respect, however, low tumor accumulation may hinder DC-mediated anti-tumor effects if the EPR effect plays a major role in CpG delivery. Although the EPR effect may play a role in the intravenous administration of DNP-CpG, distribution of DNP-CpG to the tumor was minimal upon subcutaneous administration and it is unlikely that the EPR effect offers a significant contribution to CpG delivery and subsequent anti-tumor effects. For SC administration, delivery to the lymphatics and also sustained serum levels may prove to be substantial in activating anti-tumor immune responses via DCs not residing in the tumor locale. Further studies focused on biodistribution of CpG ODNs to the draining lymph nodes will help to establish a mechanism of action. Current observations suggest that CpG delivery could involve two distinct physiological mechanisms for delivery and processing depending on the route of administration.

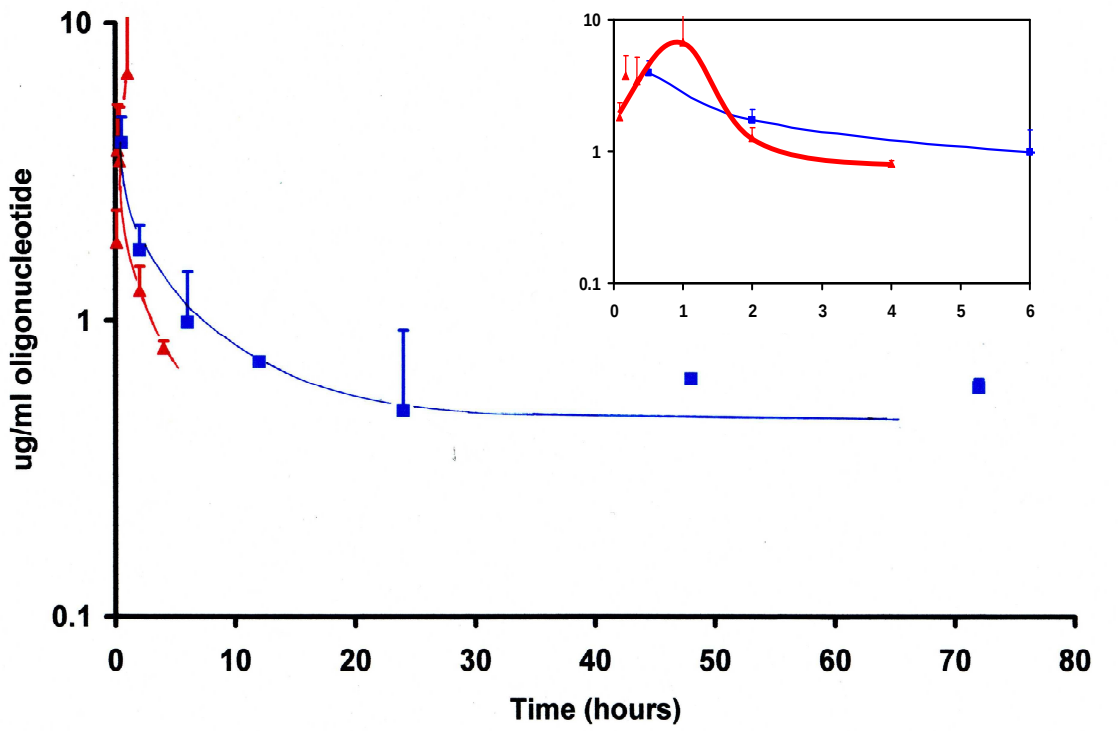

Figure 2.1. Serum pharmacokinetics of free CpG and DNP-CpG after a single subcutaneous injection. Oligonucleotides are tritium labeled. Red is free CpG. Blue is DNP-CpG. All mice were immunized against DNP. Data are presented on a log-linear scale. The inset depicts PK profiles from 0 to 6 hours. Concentrations are expressed as the mean and bars represent the standard deviation (SD). n = 4 mice per time point.

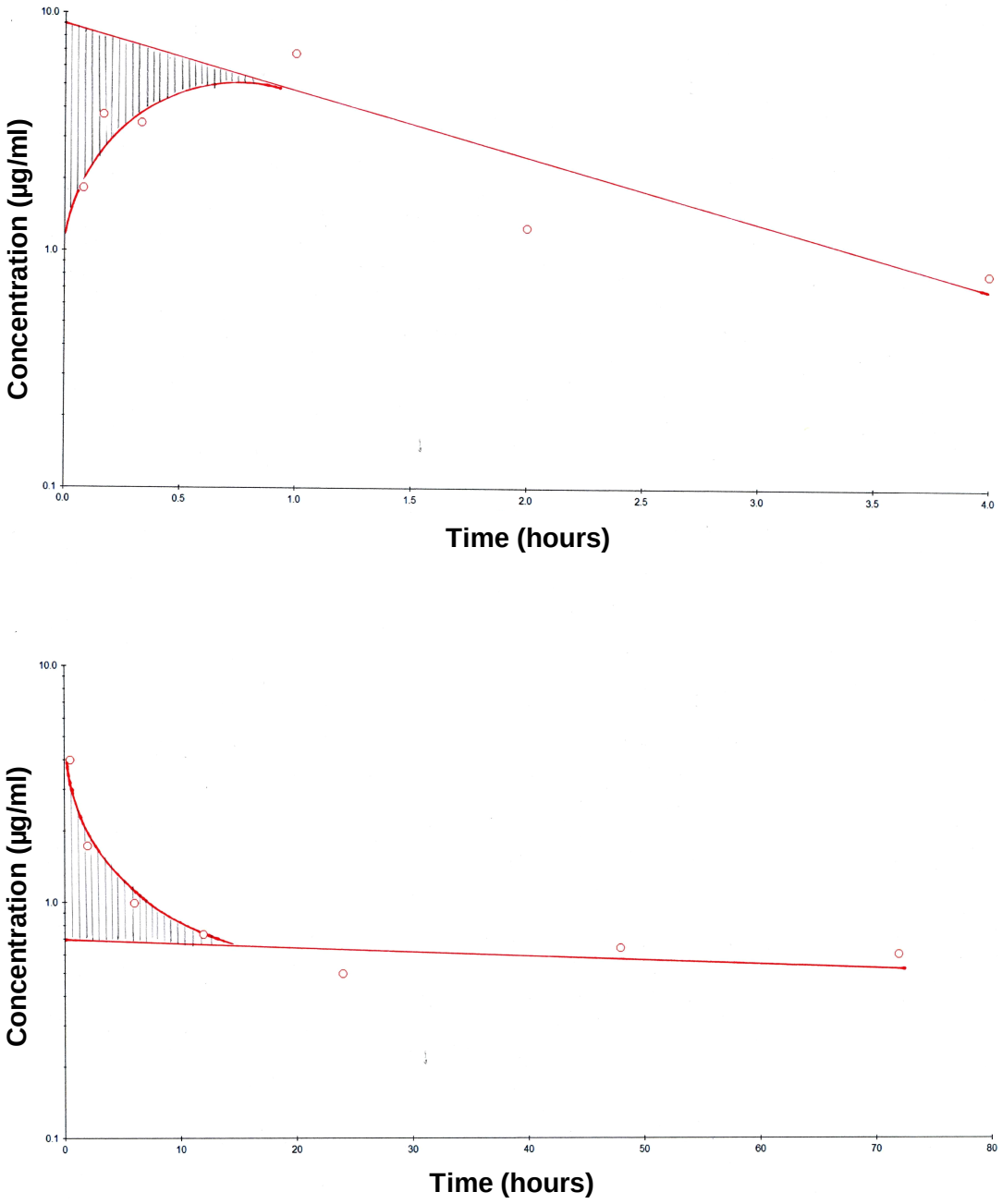

Figure 2.2. Noncompartmental analysis of serum pharmacokinetic data for [^{3}H]-CpG and DNP-[^{3}H]-CpG. The upper plot is [^{3}H]-CpG and the lower plot is DNP-[^{3}H]-CpG. Elimination and absorption rate constants were obtained, respectively, via the method of residuals using the shaded area which was determined based on the best-fit predicted line fit to the raw data (red circles).

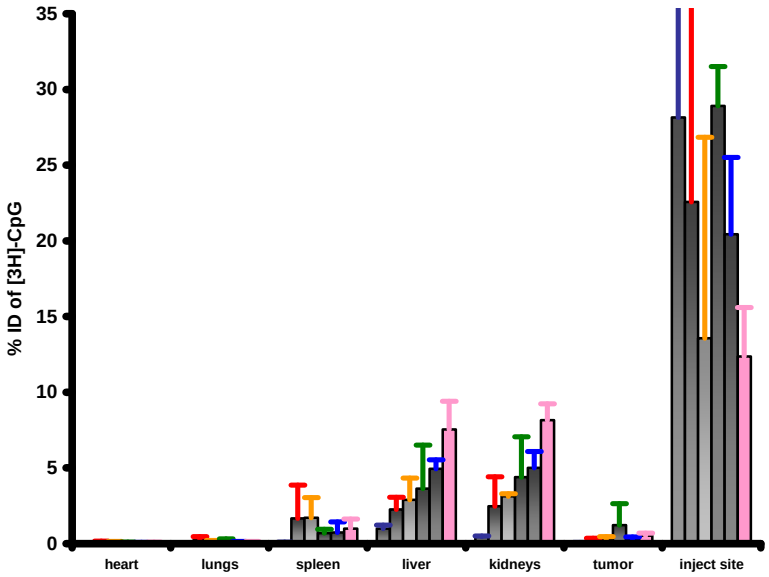

Figure 2.3. Biodistribution of free CpG after a single subcutaneous dose. Tumor-bearing mice were pre-immunized against DNP and anti-DNP antibody titer was confirmed to be high during the study. The colored bars represent the average percent of the total injected dose at given time points after the given dose ± SD. Violet, Red, Orange, Green, Blue, and Pink represent 5, 10, 20, 60, 120, and 240 minutes, respectively. n = 3 mice per time point. As expected, a large amount was retained at the injection site and a very trivial amount accumulated in tumor tissue.

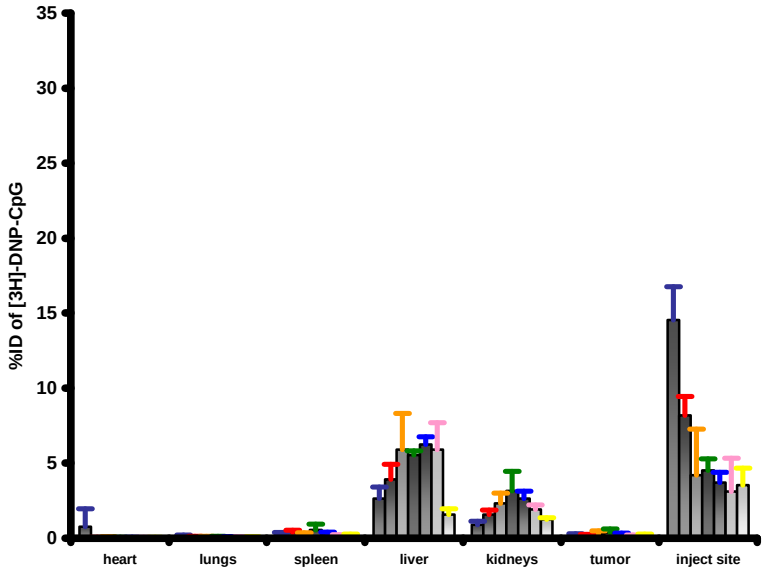

Figure 2.4. Biodistribution of DNP-CpG after a single subcutaneous dose. Tumor-bearing mice were pre-immunized against DNP and anti-DNP antibody titer was confirmed to be high during the study. The colored bars represent the average percent of the total injected dose at given time points after the given dose ± SD. Violet, Red, Orange, Green, Blue, and Pink represent 0.5, 2, 6, 12, 24, 48, 72 hours, respectively. n = 3 mice per time point.

References

(1) Krieg, A. M. Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. *Curr. Oncol. Rep.* **2004**, *6*, 88-95.

(2) Dittmer, U.; Olbrich, A. R. M. Treatment of infectious diseases with immunostimulatory oligodeoxynucleotides containing CpG motifs. *Curr. Opin. Microbiol.* **2003**, *6*, 472-477.

(3) Heckelsmiller, K.; Rall, K.; Beck, S.; Schlamp, A.; Seiderer, J.; Jahrsdörfer, B.; Krug, A.; Rothenfusser, S.; Endres, S.; Hartmann, G. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.* **2002**, *169*, 3892-3899.

(4) Palma, E.; Cho, M. J. Improved systemic pharmacokinetics, biodistribution, and antitumor activity of CpG oligodeoxynucleotides complexed to endogenous antibodies in vivo. *J. Control. Release* **2007**, *120*, 95-103.

(5) Noll, B. O.; McCluskie, M. J.; Sniatala, T.; Lohner, A.; Yuill, S.; Krieg, A. M.; Schetter, C.; Davis, H. L.; Uhlmann, E. Biodistribution and metabolism of immunostimulatory oligodeoxynucleotide CPG 7909 in mouse and rat tissues following subcutaneous administration. *Biochem. Pharmacol.* **2005**, *69*, 981-991.

(6) Krieg, A. M.; Efler, S. M.; Wittpoth, M.; Al Adhami, M. J.; Davis, H. L. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J. Immunother.* **2004**, *27*, 460-471.

(7) Srinivasan, S. K.; Iversen, P. Review of in vivo pharmacokinetics and toxicology of phosphorothioate oligonucleotides. *J. Clin. Lab. Anal* **1995**, *9*, 129-137.

(8) Geary, R. S.; Leeds, J. M.; Henry, S. P.; Monteith, D. K.; Levin, A. A. Antisense oligonucleotide inhibitors for the treatment of cancer: 1. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. *Anticancer Drug Des.* **1997**, *12*, 383-393.

(9) Stein, C. A.; Benimetskaya, L.; Mani, S. Antisense strategies for oncogene inactivation. *Semin. Oncol* **2005**, *32*, 563-572.

(10) Ghetie, V.; Popov, S.; Borvak, J.; Radu, C.; Matesoi, D.; Medesan, C.; Ober, R. J.; Ward, E. S. Increasing the serum persistence of an IgG fragment by random mutagenesis. *Nat. Biotechnol.* **1997**, *15*, 637-640.

(11) Chaudhury, C.; Mehnaz, S.; Robinson, J. M.; Hayton, W. L.; Pearl, D. K.; Roopenian, D. C.; Anderson, C. L. The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. *J. Exp. Med.* **2003**, *197*, 315-322.

(12) Bazin-Redureau, M. I.; Renard, C. B.; Scherrmann, J. M. Pharmacokinetics of heterologous and homologous immunoglobulin G, F(ab')2 and Fab after intravenous administration in the rat. *J. Pharm. Pharmacol.* **1997**, *49*, 277-281.

(13) Graham, M. J.; Freier, S. M.; Crooke, R. M.; Ecker, D. J.; Maslova, R. N.; Lesnik, E. A. Tritium labeling of antisense oligonucleotides by exchange with tritiated water. *Nucleic Acids Res.* **1993**, *21*, 3737-3743.

(14) Wilson, K. D.; Raney, S. G.; Sekirov, L.; Chikh, G.; deJong, S. D.; Cullis, P. R.; Tam, Y. K. Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles. *Int. Immunopharmacol.* **2007**, *7*, 1064-1075.

(15) Crooke, S. T.; Graham, M. J.; Zuckerman, J. E.; Brooks, D.; Conklin, B. S.; Cummins, L. L.; Greig, M. J.; Guinosso, C. J.; Kornbrust, D.; Manoharan, M.; Sasmor, H. M.; Schleich, T.; Tivel, K. L.; Griffey, R. H. Pharmacokinetic properties of several novel oligonucleotide analogs in mice. *J. Pharmacol. Exp. Ther* **1996**, *277*, 923-937.

(16) Haymann, J. P.; Levraud, J. P.; Bouet, S.; Kappes, V.; Hagège, J.; Nguyen, G.; Xu, Y.; Rondeau, E.; Sraer, J. D. Characterization and localization of the neonatal Fc receptor in adult human kidney. *J. Am. Soc. Nephrol.* **2000**, *11*, 632-639.

(17) Kobayashi, N.; Suzuki, Y.; Tsuge, T.; Okumura, K.; Ra, C.; Tomino, Y. FcRn-mediated transcytosis of immunoglobulin G in human renal proximal tubular epithelial cells. *Am. J. Physiol. Renal Physiol.* **2002**, *282*, F358-365.

(18) Løvdal, T.; Andersen, E.; Brech, A.; Berg, T. Fc receptor mediated endocytosis of small soluble immunoglobulin G immune complexes in Kupffer and endothelial cells from rat liver. *J. Cell. Sci.* **2000**, *113 (Pt 18)*, 3255-3266.

(19) Sancho, J.; González, E.; Escanero, J. F.; Egido, J. Binding kinetics of monomeric and aggregated IgG to Kupffer cells and hepatocytes of mice. *Immunology* **1984**, *53*, 283-289.

(20) Johansson, A. G.; Sundqvist, T.; Skogh, T. IgG immune complex binding to and activation of liver cells. An in vitro study with IgG immune complexes, Kupffer cells, sinusoidal endothelial cells and hepatocytes. *Int. Arch. Allergy Immunol.* **2000**, *121*, 329-336.

(21) Chen, L.; Arora, M.; Yarlagadda, M.; Oriss, T. B.; Krishnamoorthy, N.; Ray, A.; Ray, P. Distinct responses of lung and spleen dendritic cells to the TLR9 agonist CpG oligodeoxynucleotide. *J. Immunol.* **2006**, *177*, 2373-2383.

(22) Porter, C. J.; Charman, S. A. Lymphatic transport of proteins after subcutaneous administration. *J. Pharm. Sci.* **2000**, *89*, 297-310.

(23) McLellan, A. D.; Kapp, M.; Eggert, A.; Linden, C.; Bommhardt, U.; Bröcker, E.; Kämmerer, U.; Kämpgen, E. Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression. *Blood* **2002**, *99*, 2084-2093.

CHAPTER III

ANTI-TUMOR ACTIVITY OF CpG OLIGODEOXYNUCLEOTIDES UPON INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION

Abstract

Routes of administration have been a limiting factor for CpG oligodeoxynucleotide (ODN) monotherapy for tumor growth inhibition and only recently has CpG monotherapy been shown to be effective in a murine tumor model after intravenous (IV) delivery. Success has been attributed to utilization of IgG as an endogenous drug carrier for delivery of CpG ODNs to dendritic cells, especially those around tumor locales. The subcutaneous (SC) route has shown great potential, but current studies have shown poor efficacy when CpG therapy is given without additional intervention. In the following studies, we compared the anti-tumor effects between SC and IV administration of dinitrophenylated CpG (DNP-CpG) in DNP-immunized BALB/c mice bearing subcutaneous CT26 colorectal tumors. Secondly, we compared anti-tumor efficacy between free, non-haptenized CpG and DNP-CpG in the same animal model. Subcutaneous administration of DNP-CpG was highly effective in tumor inhibition and showed equivalent response to intravenous administration when anti-DNP titer was high. Increased dosing intervals resulted in tumor regression. However, loss of therapeutic efficacy was evident when antibody titer was low. Free CpG failed to show comparable anti-tumor activity when administered subcutaneously. In all cases, DNP-ss, a DNP derivative of an ODN which has an identical nucleotide composition as CpG 1826 but has a scrambled and non-immunostimulatory sequence, failed to exhibit any anti-tumor activity. Collectively, these results show the potential of the subcutaneous route of CpG ODN administration for anti-cancer monotherapy by means of *in situ* formation of immune complexes *in vivo*.

Introduction

To date, CpG ODNs used in monotherapy for tumor control suffer from lack of an effective route of administration. Although intra- and peri-tumoral injections have been effective in murine models,[1] these routes limit the use of CpG ODNs in humans to only specific types of cancer. Intravenous administration is often used because the drug achieves 100% bioavailability such that maximum absorption and distribution to target site is ensured. However, IV delivery of any ODN results in rapid clearance from the circulation to the clearing organs, particularly the liver and kidneys.[2] Intravenous dosing also subjects ODNs to dilution and non-specific binding to serum proteins, especially those with a phosphorothioate backbone.[3] Only recently, upon use of an endogenous protein carrier, have we shown that CpG can be effective in anti-cancer therapy after IV administration.[4] Our novel drug delivery strategy utilizes a chemical conjugate of CpG to a 2,4-dinitrophenyl (DNP) hapten such that it forms a monovalent immune complex (IC) with an anti-DNP IgG antibody in animals which are pre-immunized against DNP.

Since a substantial amount of endogenous IgG is found in the interstitial tissue in dynamic equilibrium with plasma, approximately at 25% of the plasma concentration[5], we reasoned that a significant amount of SC dose of DNP-CpG would exist as IC at the injection site in the tissue. These complexes will be drained to lymphatics and eventually discharged to general circulation where a high anti-DNP titer is available for further IC formation. Due to the failed attempts of intravenous free CpG ODNs delivery, the SC route of administration has been previously studied although not established for monotherapy.[6-8] Subcutaneous administration offers many practical benefits. Intravenous and intramuscular (IM) injections require skilled personnel whereas SC injections can be easily self-administered by a patient,

painless, and administered at many different sites on the body. In fact, many drugs such as morphine, insulin, and heparin have been highly successful through SC administration. The SC route is very promising especially since it allows for slow release from the injection site and for accumulation in the lymph nodes where many DCs are located.

Development of tumor vaccines using CpG ODNs monotherapy has not been successful although use of CpG ODNs as adjuvants[9-11] or in conjunction with additional therapies show clinical promise.[12,13] In exactly the same murine tumor model as ours, CpG failed to elicit anti-tumor activity after subcutaneous administration.[1] In the following studies, we utilize our novel delivery strategy in two series of experiments to compare the effectiveness between SC and IV administration for CpG monotherapy and to determine whether SC administration of DNP-CpG exhibits improved therapeutic response over free CpG in mice which were pre-immunized with DNP.

Experimental Procedures

General Procedures and Materials

All reagents were purchased from commercial sources and were used without further purification unless otherwise noted. For each experiment, 30 female BALB/c mice, five weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the UNC Eshelman School of Pharmacy Kerr Hall Animal Facility according to NIH guidelines. All procedures were approved by the UNC Institutional Animal Care and Use Committee. Molecular biology-grade water, phosphate buffered saline (PBS), sodium carbonate, thimerosal, complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA) were purchased from Sigma-Aldrich (St. Louis, MO). Hammersten-grade casein was

purchased from Research Organics (Cleveland, OH). Dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) and dinitrophenylated bovine serum albumin (DNP-BSA) were purchased from Calbiochem (San Diego, CA). Critoseal, non-heparanized microcapillary tubes, Nunc MaxiSorp 96-well flat-bottom plates, and Tween 20 were purchased from Thermo Fisher Scientific (Waltham, MA). *O*-phenylenediamine dihydrochloride (OPD) and Stable Peroxidase Substrate Buffer, and were purchased from Pierce (Rockford, IL). Goat anti-mouse IgG-peroxidase conjugate was purchased from Southern Biotech (Birmingham, AL). BALB/c-derived colon carcinoma CT26 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) with low (1 g/L) glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell culture reagents, including trypsin-EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Cell culture vented flasks and conical tubes were purchased from Corning, Inc. (Corning, NY). CpG 1826 (free CpG), dinitrophenylated CpG 1826 (DNP-CpG), and control DNP-ODN 1982 (DNP-ss) were synthesized with a phosphorothioate backbone and provided in lyophilized form by Dr. Rowshon Alam of the Nucleic Acid Core Facility of the Program Project Grant in Pharmacodynamics of Genes and Oligonucleotides at the University of North Carolina at Chapel Hill. ODNs were diluted in PBS before use.

Vaccine Preparation and Active Immunization

To prepare the primary vaccine, 20 mg of DNP-KLH was dissolved in 10 ml of endotoxin-free water to give a 2 mg/ml solution. Prior to vaccination, an equal volume of CFA was added. The mixture was emulsified by vortexing at maximum rpm for 15 minutes

using a multi-tube vortexer (VWR, West Chester, PA). Secondary vaccines were prepared similarly, but replacing CPA with IFA.

Thirty BALB/c mice were immunized intraperitoneally with 200 μl of freshly prepared primary vaccine. A booster injection was given two weeks later using 200 μl of the secondary vaccine. In the first pharmacodynamic experiment, a second booster injection was given five weeks after the first booster to maintain a high antibody titer.

Antibody Titer

In each experiment, four mice were selected for antibody titer bleeds and the same four mice were used throughout the entire duration of the experiment. Samples were collected before immunization to establish background antibody levels and were then collected every two weeks. Fifty-microliter blood samples were collected into non-heparanzied microcapillary tubes by tail clip. Blood samples were allowed to clot at room temperature (RT) for up to 1 hour and were then centrifuged at 12,700 x g for five minutes using an IECMicro-MB Centrifuge (Thermo Fisher Scientific, Waltham, MA). Serum fractions were collected and diluted 10-fold in PBS, pH 7.4. Anti-DNP IgG titers were determined by the enzyme-linked immunosorbent assay (ELISA). Nunc MaxiSorp 96-well plates were coated with 125 μl per well of a 10 μg/ml DNP-BSA solution in 0.1 M sodium carbonate buffer, pH 9.6 and incubated overnight at 4^{o}C. Wells were washed in triplicate with 250 μl per well of PBS containing 0.1% v/v Tween 20 (PBS-T washing buffer) and then blocked with a mixture of 0.5% w/v casein and 0.02% w/v thimerosal in PBS, pH 7.4 (blocking buffer) for two hours at RT. Wells were then washed again in triplicate and incubated with 50 μl of serially diluted serum samples and incubated for 2 hours at RT.

Serum samples were serially diluted from $5x10^2$ to 10^6 at half-log increments in PBS. After a triplicate wash, wells were incubated with 125 μl of goat anti-mouse IgG-peroxidase conjugate diluted in PBS for two hours at RT. Wells were washed in triplicate and incubated with 125 μl per well of a 1 mg/ml OPD solution in Stable Peroxidase Substrate Buffer for 20 minutes at room temperature. The colorimetric reaction was terminated by the addition of 125 μl per well of 1.8 N sulfuric acid. Absorbance at 492 nm was measured on a Bio-Rad 3550 Microplate Reader. Best-fit sigmoidal curves were obtained from plotting absorbances vs. log dilution factors using GraphPad Prism 4 (La Jolla, CA). Titer levels were obtained as EC_{50} values from the midpoint of each sigmoidal curve.

Tumor Inoculation, Tumor Measurements and Dosing Schedule

Four to five weeks after primary vaccination, DNP-immunized mice were inoculated with $2.5x10^5$ CT26 cells suspended in 100 μl HBSS into the right flank. Tumor growth was monitored daily. Measurements were performed using an electronic digital caliper. Tumor volumes were calculated based on the formula for an ellipsoid: length x $width^2$ x $\pi/6$. Treatments began nine days after inoculation when tumor sizes were between 75 to 150 mm^3.

In the first experiment, 24 mice were divided evenly into three test groups: DNP-ss via IV (negative control), DNP-CpG via IV (positive control), and DNP-CpG via SC (test). Here, DNP-ss is a scrambled nucleotide sequence of composition identical to that of CpG 1826 and is known as 1982 in the literature.[14] Mice dosed intravenously were given a 108 μg bolus dose of the treatment in 100 μl of PBS into the tail vein. Mice dosed subcutaneously were given a 108 μg dose of the treatment into the left flank (contralateral to the tumor). This quantity is equivalent to 100 μg of un-derivatized CpG. Three doses were given in

seven-day intervals followed by three additional doses at three-day intervals for a total of six doses. Tumors were monitored daily and measured every two or three days. Mice were euthanized when tumors exceeded 2.0 cm in any dimension.

In the second series of experiments, 29 mice were divided into three test groups: DNP-ss (negative control, n=9), free CpG (test, n=10), and DNP-CpG (test, n=10). Five 108-μg doses in 100 μl of PBS were administered subcutaneously into the left flank of each mouse every five days. Tumor monitoring, measurements, and animal euthanasia were as described above.

Statistical Analysis

Using Van der Waerden normal scores, a nonparametric pair-wise comparison test was performed to determine if there were statistical differences in tumor volumes between each group at each day. Statistical analyses were performed with the SAS statistical software by Dominic T. Moore of the UNC Lineberger Comprehensive Cancer Center Biostatistics and Data Management.

Results

In the first of two series of animal experiments, high anti-DNP antibody titer levels were established before initiation of treatments and persisted at least up to the third dose (Figure 3.1). Over a six-week period subsequent to the first booster injection, the titer continuously increased. This was in agreement with our previous finding (E. Palma, "Antibody-Mediated Systemic Delivery of CpG Oligonucleotides for Solid Tumor Immunotherapy," PhD Dissertation submitted to the University of North Carolina, 2006).

Animals were inoculated with tumor cells while the anti-DNP titer was still increasing. Figure 3.2 shows that IV dosing of the DNP-haptenized non-immunostimulatory control ODN, DNP-ss, was unable to elicit any therapeutic response. As expected, tumor growth proceeded in an exponential-like manner when no therapeutic intervention was provided. However, IV administered DNP-CpG exhibited anti-tumor efficacy. Most notable is that SC administration of DNP-CpG was not only highly effective, it was comparable to the IV route and even resulted in almost complete tumor regression after only two doses. When tumor growth rate noticeably increased, dosing frequency was increased to every three days and once again partial tumor regression was achieved. Note that in this experiment, a third immunization was made to keep anti-DNP antibody titer high throughout the treatment schedule.

In a separate, second series of experiments, the second booster immunization was omitted. Figure 3.3 shows that anti-DNP antibody titers were high at the start of dosing, but started to fall between dose 2 and 5 since the second booster was omitted. The failure of the SC administration of DNP-ss to hinder tumor development, much like the failure of the IV administration, was as expected. This negative control group of mice was sacrificed earlier than the treatment groups following UNC IACUC guidelines since tumors reached a maximum allowable size and quickly became ulcerated. A notable inhibitory response, however, was seen with SC-administered free CpG. Nonetheless, the best response came from DNP-CpG treatments. Although inhibited by free CpG treatments, tumor growth still continued to steadily increase. In contrast, DNP-CpG was clearly able to induce partial regression at early time points, an observation consistent with the previous experiment. Tumor growth was also more variable, in favor of the DNP-CpG treatments, with occasional

durations of nearly complete tumor inhibition as can be seen by the almost plateau-like areas in Figure 3.3. However, when anti-DNP titers dropped substantially, DNP-CpG treatments became less effective at tumor growth inhibition. At this point, tumor growth rate was rapid and mirrored the growth rate with the free CpG treatment group.

Discussion

DNP-ss has the same nucleotide composition as the therapeutic CpG but the sequence is arranged such that no CpG motifs are present. Since DNP-ss has no immunostimulatory function, but are structurally similar to regular CpG, it was selected as a negative control as commonly found in the literature.[15,16] As shown in Figure 3.2, IV dosing of DNP-ss failed to prevent tumor growth although it may have circulated for a long period of time, confirming the literature.[1] The SC administration of DNP-ss also failed to elicit a response. Results from IV dosing of DNP-CpG in the first series of experiments are almost identical to the results we reported recently[4] and confirms the potential use of IV administration for CpG monotherapy in a clinical setting.

The ability of SC administration to achieve such similar effects may be attributed to multiple reasons. Since plasma antibody levels are normally in constant equilibrium with tissue antibody levels, it is reasonable to believe that an IC can be readily formed at or near the injection site. An SC injection is also a type of depot injection especially for macromolecules, which would allow for a prolonged and constant release of DNP-CpG over time. Thirdly, drainage of DNP-CpG from the injection site occurs via the lymphatic system. DNP-CpG will first encounter lymph nodes before reaching the systemic circulation. Therefore, DNP-CpG can be presented in a more direct manner to dendritic cells and

macrophages in the lymph node, which would initiate the adaptive immune response leading to anti-tumor activity via its endosomal TLR9 receptors. The effect exhibited by SC administration of free CpG can be explained by similar reasoning. In contrast to IV dosing, CpG injected via the SC route is not subject to immediate systemic elimination and has the opportunity to first reach the DC targets. The only difference is that free CpG can be readily taken up by dendritic cells via fluid-phase endocytosis for processing rather than by receptor-mediated means, a subject discussed in Chapter IV.

The importance of our antibody carrier system is evident in the second series of experiments where one booster immunization was omitted (Figure 3.3). A clear inverse correlation is observed between antibody titer and tumor volume. Partial tumor regression and higher inhibition of tumor growth rate attests to the enhanced anti-tumor effects of immune complexation over free CpG treatments, the implication being the importance of maintaining a high fraction of the oligonucleotide in the form of an immune complex. Statistical analysis indicates that tumor volumes were not statistically different when treatments began. Differences were deemed significant if *p*-values were less than 0.05. DNP-CpG already showed statistical significance at day 2 when compared to DNP-ss, but no statistical difference was seen with free CpG until day 7. Except for those days and at day 16, which was borderline significant, tumor volumes were statistically significant between DNP-CpG and free CpG.

Conclusions

Our antibody-based carrier system for CpG ODN has great potential for clinical use. Not only can the intravenous route of injection now be used for effective CpG treatment, the

subcutaneous route of administration is shown to be just as effective. However, because of the possibility of highly variable titer levels, this system must be further developed to avoid inter-individual variability and even intra-individual variability depending on progression of disease. Nevertheless, these results strongly support an approach to utilize pre-existing endogenous antibodies or to devise other development means such that pre-immunization of patients can be avoided completely. The ability of SC administration of DNP-CpG to perform as effectively as the IV route is promising for CpG development and is also remarkable for many reasons. The advantages of using a long-acting depot injection include increased cost-effectiveness, medication compliance due to reduction in the frequency of dosing, as well as more consistent serum concentrations.

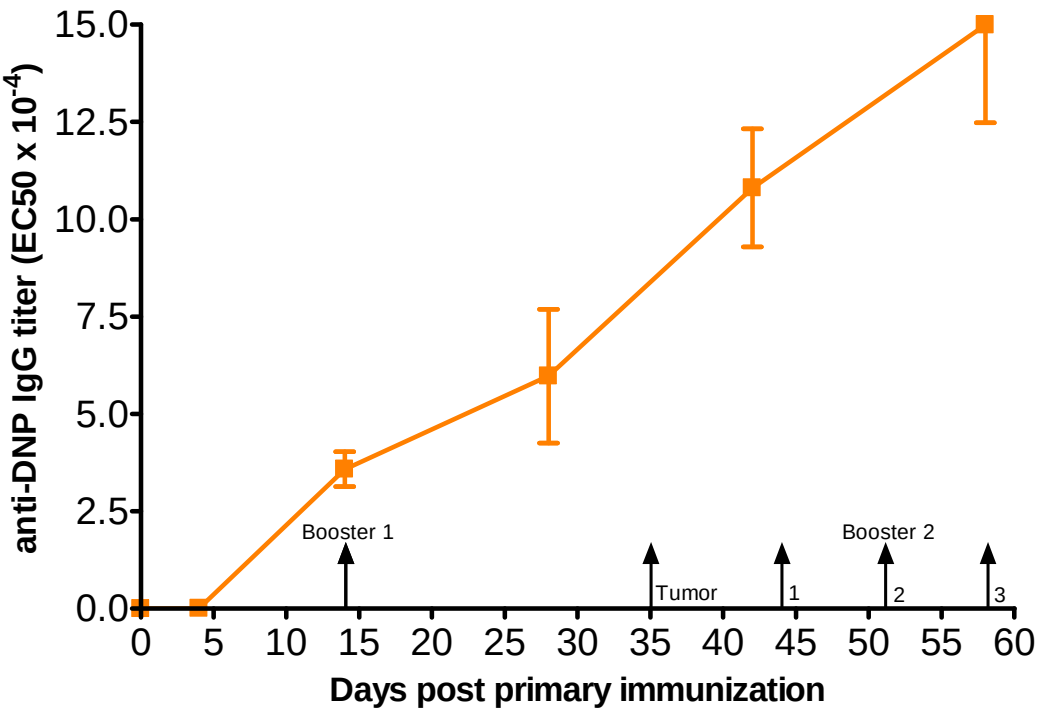

Figure 3.1. Anti-DNP antibody titer levels in BALB/c mice after immunization against DNP-KLH. A booster was given two weeks and seven weeks after the primary vaccination to maintain high antibody titer in order to ensure quantitative complexation of DNP-CpG to antibody. Titers are expressed as means ± SD. n=4.

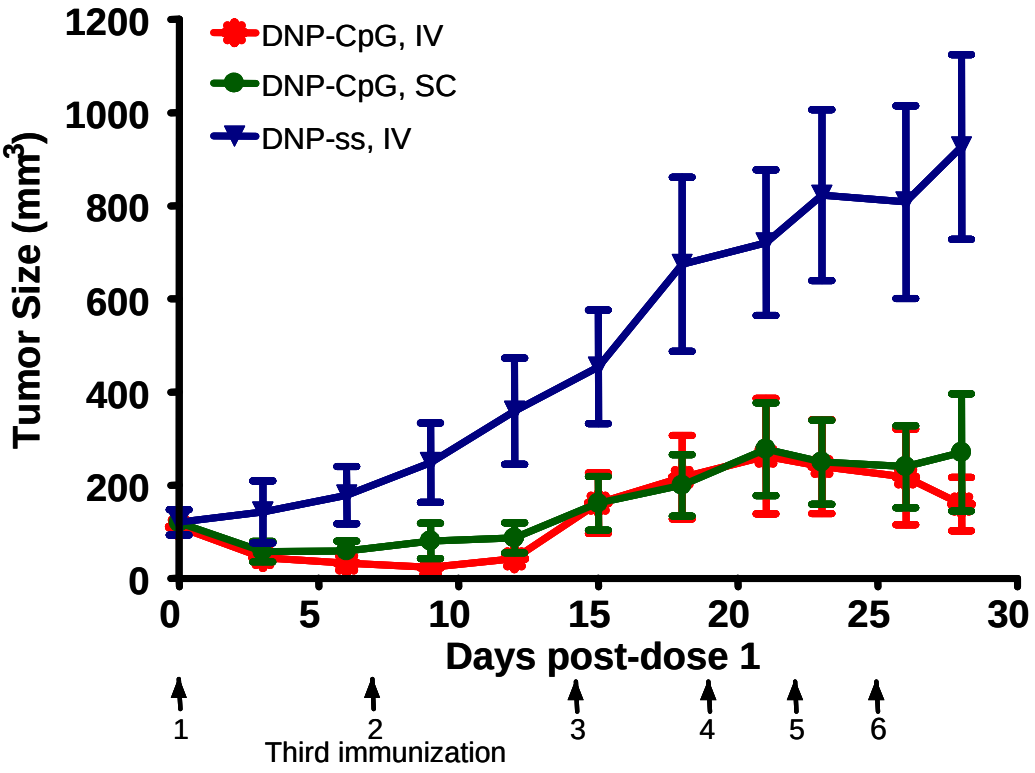

Figure 3.2. Comparison of tumor growth inhibition between subcutaneous and intravenous dosing of DNP-CpG in immunized mice. Tumor growth inhibition by intravenous DNP-ss (negative control, n=10), intravenous DNP-CpG (n=10), and subcutaneous DNP-CpG (n=10) administration in DNP-immunized, CT26 tumor-bearing mice. Tumor volumes were plotted against time in days after administration of the first dose. Data are expressed as means ± SD.

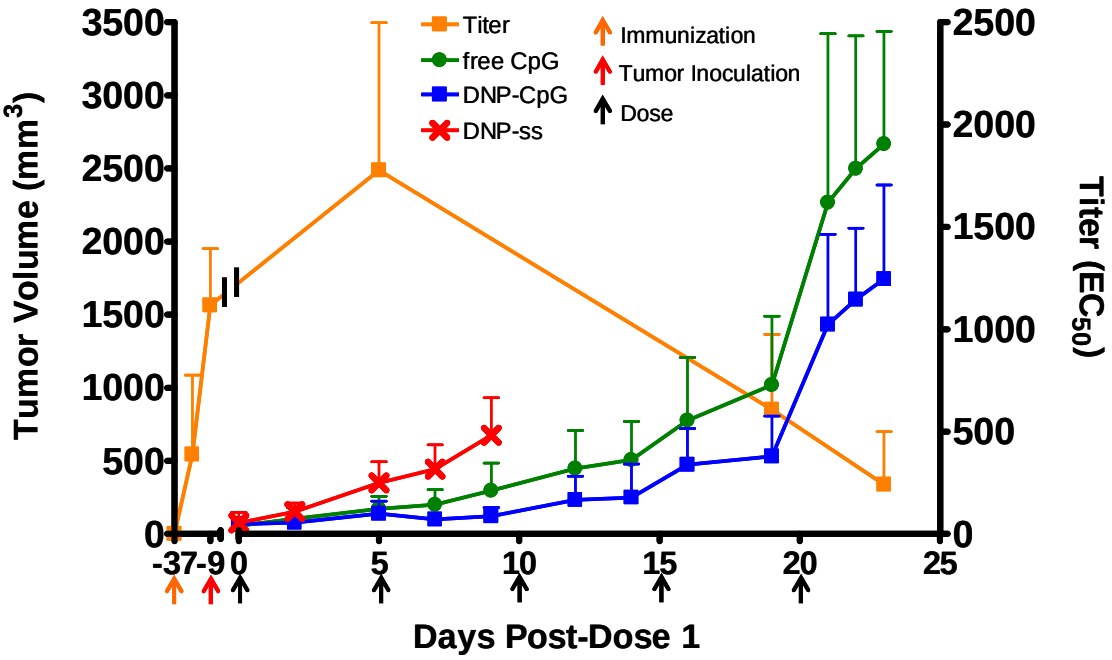

Figure 3.3. Comparison of tumor growth inhibition between free CpG and DNP-CpG during subcutaneous dosing in immunized mice. Tumor growth inhibition by subcutaneous administration of DNP-ss (negative control, n=9), free CpG (n=10), and DNP-CpG (n=10) in DNP-immunized, CT26 tumor-bearing mice. Tumor volumes were plotted against time in days after administration of the first dose. Data are expressed as means ± SD. Anti-DNP IgG titer measurements are overlaid and expressed as means ± SD.

References

(1) Heckelsmiller, K.; Rall, K.; Beck, S.; Schlamp, A.; Seiderer, J.; Jahrsdörfer, B.; Krug, A.; Rothenfusser, S.; Endres, S.; Hartmann, G. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.* **2002**, *169*, 3892-3899.

(2) Rifai, A.; Brysch, W.; Fadden, K.; Clark, J.; Schlingensiepen, K. H. Clearance kinetics, biodistribution, and organ saturability of phosphorothioate oligodeoxynucleotides in mice. *Am. J. Pathol.* **1996**, *149*, 717-725.

(3) Srinivasan, S. K.; Tewary, H. K.; Iversen, P. L. Characterization of binding sites, extent of binding, and drug interactions of oligonucleotides with albumin. *Antisense Res. Dev* **1995**, *5*, 131-139.

(4) Palma, E.; Cho, M. J. Improved systemic pharmacokinetics, biodistribution, and antitumor activity of CpG oligodeoxynucleotides complexed to endogenous antibodies in vivo. *J. Control. Release* **2007**, *120*, 95-103.

(5) Bazin-Redureau, M. I.; Renard, C. B.; Scherrmann, J. M. Pharmacokinetics of heterologous and homologous immunoglobulin G, F(ab')2 and Fab after intravenous administration in the rat. *J. Pharm. Pharmacol.* **1997**, *49*, 277-281.

(6) Krieg, A. M.; Efler, S. M.; Wittpoth, M.; Al Adhami, M. J.; Davis, H. L. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J. Immunother.* **2004**, *27*, 460-471.

(7) Murad, Y. M.; Clay, T. M.; Lyerly, H. K.; Morse, M. A. CPG-7909 (PF-3512676, ProMune): toll-like receptor-9 agonist in cancer therapy. *Expert Opin. Biol. Ther.* **2007**, *7*, 1257-1266.

(8) Wilson, K. D.; Raney, S. G.; Sekirov, L.; Chikh, G.; deJong, S. D.; Cullis, P. R.; Tam, Y. K. Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles. *Int. Immunopharmacol.* **2007**, *7*, 1064-1075.

(9) Chikh, G.; de Jong, S. D.; Sekirov, L.; Raney, S. G.; Kazem, M.; Wilson, K. D.; Cullis, P. R.; Dutz, J. P.; Tam, Y. K. Synthetic methylated CpG ODNs are potent in vivo adjuvants when delivered in liposomal nanoparticles. *Int. Immunol.* **2009**, *21*, 757-767.

(10) Salem, A. K.; Weiner, G. J. CpG oligonucleotides as immunotherapeutic adjuvants: innovative applications and delivery strategies. *Adv. Drug Deliv. Rev.* **2009**, *61*, 193-194.

(11) Chaung, H. CpG oligodeoxynucleotides as DNA adjuvants in vertebrates and their applications in immunotherapy. *Int. Immunopharmacol.* **2006**, *6*, 1586-1596.

(12) Kim, H.; Ko, H.; Ju, H.; Kim, K.; Roh, S.; Lee, H.; Im, S. CpG-ODN-based immunotherapy is effective in controlling the growth of metastasized tumor cells. *Cancer Lett.* **2009**, *274*, 160-164.

(13) Vollmer, J.; Krieg, A. M. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv. Drug Deliv. Rev.* **2009**, *61*, 195-204.

(14) Hartmann, G.; Weeratna, R. D.; Ballas, Z. K.; Payette, P.; Blackwell, S.; Suparto, I.; Rasmussen, W. L.; Waldschmidt, M.; Sajuthi, D.; Purcell, R. H.; Davis, H. L.; Krieg, A. M. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J. Immunol.* **2000**, *164*, 1617-1624.

(15) McCluskie, M. The potential of oligodeoxynucleotides as mucosal and parenteral adjuvants. *Vaccine* **2001**, *19*, 2657-2660.

(16) Utaisincharoen, P.; Kespichayawattana, W.; Anuntagool, N.; Chaisuriya, P.; Pichyangkul, S.; Krieg, A. M.; Sirisinha, S. CpG ODN enhances uptake of bacteria by mouse macrophages. *Clin. Exp. Immunol.* **2003**, *132*, 70-75.

CHAPTER IV

UPTAKE OF CpG OLIGOMERS BY DENDRITIC CELLS AND DENDRITIC CELL ACTIVATION

Abstract

Dendritic cells (DCs) are the most potent antigen-presenting cells involved in CpG oligodeoxynucleotide-based therapies. To enhance delivery of CpG to DCs, we have modified an immunostimulatory CpG through conjugation to a 2,4-dinitrophenyl (DNP) hapten such that it forms a monovalent immune complex (IC) with an anti-DNP IgG antibody. Our *in vivo* IC system has been effective in tumor growth inhibition in a murine model. We attributed the observed anti-tumor activity to the enhanced delivery of the monovalent IC to DCs via FcγRI-mediated endocytosis. In this study, DCs were treated with our IC and with free CpG to compare the extent of CpG uptake and the extent of dendritic cell activation using flow cytometry. We showed that uptake of free CpG was greater than immune-complexed CpG. However, expression of DC activation markers was greater with IC treatment. Inhibition of Fcγ receptors resulted in expression levels comparable for both IC and free CpG treatments. Taken together, the data appear to support the importance of FcγRI in the receptor-mediated uptake and processing of CpG immune complexes for the effective enhancement of DC activation.

Introduction

Plasmacytoid dendritic cells (pDCs), a subset of immature DCs, and B cells are the only human immune cells known to constitutively express TLR9.[1] When activated by CpG oligodeoxynucleotides (ODNs), pDCs excrete cytokines and chemokines which activate cells of the innate immune system which can confer direct anti-tumor effects.[2-4] DC stimulation also results in increased expression of co-stimulatory molecules such as CD80 and CD86 to signal proliferation of T-cells, particularly type 1 helper T cells (T_H1). In the presence of

tumor-associated antigens, DCs can process these antigens for major histocompatibility complex (MHC) presentation to T cells. Both the co-stimulatory and MHC signals are required for successful activation of T cells.[5] Suggestive of DC involvement in CpG therapy, intralymphatic administration considerably improved therapeutic response to CpGs[6] while another study was able to treat large murine tumors using combined DC and CpG therapy.[7] Still others have attempted to use receptor-mediated delivery of antigens to DCs for anticancer applications.[8] These findings warrant the use of CpG ODN for DC activation, ultimately resulting in T cell effector function,[9] in cancer immunotherapy. As such, delivery of CpG to DCs would benefit greatly using a drug delivery system.

A major concern with current synthetic delivery systems, especially based on macromolecular assemblies, is the inherent immunogenicity.[10] Even protein-based therapeutic ICs and antibodies can elicit immune responses and attempts have been made to minimize their immunogenicity.[11] The ideal drug delivery system must not only carry intact cargo to the target location but also avoid activating the immune system or undesired neutralization that leads to elimination from the body. Herein lies the enormous potential of endogenous antibodies in CpG and general delivery. Furthermore, DCs not only express TLR9 receptors, they also express cell-surface Fc receptors such as FcγR which bind the Fc domain of immunoglobulins. This implicates the potential for antibodies to be used for delivery to DCs.

We developed antibody-based drug delivery strategy in the treatment of solid tumors and explore the possible mechanistic effects involved. Our approach, shown to be highly effective, consists of IV bolus injection of CpG that was derivatized with 2,4-dinitrophenol (DNP) to tumor-bearing mice that had been pre-immunized against the DNP hapten.[12]

Furthermore, we will show later, contralateral subcutaneous (SC) injections of DNP-CpG were also found to be effective in the same mouse model.

Although immune complexes are known to enhance antigen presentation by DCs[13], cellular mechanisms of non-crosslinking, monovalent CpG immune complexes are unclear. One possible explanation of these successful outcomes was that we were able to provide facilitated uptake of ICs by DCs through the Fc receptor expressed on pDCs. We also propose that our ICs activate DCs more effectively than free CpG. This hypothesis arises because we expect ICs to enter the cell efficiently via FcγRI resulting in facilitated interactions with TLR-9 in the endosome. Here, we present some supportive evidence.

Experimental Procedures

General Procedures and Materials

All reagents were purchased from commercial sources and were used without further purification unless otherwise noted. C57BL/6 mice 6 to 8 weeks of age were purchased from Charles River Laboratories. All mice were housed in the UNC Eshelman School of Pharmacy Kerr Hall Animal Facility according to NIH guidelines. All procedures were approved by the UNC Institutional Animal Care and Use Committee. RPMI 1640 cell culture media was used for marrow harvesting. Dendritic cells were cultured in complete media comprised of RPMI 1640 supplemented with fetal bovine serum (FBS) (10% v/v), 1x antibiotic-antimycotic solution, 1x non-essential amino acid solution, 1000 U/ml recombinant mouse granulocyte macrophage-colony stimulating factor (rmGM-CSF), and 1000 U/ml recombinant mouse interleukin 4 (rmIL-4). All media components were from Invitrogen except for cytokines which were purchased from R&D Systems. Non-fluorescent DNP-CpG

1826 (DNP-CpG) and control ODN 1982 (DNP-scramble) were synthesized and provided lyophilized by Dr. Rowshon Alam of the Nucleic Acid Core Facility of the Program Project Grant in Pharmacodynamics of Genes and Oligonucleotides. Fluorescein isothiocyanate (FITC)-conjugated DNP-CpG was custom synthesized by Midland Certified Reagent Company (Midland, TX). ODNs were resuspended in molecular biology-grade water (Sigma-Aldrich) and concentrations were confirmed by UV spectrophotometry. Anti-DNP monoclonal IgG_1 was produced by the Tissue Culture Facility at the UNC Lineberger Cancer Center using 1B7.11 hybridoma cells and was purified before use. Purity of antibodies and oligonucleotides were confirmed by polyacrylamide gel electrophoresis. All test treatments were prepared using endotoxin-free, cell-biology grade water (Sigma-Aldrich). Lipopolysaccharide (LPS) and human serum albumin were purchased from Sigma-Aldrich. Mouse IgG_{2a} was purchased from Bethyl Laboratories (Montgomery, TX). Uptake and activation experiments were performed using a BD FACSCanto flow cytometer. Fluorophore-conjugated antibodies against DC surface markers and Fc Block, a rat anti-mouse IgG_{2b} monoclonal antibody which is used to block Fc receptors, were purchased from BD Biosciences (San Jose, CA).

Dendritic Cell Harvesting and Culture

Mouse bone marrow-derived DCs were harvested and cultured as described elsewhere[14] with some modifications. Briefly, femurs and tibias were collected from C57BL/6 mice, disinfected with 70% ethanol, washed twice with PBS, and stored in RPMI 1640. Marrow was flushed out of the bones, centrifuged at 300 x g for 5 minutes, and the supernatant was discarded. Cells were resuspended in ACK Lysis buffer (Invitrogen) by

vortexing to lyse all red blood cells followed by addition of RPMI 1640. Cells were centrifuged again and the supernatant discarded. Cells were resuspended in RPMI 1640, transferred to T-75 flasks, and incubated at 37^{o}C for 2 hours. Media was then discarded and cells were incubated with complete media. Complete media was used for subsequent media changes on day 2 and 4. On day 6, loosely adherent and suspended cells were harvested for experimental treatments. Cells were confirmed to be >95% CD11c-positive using a phycoerythrin (PE)-conjugated anti-CD11c antibody.

Dendritic Cell Uptake

Dendritic cells were transferred to a 96-well plate and seeded at a density of 2x10^{5} cells per well in 100 µl of complete media. Cells were incubated for 15-min, 30-min, 45-min, 1-hr, or 2-hr durations with either free FITC-DNP-CpG or FITC-DNP-CpG with anti-DNP mIgG$_1$. In addition, a series of cell samples were pre-incubated with 5 µl of Fc Block for 30 minutes at 4^{o}C prior to incubation with treatments. For treatments, cells were incubated in a 200 µl total volume with a 1 µg/ml equivalent to underivatized CpG. Of the total mass used, 50% was FITC-DNP-CpG and the rest was unlabeled DNP-CpG. After incubation, cells were transferred to a 5 ml Falcon tube (BD Biosciences, San Jose, CA) to which 10% v/v of 0.4% Trypan Blue solution was added to quench cell membrane-bound fluorescence. Unquenched non-fluorescent DNP-CpG was used as a negative control while unquenched FITC-DNP-CpG was used to determine maximum total fluorescence.

Dendritic Cell Activation

Dendritic cells were transferred to a 96-well plate and seeded at a density of $2x10^5$ cells per well per 100 μl complete media. Cells were incubated with a 200 μl total volume with the following final concentrations for 0, 6, 12, or 24 hours:

1. Complete media only
2. DNP-CpG (1 μg/ml relative to CpG)
3. DNP-scramble (1 μg/ml relative to CpG)
4. anti-DNP IgG (1 molar equivalent to DNP-CpG)
5. DNP-CpG + anti-DNP IgG (immune complex) 1:0.25 molar ratio
6. DNP-CpG + anti-DNP IgG (immune complex) 1:1 molar ratio
7. DNP-CpG + anti-DNP IgG (immune complex) 1:5 molar ratio
8. DNP-scramble + anti-DNP IgG 1:1 molar ratio
9. Fc Block then IC 1:1 molar ratio
10. Fc Block then DNP-scramble + anti-DNP IgG 1:1 molar ratio
11. Fc Block then media
12. DNP-CpG + Human Serum Albumin
13. Human Serum Albumin (based on mass equivalent of IgG from 1:1 IC)
14. DNP-CpG + Mouse IgG_{2a} 1:1 molar ratio
15. Mouse IgG_{2a} (1 molar equivalent to DNP-CpG)
16. LPS (0.1 μg/ml)

For IC formation, DNP-CpG was incubated with anti-DNP antibody for 1 hour at 37^oC. At the end of the incubation intervals, all samples were centrifuged at 300 x g for 5 minutes and the supernatant was decanted. Cells were washed with two 500 μl volumes of

FBS Stain Buffer (BD Biosciences) and resuspended in 200 μl of the buffer. Cells were then incubated with 5 μl of Fc Block for 30 minutes at 4^{o}C followed by addition of various fluorophore-conjugated antibodies against various DC surface activation markers. One series of cells was then incubated with 1 μl of FITC-conjugated anti-CD86 antibody, 1 μl of PE-conjugated anti-CD80 antibody, and 1 μl of allophycoerythrin-conjugated anti-CD40 antibody. Another series of cell samples were incubated with 1 μl of FITC-conjugated anti-CD86 antibody and 1 μl PE-conjugated anti-I-A^{b} (MHC II marker for the C57BL/6 mouse strain). Cells were allowed to incubate in the dark for 30 minutes at 4^{o}C followed by two 500 μl washes with buffer. Cells were finally resuspended in 500 μl of buffer for flow cytometry. In all cases, we ran isotype controls with rat $IgG_{2a,\kappa}$-FITC and rat $IgG_{1,\kappa}$-APC to establish the absence of non-specific staining of DCs in all of the treatments.

Results

Dendritic Cell Uptake

As shown in Figure 4.1, non-fluorescent DNP-CpG treatments resulted in no levels of fluorescence while unquenched FITC-DNP-CpG established maximum possible fluorescence levels for each incubation period. Cells incubated with FITC-DNP-CpG at 4^{o}C followed by quenching were used to establish baseline levels of intracellular fluorescence. Maximum uptake of FITC-DNP-CpG and FITC-IC occurred around 1 hour. FITC-DNP-CpG treatments exhibited the most intracellular fluorescence across all time points. Across all incubation durations, except for the 2-hour incubation, the FITC-IC also had high intracellular fluorescence although approximately 10% lower than FITC-DNP-CpG.

Incubation of cells with Fc Block prior to treatments resulted in drastic decreases in intracellular fluorescence levels for both FITC-DNP-CpG and FITC-IC treatments.

Dendritic Cell Activation

For activation studies, only CD86 activation results are shown in Figure 4.2, as they were also representative of those obtained for all aforementioned other markers. Since LPS is known as a potent activator of DCs and can fully activate DCs between 16 to 24 hours, it was used as a positive control. Cells incubated with media only had a maximum baseline activation level around 35%. Free CpG and free anti-DNP antibody can independently activate DCs, but the non-immunostimulatory ODN, DNP derivative of the scrambled sequence 1982, does not. Incubation of DNP-CpG with either albumin or IgG_{2a} only activate to free DNP-CpG levels. Pre-incubation of cells with Fc Block notably decreases DC activation levels equivalent or lower than free CpG levels. Most notable, however, is that our immune complex (IC) shows increased dendritic cell activation over free DNP-CpG. A 1:1 immune complex was found to be the most effective while a large excess of antibody displayed levels of activation similar to free CpG and free antibody.

Discussion

Dendritic Cell Uptake

FITC-DNP-CpG showed highest intracellular fluorescence across all time points (Figure 4.1). This is most likely due to the high endocytic capacity of dendritic cells.[15] Although slightly lower, FITC-IC also displayed great intracellular uptake. A lower intracellular fluorescence may be explained as its internalization could have been restricted to

FcγRI-mediated endocytosis. Lower IC fluorescence levels are not thought to be a result of complexation with the antibody, but this has yet to be confirmed. Nonetheless, the high intracellular fluorescence, especially after 1 hour, can attest to the efficient uptake of ICs. After two hours, intracellular levels of FITC-DNP-CpG and FITC-IC drop dramatically. Cellular processing and degradation of treatment compounds can be expected at this point. Increase in fluorescence of both Fc Block treatments at two hours appears to be also consistent with the possibility that the cells are likely eliminating Fc Block and restoring normal endocytic function. The decrease seen with FITC-DNP-CpG may be due to the hindrance of ODN in accessing endocytic routes. Use of Fc Block dramatically decreases fluorescence for FITC-IC as expected.

Dendritic Cell Activation

The increased activation of the IC over free DNP-CpG is not considered to be simply due to an additive effect since levels of free DNP-CpG alone and anti-DNP antibody alone are rising from 12 to 24 hours whereas IC levels from 12 to 24 are very similar (Figure 4.2). In addition, IC activation should not decrease with increasing antibody concentration if activation is thought to be an additive effect of antibody and DNP-CpG. DNP-CpG with either human serum albumin or an unrelated antibody (mouse IgG_{2a}) only has the same level of activation as DNP-CpG alone. This is consistent with the finding that albumin and IgG_{2a} alone only exhibit baseline activation. Therefore, our specific anti-DNP IgG antibody is vital for the increased activation which also suggests that FcγRI-mediated endocytosis is involved.

Multiple treatments were included to specifically elucidate the importance of our hypothesized FcγRI-mediated uptake. At a 1-to-0.25 molar ratio of DNP-CpG to anti-DNP

antibody, enhanced DC activation was seen. However, except for the positive control, the 1:1 molar ratio gave the maximum DC activation. The data shows that an excess (5 molar equivalent) of anti-DNP antibody resulted in the worse activation response between all three ratios examined. At a lower antibody ratio, a lower amount of DNP-CpG existed as an immune complex and, therefore, was not efficiently endocytosed and processed by DCs. At the other extreme, an excess of antibody resulted in low activation most likely due to competitive inhibition. Based on this reasoning, a 1:1 ratio would be ideal since a quantitative amount of DNP-CpG would be bound and mainly immune complexes are occupying FcγRI receptors. Results also further indicate the involvement of FcγRI since Fc Block also significantly lowers the activation of both DNP-CpG + anti-DNP antibody and DNP-scramble + anti-DNP antibody. When comparing DNP-scramble + anti-DNP-antibody with Fc-block + DNP-scramble + anti-DNP antibody, lower activation is also noted for the latter treatment. These findings all independently suggest that FcγRI must be important for processing of ICs.

As a footnote, the data presented in this section as well as the previous section were obtained from single determination of each activation marker albeit each entailed counting of over 10^4 cells. Ideally, the effect of each treatment should have been assessed at least triplicate determinations. In summary, the present data are preliminary in nature and warrant further investigation.

Conclusions

DNP-CpG can freely be taken up by DCs via macropinocytosis whereas ICs must enter the cell via FcγRI. This is supported by our finding that free FITC-DNP-CpG treatment

had a higher intracellular fluorescence compared to IC treatment. However, although ICs are limited to this single uptake mechanism and intracellular fluorescence was lower, we find that ICs are able to stimulate DC activation better than free DNP-CpG. It is also important to note that even though free DNP-CpG exhibits good uptake and activation *in vitro*, these promising results are not translated to *in vivo* therapeutic effects.[12] Taken together, our findings suggest that FcγRI plays an important role in the cellular uptake of ICs and that our IC is effective in enhancing levels of dendritic cell activation.

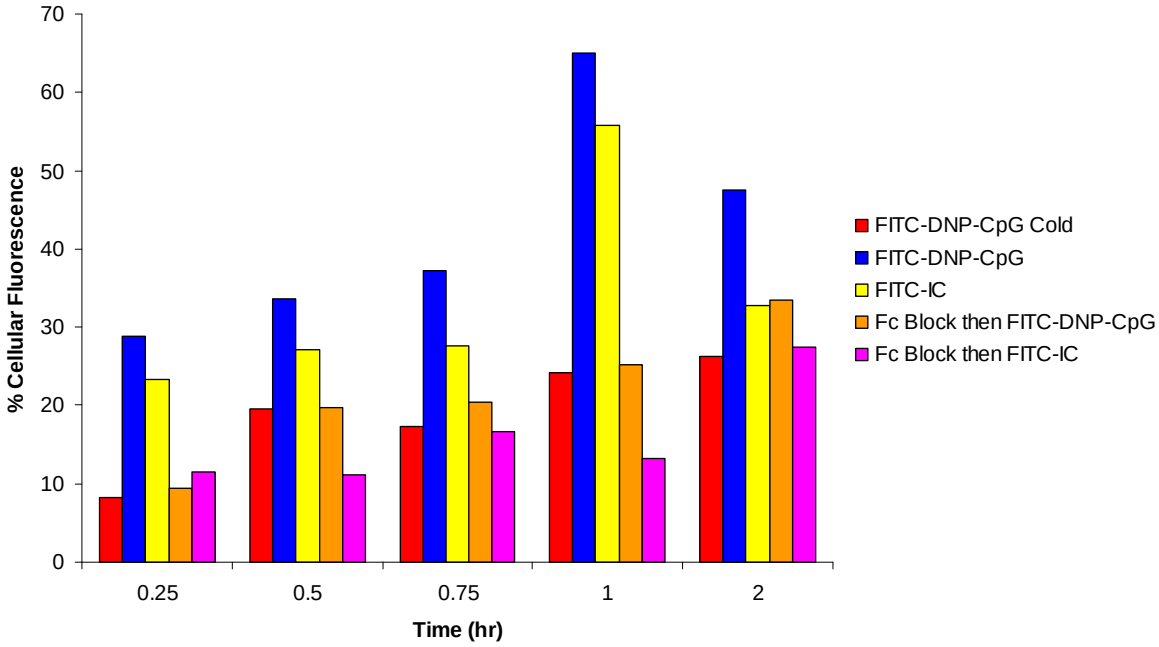

Figure 4.1. Dendritic cell uptake of free CpG and DNP-CpG. Dendritic cell uptake as measured by fluorescence in dendritic cell samples after 0.25, 0.5, 0.75, 1, and 2-hour incubations with: free FITC-DNP-CpG on ice (red); free FITC-DNP-CpG (blue); FITC-IC (yellow); Fc Block then free FITC-DNP-CpG (orange); Fc Block then FITC-IC (pink). Samples were quenched with 10% v/v of a 0.4% Trypan Blue solution to examine intracellular fluorescence. Cells were incubated at 37^{o}C unless otherwise noted.

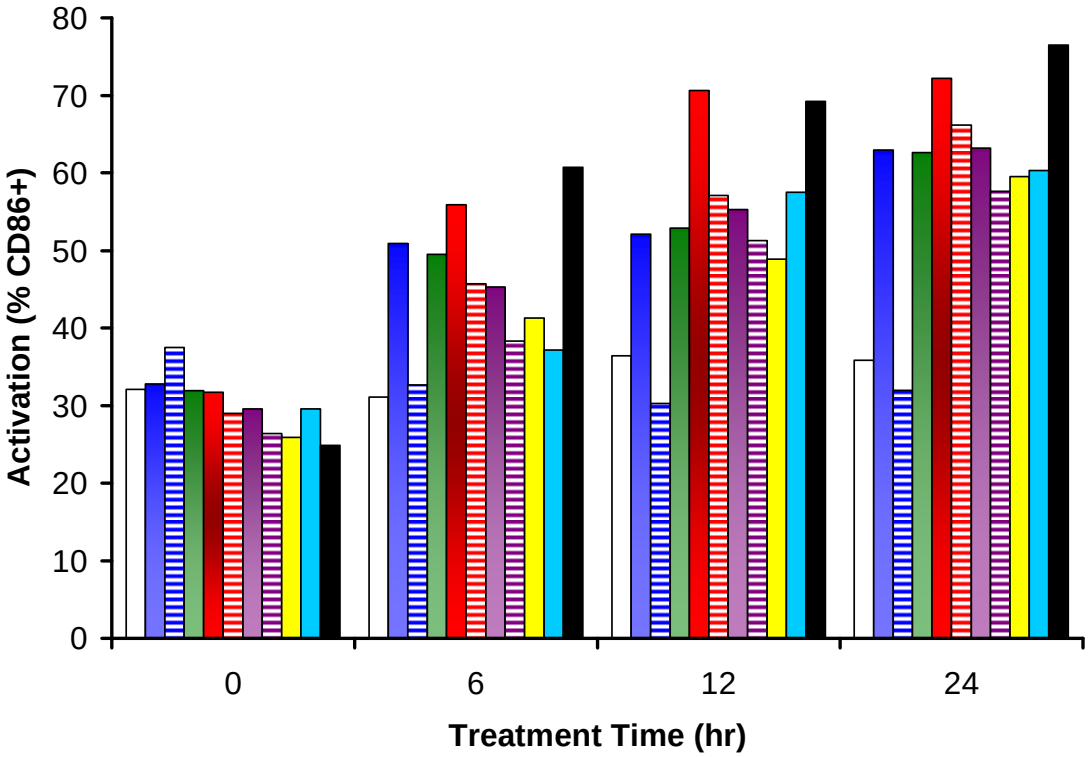

Figure 4.2. Dendritic cell activation by free CpG and DNP-CpG with and without anti-DNP antibodies. These CD86 results are also representative of those obtained from CD40, CD 80, and MHC II. Dendritic cell activation as measured by surface activation marker expression levels after 0, 6, 12, and 24-hour incubation periods with: media only (white); DNP-CpG (blue); DNP-scramble (blue lines), anti-DNP antibody alone (green); ICs with 1:1 DNP-CpG:antibody ratios (red); DNP-scramble with antibody (red lines); Fc Block followed by IC (violet); Fc Block followed by DNP-scramble with antibody (violet lines); DNP-CpG with albumin (yellow); DNP-CpG with IgG_{2a} (turquoise); and LPS (black). In all cases, 1.08 μg/ml DNP-CpG was used to activate cells. Negative controls with Fc block alone, albumin alone, and IgG_{2a} alone yielded baseline activation at all time points studied (data not shown).

References

(1) Iwasaki, A.; Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **2004**, *5*, 987-995.

(2) Ikeda, H.; Chamoto, K.; Tsuji, T.; Suzuki, Y.; Wakita, D.; Takeshima, T.; Nishimura, T. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. *Cancer Sci.* **2004**, *95*, 697-703.

(3) Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **2003**, *3*, 133-146.

(4) Dredge, K.; Marriott, J. B.; Todryk, S. M.; Dalgleish, A. G. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. *Cancer Immunol. Immunother.* **2002**, *51*, 521-531.

(5) Bretscher, P. The two-signal model of lymphocyte activation twenty-one years later. *Immunol. Today* **1992**, *13*, 74-76.

(6) von Beust, B. R.; Johansen, P.; Smith, K. A.; Bot, A.; Storni, T.; Kündig, T. M. Improving the therapeutic index of CpG oligodeoxynucleotides by intralymphatic administration. *Eur. J. Immunol.* **2005**, *35*, 1869-1876.

(7) Heckelsmiller, K.; Beck, S.; Rall, K.; Sipos, B.; Schlamp, A.; Tuma, E.; Rothenfusser, S.; Endres, S.; Hartmann, G. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. *Eur. J. Immunol.* **2002**, *32*, 3235-3245.

(8) Proudfoot, O.; Apostolopoulos, V.; Pietersz, G. A. Receptor-mediated delivery of antigens to dendritic cells: anticancer applications. *Mol. Pharm.* **2007**, *4*, 58-72.

(9) Brunner, C.; Seiderer, J.; Schlamp, A.; Bidlingmaier, M.; Eigler, A.; Haimerl, W.; Lehr, H. A.; Krieg, A. M.; Hartmann, G.; Endres, S. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. *J. Immunol.* **2000**, *165*, 6278-6286.

(10) Jiskoot, W.; van Schie, R. M. F.; Carstens, M. G.; Schellekens, H. Immunological risk of injectable drug delivery systems. *Pharm. Res.* **2009**, *26*, 1303-1314.

(11) Onda, M. Reducing the immunogenicity of protein therapeutics. *Curr. Drug Targets* **2009**, *10*, 131-139.

(12) Palma, E.; Cho, M. J. Improved systemic pharmacokinetics, biodistribution, and antitumor activity of CpG oligodeoxynucleotides complexed to endogenous antibodies in vivo. *J. Control. Release* **2007**, *120*, 95-103.

(13) Schuurhuis, D. H.; Ioan-Facsinay, A.; Nagelkerken, B.; van Schip, J. J.; Sedlik, C.; Melief, C. J. M.; Verbeek, J. S.; Ossendorp, F. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *J. Immunol.* **2002**, *168*, 2240-2246.

(14) Inaba, K.; Inaba, M.; Romani, N.; Aya, H.; Deguchi, M.; Ikehara, S.; Muramatsu, S.; Steinman, R. M. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **1992**, *176*, 1693-1702.

(15) Levine, T. P.; Chain, B. M. Endocytosis by antigen presenting cells: dendritic cells are as endocytically active as other antigen presenting cells. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8342-8346.

CHAPTER V

CONCLUSIONS AND FUTURE STUDIES

Recent promising results obtained with intravenous delivery of CpG oligodeoxynucleotides (CpG-ODNs) for solid tumor immunotherapy[1] warranted further study to establish additional means of administration and to elucidate mechanistic immune responses. In the present studies, we have analyzed the pharmacokinetic and pharmacodynamic effects of dinitrophenylated CpG (DNP-CpG) after subcutaneous administration into DNP-immunized, tumor-bearing mice. We found a sustained release of DNP-CpG from the injection site and enhanced circulation in the serum. Although there was low accumulation of DNP-CpG in tumor tissue, significant anti-tumor activity was observed. Here we confirmed that antibody complexes with haptenized CpG ODNs exhibit anti-cancer effects in pre-immunized animals. Low antibody titers resulted in significant loss of therapeutic activity by DNP-CpG thereby confirming the important role of antibodies to mediate CpG delivery. These studies establish the opportunity for SC administration in CpG ODN monotherapy. We also propose based on these results that a lower dose of DNP-CpG per injection could be used to achieve similar anti-tumor efficacy.

To determine possible mechanistic means by which anti-tumor activity ensued upon SC administration of our treatment, we examined the ability of our monovalent immune complex to promote uptake of DNP-CpG into dendritic cells through FcγRI-mediated endocytosis and to subsequently enhance activation of dendritic cells (DCs). We found that while free CpG uptake was higher than IC uptake, DC activation was enhanced in the latter case. Blocking of Fc receptors using the Fc block reagent resulted in a significant decrease in IC uptake by DCs. Our study also confirms that DC activation by our system is specific and activation enhancement requires DNP-CpG with an anti-DNP antibody. Attempts to

activate DCs using non-immunostimulatory ODNs or other proteins were not as effective as the IC. The decrease in DC activation markers after blocking FcγRI-receptor uptake supports the involvement of FcγRI-mediated processing of ICs.

Although we previously reasoned that the success of our delivery strategy could be partially attributed to the enhanced permeability and retention (EPR) effect, pharmacokinetic and pharmacodynamic results, such as the low levels of DNP-CpG detected in the tumor tissue and the ability of free CpG to hamper tumor growth, suggest otherwise. In the case of intravenous administration, the EPR effect may be the mechanism although activation of DCs in the spleen may contribute to anti-tumor effects. In the case of subcutaneous administration, no EPR effect is expected because the DNP-CpG levels in the systemic circulation at any given time remains low and accumulation in the tumor periphery is also very low. We propose that the mechanism of action may be the direct activation of DCs in the draining lymph nodes. Elevated interstitial fluid pressure (IFP) in the tumor vicinity is a barrier to macromolecular drug delivery which means that IC accumulation in the tumor periphery could be limited.[2] Any accumulation would also only be transient and retention would require receptor-ligand binding.[3] Even if a sufficient level of binding was to occur, chaotic and impaired tumor lymphatics could limit dendritic cell migration from the tumor periphery to the lymph nodes, thus preventing antigen-presentation to T cells. As such, the ability of the immune complex to target DCs through general lymphatic and/or systemic circulation may be a more reasonable explanation. Dendritic cells, in this case, can find tumor antigen released into the systemic circulation due the IFP phenomenon. CpG stimulation of DCs also leads to the stimulation of natural killer cells which may also be involved in anti-tumor activity. Since there is still much speculation as to the processes

involved in the immune response upon CpG stimulation, further mechanistic studies are necessary. Nonetheless, our results lead to the important discovery that the mechanism of action in CpG processing is dependent on the route of delivery.

These promising results warrant further development of antibody-based systems for CpG delivery. For patients who have compromised immune function due to prior treatments or due to the negative effects of disease progression itself, pre-immunization against a hapten may not be acceptable. *Ex vivo* complexation is a possible alternative although previous unpublished preliminary data from our lab has suggested that the IC may not be maintained in a dynamic *in vivo* setting. An alternative would then be to chemically conjugate a CpG ODN directly to an antibody molecule. However, this may only work using the host's own endogenously produced antibodies since use of *ex vivo* murine and chimeric monoclonal antibodies can exhibit poor half-lives[4,5] or altered affinity to Fc receptors upon storage.[6] The ideal method of development would then be to use an already existing endogenous antibody which is known to recognize a particular hapten or epitope. As a result of vaccines received during childhood and adulthood, antibodies against certain pathogens are ever-present in the systemic circulation although concentrations may be insufficient. The anti-Gal antibody is the only naturally occurring antibody known to exist in large amounts in all humans.[7] The anti-Gal antibody recognizes the α-gal carbohydrate epitope which is normally absent in humans. A system can be envisioned then in which CpG is conjugated to the α-gal epitope instead of DNP. In fact, recent work has utilized *in vivo* anti-Gal-based immune complexes for DC delivery of a vaccine.[8] Further development would also allow co-administration of CpG with tumor antigen as a therapeutic anti-cancer vaccine.

Through the present studies it has become apparent that multiple aspects must be investigated to establish primary mechanistic processes involved, particularly with the relevance of the EPR effect towards *in vivo* systems. Mechanistic studies can be accomplished by multiple techniques. Rather than *in vitro* activation, DC activation levels after treatment can be studied from acquisition of DCs from lymph nodes for flow cytometry. Immunohistochemistry of normal and tumor tissue can reveal the type and extent of lymphocyte involvement. Additionally, studying the impact of FcγRI-deficiency on IC processing in a pre-immunized, tumor-bearing mouse model would be of great interest. FcγR-knockout mice are available for such studies. IC uptake and activation of DCs can be further confirmed by utilizing FcγRI-deficient DCs cultured from the bone marrow of the same mouse strain.[9] Effector cell involvement can be analyzed by looking at T-cell proliferation in spleens and infiltration into tumor tissue.

Collectively, our findings promote the use of CpG monotherapy for clinical use in cancer therapy although cellular toxicity and immunological memory should first be studied. Additional routes of administration could be established, such as intranasal or intradermal delivery where DCs are definitively located as a first-line defense against pathogenic invasion, for treatment of various diseases. Our systemically delivered ICs could result in reduced CpG ODN therapeutic dose and dosing frequency. Treatment of occluded and metastasized tumors may also be possible without further intervention with invasive surgery or toxic chemotherapy. Successful outcomes of these studies will undoubtedly bring about an accelerated development of CpG-based immunotherapy, possibly in synergy with other

treatment modalities, for various human diseases in addition to cancer such as infectious diseases, allergic conditions, and asthma.[10] We also envision a general application of using antibodies as a drug carrier, not as a targeting ligand, to delivery of other small nucleic acids.

References

(1) Palma, E.; Cho, M. J. Improved systemic pharmacokinetics, biodistribution, and antitumor activity of CpG oligodeoxynucleotides complexed to endogenous antibodies in vivo. *J. Control. Release* **2007**, *120*, 95-103.

(2) Stohrer, M.; Boucher, Y.; Stangassinger, M.; Jain, R. K. Oncotic pressure in solid tumors is elevated. *Cancer Res.* **2000**, *60*, 4251-4255.

(3) Berk, D. A.; Yuan, F.; Leunig, M.; Jain, R. K. Direct in vivo measurement of targeted binding in a human tumor xenograft. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1785-1790.

(4) Ober, R. J.; Radu, C. G.; Ghetie, V.; Ward, E. S. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *Int. Immunol.* **2001**, *13*, 1551-1559.

(5) Goodman, G. E.; Beaumier, P.; Hellström, I.; Fernyhough, B.; Hellström, K. E. Pilot trial of murine monoclonal antibodies in patients with advanced melanoma. *J. Clin. Oncol.* **1985**, *3*, 340-352.

(6) Bertolotti-Ciarlet, A.; Wang, W.; Lownes, R.; Pristatsky, P.; Fang, Y.; McKelvey, T.; Li, Y.; Li, Y.; Drummond, J.; Prueksaritanont, T.; Vlasak, J. Impact of methionine oxidation on the binding of human IgG1 to Fc Rn and Fc gamma receptors. *Mol. Immunol.* **2009**, *46*, 1878-1882.

(7) Galili, U.; Rachmilewitz, E. A.; Peleg, A.; Flechner, I. A unique natural human IgG antibody with anti-alpha-galactosyl specificity. *J. Exp. Med.* **1984**, *160*, 1519-1531.

(8) Abdel-Motal, U. M.; Wigglesworth, K.; Galili, U. Mechanism for increased immunogenicity of vaccines that form in vivo immune complexes with the natural anti-Gal antibody. *Vaccine* **2009**, *27*, 3072-3082.

(9) Barnes, N.; Gavin, A. L.; Tan, P. S.; Mottram, P.; Koentgen, F.; Hogarth, P. FcγRI-Deficient Mice Show Multiple Alterations to Inflammatory and Immune Responses. *Immunity* **2002**, *16*, 379-389.

(10) Dittmer, U.; Olbrich, A. R. M. Treatment of infectious diseases with immunostimulatory oligodeoxynucleotides containing CpG motifs. *Curr. Opin. Microbiol.* **2003**, *6*, 472-477.

APPENDIX

CYTOKINE LEVELS DURING CpG TREATMENTS

Previous investigations have showed that alteration of cytokine genes or direct administration of various cytokines could result in anti-cancer effects. However, certain dismal results have been consistent with the difficulties associated with cytokine cancer therapy, where systemic administration of cytokines produces significant toxicity side effects at an effective dose. Success may be obtained by mimicking the paracrine functions of cytokines in the tumor microenvironment.[1,2] We attempted to demonstrate that our delivery strategy mimics local infection or inflammation in the tumor tissue. This is a reasonable hypothesis given that CpG is derived from bacterial DNA and that we measured as much as 20% of the total intravenously injected dose in the tumor tissue.[3] Since SC administration showed little tumor accumulation, we were curious to see whether the anti-tumor activity could have been due to a cytokine effect. Nonetheless, it has proved to be rather difficult to present experimental evidence.

Elevated level of serum cytokines from CpG administration may not be directly related to anti-tumor activity.[1,2] This notion was experimentally supported as follows. Three groups of BALB/c mice each with 5 to 6 mice received either: (A) contralateral subcutaneous (SC) dosing of DNP-CpG in DNP-immunized mice, (B) SC dosing of free CpG to non-immunized mice, and (C) peritumoral injection of CpG also to non-immunized mice (positive control). Four hours after each of the four weekly administrations, approximately 50 μl of blood was withdrawn via tail nick and processed to serum. Then IL-12 concentrations were determined using a commercially obtained BD OptEIA® kit (BD Biosciences).

Although the data presented in Figure A.1 are on only one cytokine, the following conclusion can be made. In all cases, a tremendous IL-12 response was seen compared with the basal level (the first small bar only visible on Group C). Although subcutaneous administration of free CpG generates a higher level of IL-12 compared to DNP-CpG, tumor inhibition studies showed that DNP-CpG treatment results in a better anti-tumor response. This finding supports the notion that local production of cytokines, not their systemic levels, is the key to anti-tumor activity.

The above set of data supports only a negative conclusion. Next, we sought after direct evidence for the very premise upon which the proposed studies are developed: i.e., our CpG delivery systems are able to induce proinflammatory cytokines locally in tumor tissue although the CpG is administered via bolus IV or SC. In essence, our delivery system mimics local bacterial infection that would induce immediate innate immune response. The experiments dealt with 3 groups of CT26 tumor-bearing BALB/c mice, each with 4 animals.

Group A consisted of DNP-immunized mice receiving DNP-CpG intravenously (test). In B non-immunized mice received free CpG intravenously (negative control) whereas Group C received free CpG peritumorally (positive control). A single dose of 100 μg IV or 20 μg PT was administered when tumor becomes approximately 30~100 mm^3. The latter dose was chosen based on the fact that in the literature PT dose typically ranges from 3~10 μg[4], 10 μg[5], 20 μg[6], 20~40 μg[7], to as high as 100 μg[8] and the finding that our formulation delivered to tumor tissue approximately 20% ID at the time of cytokine assay, i.e., 4 hrs after administration.[3]

Surgically removed tumor and normal tissue contralateral to tumor were homogenized with T-PER® Tissue Protein Extraction Reagent, 10 ml per gram tissue, supplemented with protease inhibitor cocktail (Pierce, Rockford, IL), as described elsewhere.[5] The supernatant and serum were serially diluted in PBS for ELISA with BD OptEIA® ELISA Kits: TNF-α, IFN-γ, IL-6, IL-12, and GM-CSF. It is known that these cytokines become overexpressed in tumor tissue upon repeated PT daily dosing of free CpG especially when tumor burden is low.[5] Briefly these cytokines are indicative of the following phenomena in innate immunity triggered by CpG ODNs[9]: TNF-α and IL-12, DC activation; IFN-γ, NK cell activation; IL-6, B cell growth and differentiation; and GM-CSF, DC stimulation.

As shown in Figure A.2, there is some indication our approach produced more pro-inflammatory cytokines in tumor tissue than free CpG injected IV. Due to a small number of animals tested in each group (n = 4) and wide inter-subject variations, however, there were no statistically significant differences based on t-test analysis among the test treatments for all five cytokines measured ($p>0.05$). This must be because we analyzed the sample just after one treatment. The amount of CpG delivered via ICs was simply insufficient to create a situation to mimic local inflammation.

In the prior study, we attributed the lack of statistically significant differences in various cytokine levels among the three treatments to the fact that we measured them after only one single dosing. Since then we have repeated the experiment with five daily doses.

When the tumor volume was between 20 to 75 mm^3, four mice in each of three groups received a daily dose of: test group A (red), 50 μg IV to immunized mice; negative control B (green), 50 μg IV; and positive control C (gray), 10 μg intratumoral. Four hours after the fifth and final dose, animals were sacrificed and the tumor was processed for ELISA.

As shown in Figure A.3, IL-12 is the only cytokine that appeared to be significantly more produced in the tumor with the test formulation. Even here, the standard deviation was so large and the number animals used was small (n=4), the p value was as high as 0.31 for the test formulation-negative control comparison. Similarly TNF-α production in tumor with the test formulation is greater than that of negative control. However, once again, the p value was close to 0.38. Within experimental error, the other two cytokines did not show any modality-dependence.

The inter-subject variations we observed were in the same magnitude as previously reported.[5] The cytokine study in this manuscript was what prompted us to analyze cytokine levels in our own model. In retrospect, we should not have run this series of experiments. Instead, we should have characterized cell infiltration via immunohistochemistry, an important subject of investigation in the future. The complexities involved with cytokine management suggest that this method of anti-cancer treatment may not be the ideal treatment modality.

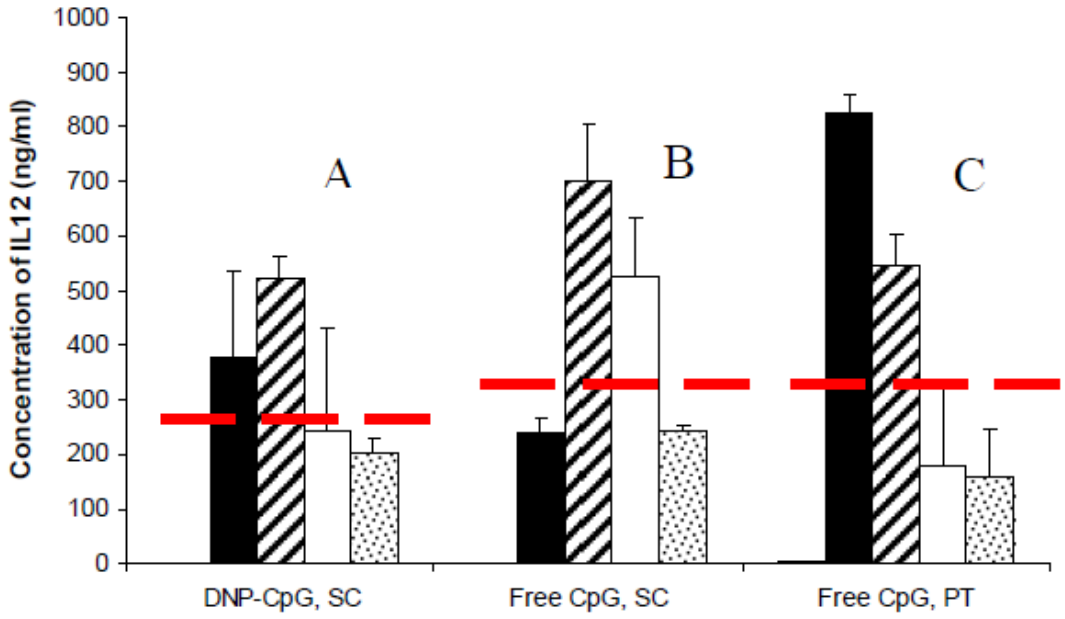

Figure A.1. Comparison of serum IL-12 concentrations upon various single-dose CpG treatments. Serum concentration of IL-12 found 4 hrs after dosing of; (A) DNP-CpG in DNP-immunized mice via SC route, (B) CpG to non-immunized mice via SC route, and (C) CpG to non-immunized mice via PT route. In all cases, mice harbored CT26 tumors of 50 to 150 mm^3 of starting size. In each group there are 5 bars: the first one represents IL-12 of mice not dosed (baseline level). It is almost non-detectable but barely visible in Group C. The 2nd (filled in black) through the 5th bars (filled with dots) represent the IL-12 level after the 1st through the 4th weekly dosing, respectively. The red horizontal lines represent the average values in each group of 5 or 6 mice.

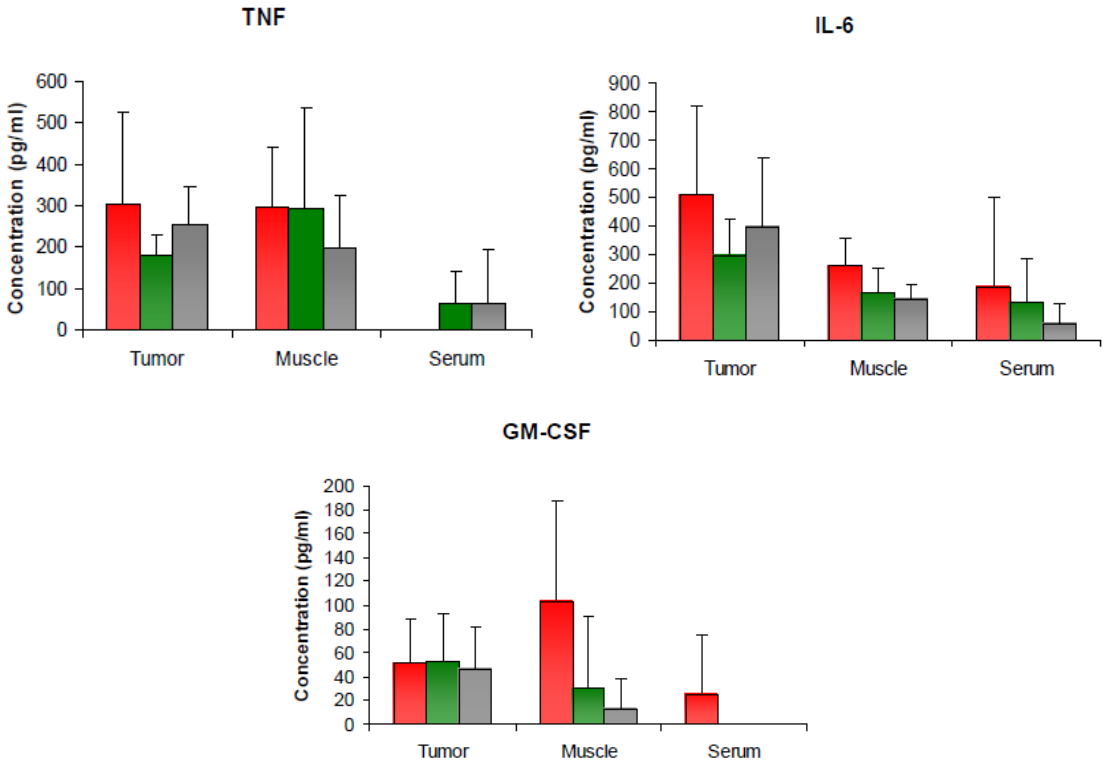

Figure A.2. Comparison of cytokine levels in tumor, muscle, and serum upon single-dose CpG treatments. Three pro-inflammatory cytokine levels found in tumor tissue, contralateral muscle tissue, and serum after single administration of: A (red), DNP-CpG to pre-immunized mice IV; B (green), free CpG IV; and C (grey), free CpG PT. In all cases, n = 4 and tumor was 30~100 mm^3 in size when injection took place. Normalization is in terms of tumor size based on the size-dependent T-PER buffer volume used.

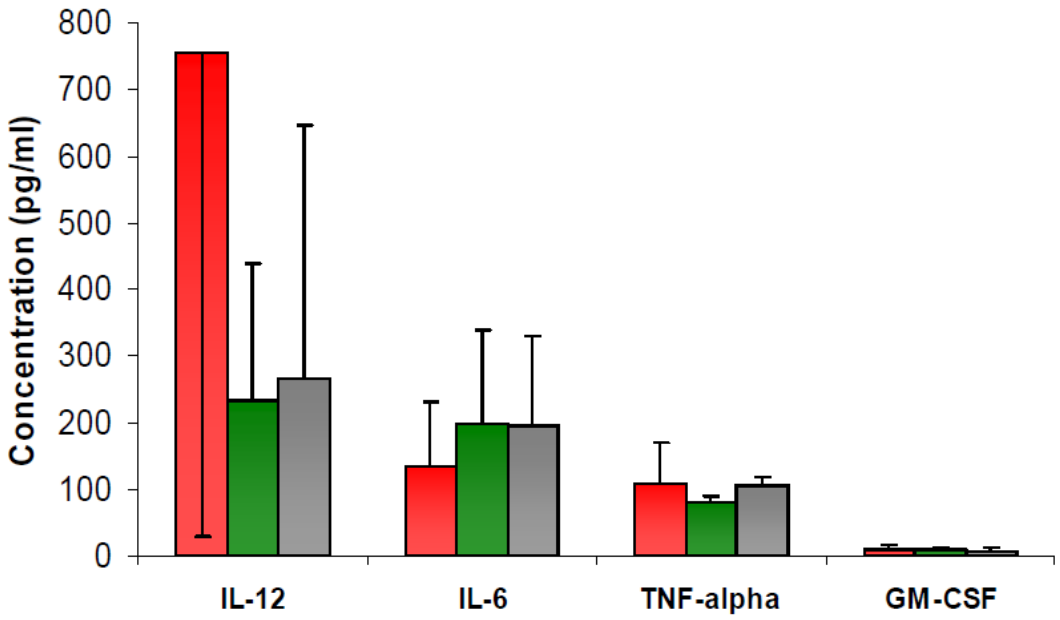

Figure A.3. Comparison of cytokine levels in tumor tissue after multiple dosing of CpG treatments. Four pro-inflammatory cytokine levels found in tumor tissue after five daily administrations of: A (red), DNP-CpG to pre-immunized mice IV; B (green), free CpG IV; and C (grey), free CpG IT. In all cases, n = 4 and tumor was 20-75 mm^3 in size when injections took place. Four hours after the fifth/final dose, animals were sacrificed and the tumor was processed for ELISA. Normalization is in terms of tumor size based on the size-dependent T-PER buffer volume used.

References

(1) Dranoff, G. Cytokines in cancer pathogenesis and cancer therapy. *Nat. Rev. Cancer* **2004**, *4*, 11-22.

(2) Li, C.; Huang, Q.; Kung, H. Cytokine and immuno-gene therapy for solid tumors. *Cell. Mol. Immunol.* **2005**, *2*, 81-91.

(3) Palma, E.; Cho, M. J. Improved systemic pharmacokinetics, biodistribution, and antitumor activity of CpG oligodeoxynucleotides complexed to endogenous antibodies in vivo. *J. Control. Release* **2007**, *120*, 95-103.

(4) Kunikata, N.; Sano, K.; Honda, M.; Ishii, K.; Matsunaga, J.; Okuyama, R.; Takahashi, K.; Watanabe, H.; Tamura, G.; Tagami, H.; Terui, T. Peritumoral CpG oligodeoxynucleotide treatment inhibits tumor growth and metastasis of B16F10 melanoma cells. *J. Invest. Dermatol* **2004**, *123*, 395-402.

(5) Sharma, S.; Karakousis, C. P.; Takita, H.; Shin, K.; Brooks, S. P. Cytokines and chemokines are expressed at different levels in small and large murine colon-26 tumors following intratumoral injections of CpG ODN. *Neoplasia* **2004**, *6*, 523-528.

(6) Kawarada, Y.; Ganss, R.; Garbi, N.; Sacher, T.; Arnold, B.; Hämmerling, G. J. NK- and CD8(+) T cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides. *J. Immunol.* **2001**, *167*, 5247-5253.

(7) Sfondrini, L.; Besusso, D.; Rumio, C.; Rodolfo, M.; Ménard, S.; Balsari, A. Prevention of spontaneous mammary adenocarcinoma in HER-2/neu transgenic mice by foreign DNA. *FASEB J* **2002**, *16*, 1749-1754.

(8) Heckelsmiller, K.; Rall, K.; Beck, S.; Schlamp, A.; Seiderer, J.; Jahrsdörfer, B.; Krug, A.; Rothenfusser, S.; Endres, S.; Hartmann, G. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.* **2002**, *169*, 3892-3899.

(9) Krieg, A. M. Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug Discov.* **2006**, *5*, 471-484.