THE EFFECTS OF PLASMID DNA AND IMMUNOSTIMULATORY CpG MOTIFS ON IMMUNE SURVEILLANCE IN SHEEP LYMPH NODES

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ABSTRACT

This Ph.D. dissertation examined the effects of bacterial DNA on immune surveillance in ovine lymph nodes (LN). The first study identified long-term changes in lymph nodes following exposure to plasmid DNA. Treated lymph nodes were heavier and had larger medullary areas. Furthermore, medullary cord thickness and medullary sinus width as well as germinal center size and number were increased in plasmid treated lymph nodes.

To determine whether bacterial DNA altered cell trafficking through lymph nodes, the efferent lymphatic of the prescapular LN of sheep was cannulated and cells were collected. Intradermal injection of as little as 4 μg of plasmid DNA expressing the green fluorescent protein of jellyfish (eGFP) caused a marked increase in the cell trafficking through the prescapular lymph node. A dose-dependent facet existed for this response, as the increase in cell trafficking response persisted longer with 40 μg or 400 μg of plasmid-eGFP than with 4 μg. This increased cell trafficking was independent of green fluorescent protein expression as both pCAN1-eGFP and pCAN1 induced similar responses.

Increased cell traffic induced by bacterial DNA was further characterized by determining whether bacterial DNA form was critical for this response. Treatment with intact plasmid (circular) DNA induced the greatest increase in cell traffic. In contrast, when plasmid DNA was digested with restriction enzymes into linear fragments of DNA, then cell trafficking was not significantly increased.

Numerous studies have shown that immunostimulatory guanosine-cytosine (CpG) motifs activate cells *in vitro* and can improve protective immune responses *in vivo*. Therefore we tested the hypothesis that the presence of immunostimulatory CpG motifs within plasmids was responsible for altering cell trafficking. It was shown

that plasmids with added immunostimulatory CpG sequences affected cell trafficking in a dose-dependent manner. The injection of 40 μg of pBISIA-88 caused an increase in cell trafficking while injecting 400 μg pBISIA-88 failed to increase cell flow above control levels.

Analysis of cell populations collected in efferent lymph gives insight into treatment effects on immune surveillance. This is well-studied following treatment with antigen and in general, antigen induces a selective movement of cells into lymph that occurs 24 hours post-treatment. In contrast, plasmid DNA induced a rapid, non-selective movement of lymphocytes through the lymph node, suggesting that bacterial DNA and antigen may affect cell trafficking by different mechanisms and thereby have distinct effects on immune surveillance.

Short-term effects of bacterial DNA on lymph node architecture and cellular composition were also investigated to determine whether an increase in cell trafficking was associated with structural changes within the lymph node. While it was shown that bacterial DNA induced changes within a lymph node, including an increased number of germinal centers and an increased frequency of $CD72^{+}CD21^{+}$ B cells, these changes were not correlated to increased cell trafficking.

In summary, bacterial DNA altered immune surveillance in sheep lymph nodes by changing cell traffic and lymph node architecture and composition. Many of these responses differed from responses observed by others following antigen treatment. The present observations suggest that bacterial DNA can function as an important signal to modulate immune surveillance and host responses to infections by pathogens.

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1-TAC	Interferon inducible T cell α-chemoattractant
AmpR	Ampicillin resistance gene
APC	Antigen presenting cell
β-GalN	β-galactosamine
BLC	B lymphocyte chemoattractant chemokine
BSA	Bovine serum albumin
BGH poly[A]	Bovine growth hormone poly [A] sequence
BHV-1	Bovine herpesvirus-1
C3H/HeJ mice	Lipopolysaccharide unresponsive strain of mice
CD	Cluster of differentiation
CMI	Cell-mediated immunity
CoL E1	Colicinogenic factor E1
COS 7 cells	COS 7 fibroblast cell line
CpG	Guanosine-cytosine hexamer
CRP	C-reactive protein
CTL	Cytotoxic T cells
CTLA4	Cytotoxic T lymphocyte antigen 4
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
EC	Extracellular
ELC	Epstein-Barr virus-induced gene 1 ligand chemokine
eGFP	Variant form of green fluorescent protein of jellyfish
FCRγ	Complement receptor-γ found on neutrophils, lymphocytes and macrophages
FDC	Follicular dendritic cell
FRC	Fibroblastic reticular cell
FIV	Feline immunodeficiency virus
gB and gD	Glycoprotein B and D of Bovine herpesvirus-1
GC	Germinal center
GFP	Green fluorescent protein of jellyfish
GM-CSF	Granulocyte-macrophage colony stimulating factor
HA	Hemagglutinin antigen of influenza virus
HBSAg	Hepatitis B surface antigen
HCC	Human C-C chemokine
H+E	Hematoxylin and eosin stain
HEV	High endothelial venule
HIV-1	Human immunodeficiency virus-1
HCMV IE1	Human cytomegalovirus immediate-early promoter
MHC-I and -II	Major histocompatibility complex-I and –II
HSV-1	Herpes simplex virus-1
ICAM-1	Intercellular adhesion molecule-1
id	Intradermal
IgA, IgE, IgG, and IgM	Immunoglobulin classes A, E, G and M
IgG1, IgG2a	Isotypes of immunoglobulin G
IL-1 through -18	Interleukin-1 through -18

IL2Rα	Interleukin-2 receptor α subunit
im	Intramuscular
IFN-α, -β and -γ	Interferon-α, -β and -γ
Intron A	Largest intron of human cytomegalovirus
INODN	Immunoneutralizing oligodeoxyribonucleotide
inv	Intravaginal
ip	Intraperitoneal
IP10	Interferon-inducible protein of 10 kd
ISODN	Immunostimulatory oligodeoxyribonucleotide(s)
iv	Intravenous
LN	Lymph node(s)
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4
MCP	Monocyte chemoattractant protein
MDC-1	Macrophage-derived chemokine-1
MFI	Mean fluorescence intensity
Mig	Monokine induced by IFN-γ
MIP	Macrophage inflammatory protein
MY-1	Immunostimulatory cell fraction of *M. bovis*
MyD88	Myeloid differentiation marker 88
NK cells	Natural killer cells
NP	Nucleoprotein of influenza virus
NZB/NZW mice	New Zealand black/white mouse
ODN	Oligodeoxyribonucleotide(s)
ODN 2135	5'-TCGTCGTTTGTCGTTTTGTCGTT-3'
ORI	Origin of replication
pBIS-88	pBIS-88 plasmid
pBISIA-88	pBISIA-88 plasmid
pCAN1	pCAN1 plasmid
pCAN1-eGFP	pCAN1 encoding eGFP
pCAN1-GFP	pCAN1 plasmid encoding GFP
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
pMASIA	pMASIA plasmid
PI_3 virus	Parainfluenza-3 virus
Poly[A] tails	Polyadenylation tails
PSA	Prostate specific antigen
pSLIAgD	pSLIA plasmid encoding glycoprotein D of bovine herpesvirus-1
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
pUC19	pUC19 plasmid
pUk21-A2	pUk21-A2 plasmid
PWM	Pokeweed mitogen
RANTES	Regulated on activation normal T cell expressed and secreted
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RSV LRT	Rous sarcoma virus long terminal repeats
sc	Subcutaneous

SDF-1α	Stromal-derived factor-1α
SIV	Simian immunodeficiency virus-1
SEM	Standard error of the mean
SLC	Secondary lymphoid tissue chemokine
SLE	Systemic lupus erythematosus
SV40 promoter	Simian virus 40 early promoter
TARC	Thymus and activation-regulated chemokine
TBM	Tingible-body macrophage
TECK	Thymus-expressed chemokine
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
TNF-α	Tumor necrosis factor-α
TLR-9	Toll like receptor-9
TRAF6	Tumor necrosis receptor-associated factor 6
VIP	Vasoactive intestinal peptide
VP4 and 7	Rotavirus protein 4 and 7

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

For centuries it has been known that protective immunity can develop following an exposure to an infectious agent. Early clinical studies showed that the injection of attenuated or killed pathogens could prevent disease in individuals subsequently exposed to the same organism. Pasteur coined the term vaccines to describe the immunization procedure with agents (antigens) that could promote protection against disease (Silverstein, 1989). Since then, the development of vaccines has grown and the administration of either killed or modified-live vaccines is used routinely for the immunoprophylaxis of both veterinary and human diseases.

Plasmid-based vaccines are a recent technology developed for the induction of disease protection. The ability to insert gene sequences that encode for almost any known protein potentially makes DNA vaccines an ideal method for immunoprophylaxis. Methods to improve DNA vaccine efficacy, including improved DNA vaccine adjuvanticity by the addition of immunostimulatory guanosine-cytosine (CpG) motifs, the coexpression of immunomodulatory proteins such as cytokines, and the coadministration of antigen to bolster immune responses, are presently being studied. Currently, DNA vaccines are not being implemented in human or animal vaccination programs. The infancy of DNA vaccine technology, its inability to outperform conventional vaccines and a potential lack of acceptance by the public, are barriers that may impede DNA vaccine use. To overcome these problems, DNA

vaccines are currently undergoing intensive research to improve vaccine efficacy. A major finding is that small sequences of DNA with a central CpG motif flanked by two purine and pyrimidine nucleotides can markedly influence both innate and adaptive immune responses. It has become evident that plasmid (bacterial) DNA, and in particular these CpG rich DNA sequences, can activate the cells involved in the induction of an immune response. These cells, namely B cells, natural killer cells, dendritic cells and macrophages, respond to CpG stimulation *in vitro* with enhanced proliferative responses, decreased apoptosis, cell maturation and the secretion of a plethora of cytokines and chemokines (Krieg *et al.*, 1995; Ballas *et al.*, 1996; Yi *et al.*, 1998, Takeshita *et al.*, 2000) . Plasmids with added immuno-stimulatory CpG sequences can induce strong humoral and cell-mediated immunity i*n vivo*, as well as protect animals against challenge with various microorganisms (Klinman *et al.*, 1999; Weighardt *et al.*, 2000).

To date, little research has examined the effects of either plasmid DNA or immunostimulatory DNA sequences that contain CpG motifs (oligodeoxyribonucelotide; ODN) on immune surveillance. The recirculation of lymphocytes through secondary lymphoid tissues plays a critical role in the induction of an adaptive immune response by providing a mechanism for antigen presenting cells to screen the T and B cell antigen receptor repertoire. This screen of the receptor repertoire enhances the level of immunosurveillance performed by the adaptive immune system. Therefore, I hypothesized that one mechanism by which immunostimulatory CpG motifs can enhance adaptive immune responses is by altering cell trafficking.

In this work, which used the ovine lymph node model, immunosurveillance was monitored by analyzing the dynamics of cell migration through a lymph node and then determining whether these changes in cell trafficking were associated with morphological changes within the lymph node. The following sections contain a review of the literature

on: DNA vaccines, immunostimulatory CpG motifs, lymph node structure and cell trafficking to provide a background and rationale for the research conducted.

1.1.1 Similarities and Differences Between Eukaryotic and Prokaryotic DNA

Eukaryotic and prokaryotic DNA are similar, yet in some respects differ in physical properties and chemical composition. For a comprehensive review on eukaryotic and prokaryotic DNA, see Alberts *et al.* (1994). Briefly, both prokaryotic and eukaryotic DNA contain the four nucleotides of adenine, guanine, cytosine and thymine, covalently bonded by phosphodiester linkages that join the 5' carbon of a deoxyribose sugar of a nucleotide to the 3' carbon of another. Strands of DNA are held together by hydrogen bonds and form an α helix with one turn every 3.4 nm. The molecular packaging differs between prokaryotic and eukaryotic DNA. Prokaryotic DNA is circular and located within the cytoplasm, while eukaryotic DNA is wrapped around protein scaffolds (nucleosomes) to form chromosomes located within the nucleus. The eukaryotic genome is 1000-10000 times larger than prokaryotic, which necessitates it needs to be tightly wrapped into chromosomes. The location of DNA within a nucleus in higher organisms offers protection from cytoplasmic metabolites and permits a special form of regulation for DNA replication. Unlike prokaryotic DNA that undergoes both transcription and translation simultaneously, eukaryotic DNA is transcribed within the nucleus, with translation and post-translation modifications occurring within the cytoplasm.

There are also significant differences between eukaryotic and prokaryotic DNA at the molecular level. Firstly, prediction of nucleotide frequencies by 'nearest neighbor analysis' indicates that the base pair arrangement in eukaryotic DNA is non-random, as base pair frequencies found in eukaryotes do not correspond with predicted frequencies. Swartz *et al.* (1962), demonstrated that the CpG frequency in eukaryotic DNA was one-third the predicted frequency calculated from base pair composition. Secondly, the majority of vertebrate DNA is methylated while the methylation of invertebrate DNA is

limited or nonexistent (Bird, 1987). Methylation of eukaryotic DNA occurs by transferring a methyl group from s-adenosyl-methionine to cytosine to form 5-methylcytosine (Hergersberg, 1991).

Approximately 1% of eukaryotic DNA is unmethylated and is located in discrete sites such as 'CpG islands' and areas of regulation that include promoters (Bird *et al.*, 1985). This lack of methylation and an increased frequency of CpG motifs in 'CpG islands' and the under-representation of CpG motifs in the majority of eukaryotic genomic DNA have sparked theories endeavoring to explain the CpG discrepancies present in vertebrate DNA. A popular theory suggests that cytosine is prone to deamination and that deamination of 5'-methylcytosine leads to the formation of thymine. This newly formed thymine causes a base pair mismatch leading to excisional repair of the nucleotide and loss of the original cytosine residue. The consequence is an overall reduction in CpG frequency within the genome (Bird *et al.*, 1985).

Lack of methylation of cytosine residues in areas of DNA regulation such as promoter regions may ensure that only selected regions of DNA are transcribed. It is speculated that methylated nucleotides can either prevent protein-DNA interaction or alter chromatin structure. The presence of methylated bases in large areas of genomic DNA, would prevent polymerases from randomly binding to DNA regulatory proteins and transcribing gene products (Hergersberg, 1991).

1.1.2 Characteristics of Plasmids Used in DNA Vaccines

A circular plasmid is the primary construct used in DNA-based immunization. Most plasmids contain an origin of replication, antibiotic resistance gene, a multiple cloning site, a promoter/enhancer region and a messenger RNA (mRNA) polyadenylation (poly [A]) sequence. The insertion of intron sequences into a plasmid for improved gene expression is optional.

1.1.2.1 Origin of Replication

An origin of replication (ORI) is needed within a plasmid to produce large copy numbers of plasmid in bacteria. The colicinogenic factor E1 (CoL E1) exists in *E.coli* as an autonomous replicating circular plasmid. In an experiment by Clewell (1972), bacteria containing CoL E1 plasmid were grown in media treated with chloramphenicol. DNA analysis indicated that bacterial chromosomal DNA did not increase in content indicating a cessation of host cell DNA synthesis. In contrast the CoL E1 plasmid continued to replicate long after bacterial chromosomal DNA synthesis had ceased (Clewell, 1972). Plasmid numbers increased by 125-fold and the self-replicating plasmids were virtually unaffected by antibiotics. The ability of CoL E1 to replicate autonomously makes it a good candidate for use within the ORI. Indeed, a portion of CoL E1 is used as the ORI in many commercially available plasmids.

1.1.2.2 Antibiotic Resistance

To produce large quantities of a plasmid, bacteria containing the plasmid must be grown in culture and be 'positively selected' from contaminating bacteria. This can be accomplished by inserting an antibiotic resistance gene into the plasmid. The plasmid will express the protein for antibiotic resistance and therefore all bacteria containing that protein will survive and propagate in culture supplemented with the specific antibiotic. In contrast, bacteria lacking the plasmid cannot produce its antibiotic resistance protein and will die in culture. Antibiotic resistance genes used in plasmid isolation express proteins that interfere with the activities of a specific antibiotic, including ampicillin, kanamycin, neomycin, and chloramphenicol.

1.1.2.3 Promoter/Enhancer Regions

To generate high-level expression of an inserted gene product, viral promoters/ enhancers are often added to plasmids upstream from the inserted gene. The three main promoters/enhancers used in plasmids are, the early simian virus (SV40) promoter,

the Rous sarcoma virus long terminal repeats (RSV LTR), and the immediate-early promoter of human cytomegalovirus (HCMV IE1).

The early SV 40 promoter was one of the first promoters to demonstrate enhanced protein expression in cell culture. Moreau *et al.* (1981) showed that removal of a 72-base pair repeat within the SV 40 promoter caused a marked decrease in gene expression. In comparison, the addition of this sequence to other viral promoters caused a significant increase in reporter gene expression.

The RSV LTR are conserved sequences involved in the initiation of transcription and include sequences found in TATA boxes. Plasmids with the RSV LTR promoter can express high levels of protein: compared to plasmid lacking the promoter, plasmids with the RSV LTR promoter displayed a three and ten-fold increase in gene expression in mouse kidney cells and HELA cells, respectively (Gorman *et al.*, 1982).

The IE1 promoter/enhancer from HCMV also has strong promoter activity *in vitro*. Boshart *et al.* (1985) compared gene expression in plasmids encoding HCMV IE1 or other viral promoters. Northern blot analysis indicated that a plasmid with HCMV IE1 had a three- to five-fold increase in gene expression compared to one with the SV 40 promoter. Similarly, plasmids with the HCMV IE1 had the greatest level of gene expression when compared to RSV LTR and other viral promoters that included those from hepatitis B virus, herpesvirus saimiri and murine cytomegalovirus (Boshart *et al.*, 1985). The HCMV IE1 promoter contains four copies of which three repeating CCCGCC base pair sequences provides a binding site for SP1 transcription factor (Boshart *et al.*, 1985). This multiple repeating binding site may explain the enhanced gene expression with plasmids encoding HCMV IE1.

To enhance gene expression in selected tissue, tissue specific promoters and enhancers can be added to plasmid DNA. It is believed that targeting a plasmid to the desired tissue should ensure that genes are expressed only within that tissue. This is

particularly important in cancer therapy, as targeting plasmids expressing cytotoxic gene products to cancer cells could reduce systemic toxicity. The addition, for example, of a prostate specific antigen (PSA) tissue promoter and enhancer to plasmids that express p53, preferentially killed transfected prostate cancer cells while sarcoma cells exposed to the same plasmid survived (Lee *et al.*, 2000). The use of tissue-specific promoters and enhancers may also help improve DNA vaccine efficacy. Plasmids designed to specifically express antigen in immunologically active cells such as dendritic cells may therefore improve the induction of a desired immune response.

1.1.2.4 Polyadenylation Tails

To produce large quantities of expressed protein, a constant supply of mRNA must be produced and maintained within the cell. To maintain high levels of mRNA by enhancing mRNA stability within cells, poly [A] tails are added to the 3' end of the gene transcript. Pfarr *et al.* (1986) demonstrated that the poly [A] tail of mRNA improves protein production and that poly [A] tails of different species have different levels of activity. Plasmids with or without the bovine growth hormone poly [A] sequence (BGH poly [A]) were compared and it was observed that protein expression was 60 fold higher in cells transfected with plasmids containing BGH poly [A] than cells transfected with plasmid lacking BGH poly [A] sequence. Cells transfected with a BGH poly [A]-containing plasmid also had a three-fold increase in protein expression when compared to cells transfected with plasmid containing human collagen poly [A] (Pfarr *et al.*, 1986).

1.1.2.5 Intron A

The largest intron from HCMV, intron A, has been added to plasmids to improve protein production. Intron A has five binding sites for strong nuclear factor 1 (NF1) regulator genes and it is believed that these binding sites help improve plasmid transcription. Chapman *et al.* (1991) showed that cells transfected with plasmids containing intron A demonstrated a 98% increase in gene expression compared to cells

transfected with plasmid that lacked intron A. To determine if the NF1 site within intron A is essential for enhanced gene expression, three bases within the NF1 site were mutated and the modification of these three bases was found to cause a marked reduction in protein expression (Chapman *et al.*, 1991).

1.1.3 Immune Stimulatory Properties of Bacterial DNA

The immunostimulatory effects of bacterial DNA were first discovered in the mid-1980s by several Japanese research groups. Tokunaga *et al.* (1984) digested *Mycobacterium bovis* and found that the streptomycin sulfate (SM) precipitated fraction displayed anti-tumor activity *in vitro*. This SM fraction was further purified into several sub-fractions and the MY-1 sub-fraction was found to be immunologically active. The composition of MY-1 fraction is: 70% DNA, 28% RNA, 1.3% protein, 0.2% saccharides, and 0.1% lipid. Multiple injections of the MY-1 fraction caused tumor regression in guinea pigs. To determine which component of MY-1 induced immune stimulation, Tokunaga *et al.* (1984) digested the fraction with DNase and RNase enzymes. They demonstrated anti-tumor activities in RNase treated samples while, in contrast, the anti-tumor activity was abolished following DNase treatment. This clearly demonstrated that DNA was the active component of MY-1.

To characterize its activity further, Shimada *et al.* (1985) examined the effects of MY-1 in mice. They showed that mice injected with MY-1 demonstrated a marked reduction in both tumor size and metastases. Also illustrated was a 100% survival rate in mice treated with repeated injections of high concentration of MY-1 into subcutaneous tumors and the numbers of tumors at distant sites were reduced after subcutaneous injections of MY-1. Interestingly, the researchers showed that the MY-1 activity was not due to direct cytotoxicity, as incubation of the fraction with both normal cells and various tumor cells caused no increase in cell death in either group.

To determine which sequences of bacterial DNA were immunostimulatory, Tokunaga *et al.* (1992) digested the DNA and then examined the effects of digested DNA on natural killer (NK) cell activity *in vivo*. They showed that only oligomers greater than 15 bases were active and that these immunostimulatory sequences were palindromes that centered on a CpG motif. These initial experiments demonstrated the ability of bacterial DNA, rather than other biologically active bacterial components, to stimulate cells and that activity of bacterial DNA was likely related to the presence of short DNA sequences containing CpG motifs.

1.2 Introduction to DNA Vaccines

The development of DNA vaccines was a consequence of work directed at improving post-natal gene therapy. Early gene therapy research was aimed at removing cells from tissue, transfecting these cells with viral vectors and then re-implanting them into the host (Friedmann, 1989). It was during this period that the seminal experiment by Wolff *et al.* (1990), demonstrated the uptake of naked DNA and the expression of encoded genes *in vivo*. The researchers injected DNA vectors expressing different reporter genes into skeletal muscle of mice. It was shown that the level of gene expression in muscle fibers was strong and comparable to gene expression in fibroblasts, with protein expression within muscle persisting for at least two months.

The definitive experiment, proving that injection of plasmid vectors was a suitable method for inducing immunity, was performed by Ulmer *et al.* (1993). Mice injected intramuscularly (im) with plasmid encoding influenza A nuclear protein developed protective immunity against an aerosolized viral challenge. The plasmid treatment generated both antigen-specific cell-mediated (CMI) and humoral immune responses, with the CMI response providing disease protection. These initial experiments demonstrated that injected plasmid DNA could indeed express encoded genes at a level sufficient to induce protective immunity.

1.2.1 Potential Benefits of DNA Vaccination

DNA vaccines offer several distinct advantages over current killed and attenuated (modified live) vaccines. These benefits include the development of strong humoral and cell-mediated responses. The ability to immunize against multiple pathogens will be discussed in the following section. It is noteworthy that, although DNA vaccination is a potential weapon in the arsenal for disease prevention, currently no DNA vaccine has out-performed any conventional vaccine (Manickan *et al.*, 1997). Although there are many examples of DNA vaccines inducing strong humoral and CMI responses as well as generating protective immunity, it will be shown that there are also examples of DNA vaccine failure.

1.2.1.1 Antigen-specific Humoral and Cell-Mediated Responses

DNA vaccines can induce high antibody titers that persist for long periods of time. Using a vector encoding influenza A nuclear protein, Ulmer *et al.* (1993) injected 100 μg of plasmid into the quadriceps muscles of mice at birth and at three and six weeks of age. Enzyme linked immunosorbent assay (ELISA) for nuclear protein (NP) demonstrated a three-log increase in serum antibody titer at the fifth week of age. To determine whether anti-NP antibodies were protective, the researchers took serum antibodies from plasmid DNA treated mice and passively immunized naive mice exposed to live virus. A reduction in virus load was not detected in mice following viral challenge suggesting that antibodies generated after plasmid injection were ineffective at controlling virus growth. In contrast, the transfer of serum antibodies from mice exposed to whole virus to naive mice resulted in a marked reduction in virus load following challenge. The likely explanation for this observation is that neutralizing antibodies were specific to viral proteins other than the internal structural NP.

In further studies to determine whether plasmid-based vaccines can generate protective antibodies, mice were injected with a plasmid expressing a strain-specific

hemagglutinin A (HA) protein. In these mice, antibody titers remained elevated for at least 1.5 years and after several 1 μg injections of DNA encoding HA, all mice survived viral challenge (Deck *et al.*, 1997). This study, however, did not determine if a CMI developed after injection and therefore it cannot be assumed that protection arose from humoral immunity alone.

Similar to viral infections or vaccination with live attenuated vaccines, DNA vaccines can generate antigen-specific CMI *in vivo*. Protective cell-mediated immunity was highlighted in a vaccine study in mice conducted by Ulmer *et al.* (1993). To determine whether protective immunity was caused by production of antigen-specific cytotoxic T lymphocytes (CTL), spleen T cells were isolated from DNA-immunized mice and stimulated with IL-2. These cells were then incubated with either virally-infected autologous cells or cells loaded with MHC-restricted NP peptides. There was marked lysis of spleen cells with both treatments, indicating that CTL responses occurred and were indeed antigen-specific.

The experiments by Deck *et al.* (1997) and Ulmer *et al.* (1993) indicated that DNA based vaccines have the ability to generate long-term protection in mice. Although DNA vaccines can induce protective immunity against certain pathogens, DNA vaccines have not been able to induce sustained immune responses and protection against all pathogens as initially anticipated. As an example, injection of a DNA vaccine against rotavirus in mice, was ineffective in disease protection. Choi *et al.* (1998), showed that the intradermal injection of plasmids encoding either the viral protein (VP) 4 or VP7 of rotavirus produced moderate serum IgG antibody titers and low levels of neutralizing fecal IgA antibodies. Neither plasmid treatment, however, reduced viral shedding during the 7-day challenge period.

Immune responses induced by DNA vaccines can also vary substantially among different species. DNA vaccines expressing the gene for the sporozoite protein of

Plasmodium falciparum were injected into inbred and outbred strains of mice and into 20 humans (Le *et al.*, 2000). All treated mice developed robust antibody responses as early as 10 days post-treatment. In comparison, the human volunteers failed to develop detectable antibody titers over a 12-month period. These two examples illustrate the inability of some antigens to elicit adequate protective immune responses, and the variability of immune responses generated to DNA vaccines between two different species.

1.2.1.2 Immunization Against Multiple Antigens

Another potential advantage of using DNA vaccines to protect against disease is the relative ease of combining plasmids that express various antigens into a single vaccine formulation. Using such a vaccine, a clinician could immunize individuals against multiple pathogens with one injection. A detailed study by Braun *et al. (*1998) examined the effects of combining different DNA vaccines on antibody production. Mice were given a single intradermal injection of individual plasmids expressing glycoprotein B (gB) and D (gD) of bovine herpesvirus-1 (BHV-1), the hemagglutinin/neuraminidase of bovine parainfluenza-3 virus (PI_3) or the hemagglutinin of human influenza virus (HINF). Antibody levels generated by these mice peaked between Day 70 and Day 95 post-vaccination and were primarily IgG1 isotype, with the exception that immunization with plasmid expressing HINF generated IgG2a antibodies. To determine if the addition of a second plasmid would alter antibody titers, mice were injected simultaneously with two different plasmids. The data demonstrated that a slight decrease in total antibody production levels occurred suggesting that co-administration of plasmid has little adverse effect on total antibody production. In addition, the co-administration of different vectors did not change the isotype of antibodies produced. There was one exception, however, where the mixing of plasmids expressing HINF and gD modified the predominantly IgG2a antibody response to a predominantly IgG1 response in treated mice (Braun *et al.*, 1998).

Co-administration of plasmids encoding different antigens therefore was shown to elicit strong antibody responses. Unfortunately, this study did not determine if antibody responses were protective.

Neonates of domestic species and human babies are highly susceptible to infectious agents and often succumb to disease. It would therefore be beneficial to vaccinate newborns early in life and render them less susceptible to infection. The presence of maternal antibodies and the perception that neonates are susceptible to antigen-specific immunotolerance have created obstacles to the development of vaccines for the neonate. DNA vaccines appear able to surmount these problems and induce both antigen-specific humoral and CMI. A study by van Drunen Littel-van den Hurk et al. (1999) showed that lambs can overcome the presence of maternal antibodies and develop immune responses after DNA vaccination. Ewes, between 75 to 95 days gestation, were vaccinated with either a plasmid expressing gD (pSLIAgD) or a null plasmid control. The newborn lambs were then vaccinated with pSLIAgD at three days of age and antibody levels were evaluated. All lambs were shown to be immunologically competent, as lambs from ewes treated with the null plasmid generated modest levels of gD specific antibody titers. The lymphocytes of lambs from pSLIAgD immunized ewes also generated strong antigen-specific T cell proliferative responses *in vitro*. This investigation indicated that the presence of maternal antibody did not prevent the development of a CMI response (van Drunen Little-van den Hurk et al., 1999).

Not all DNA vaccines will generate protective immunity in neonates in the presence of maternal antibodies. As an example, neonatal mice from hyper-immune dams were injected with plasmids that express HA and NP of influenza virus. The neonates failed to generate HA specific antibodies but could develop high levels of NP specific antibodies. Strong CTL responses were induced by both antigens, however mice with low levels of HA antibodies succumbed to virus challenge. It was suggested

that the development of protective antibody was essential for survival and the presence of maternal antibodies specific for HA antigen lead to vaccine failure (Pertmer *et al.*, 2001).

1.2.2 Enhancement of Immune Responses to DNA Vaccines

For an extensive review on methods to enhance efficacy of DNA vaccination see Gurunathan *et al.* (2000). Ultimately, an effective vaccine should stimulate long-term immunity and memory that provides disease protection. Unfortunately, DNA vaccines have not been the 'magic bullet' as many scientists hoped and there are many examples of vaccine failure after inoculation (Richardson *et al.*, 1997; Choi *et al.*, 1998; Le *et al.*, 2000). Various approaches to improve vaccine efficacy have therefore been undertaken. These include the addition of viral promoters and intron sequences to vectors (section 2.2.3 and 2.2.5), the addition of CpG immunostimulatory sequences to plasmids (section 2.10.2), the expression of cytokines and co-stimulatory molecules, vaccine boosting, enhanced delivery to MHC, and gene manipulation.

1.2.2.1 Plasmid Encoding Cytokines and Co-stimulatory Molecules

Plasmid-based vaccines encoding cytokines or antigen presenting cell co-stimulatory surface molecules can be used to improve immune responses. This can be accomplished by either co-administrating two or more plasmids, or pre-immunization with plasmids used to improve adjuvanticity. Leachman *et al.* (2000) demonstrated that pre-injection of plasmid DNA expressing granulocyte-macrophage colony stimulating factor (GM-CSF), improved immune responses to subsequent DNA vaccination against rabbit papillomavirus. Immunization with the plasmid-expressing antigen alone caused a 50% reduction in skin lesions of rabbits challenged with virus. In comparison, pre-treatment with a GM-CSF expressing plasmid followed by injection of the plasmid encoding viral antigen prevented the formation of skin lesions in 67% of the challenged animals.

Furthermore, there was a delay in the onset of skin lesions and a 99% reduction in the total volume of papillomas in the pre-treatment group.

In experiments by Tripp *et al.* (2000), mice were treated with plasmids expressing CD40 ligand and G protein of respiratory syncytial virus (RSV). These mice were then challenged with RSV and their immune functions assessed. *In vitro* assays on pulmonary T lymphocytes demonstrated an approximate two to four-fold increase in the production of Th1 cytokines (IL-2 and INF-γ) and a 30% increase in nitrous oxide levels. There were also improved CTL responses, a seven-fold increase in antigen-specific antibody titers and a 50% increase in virus clearance.

1.2.2.2 Boosting with Protein or Live Virus

Although DNA vaccines can generate both antigen-specific antibody and CTL responses *in vivo*, these responses often do not provide disease protection (Choi *et al.*, 1998; Le *et al.*, 2000). Immune responses can be enhanced by boosting DNA-immunized animals with either protein or live virus. Monkeys inoculated with DNA vectors expressing HIV-1 envelope protein developed modest levels of neutralizing antibody and weak *in vitro* CTL responses. In comparison, if DNA-primed monkeys were subsequently given HIV-1 glycoprotein, then there was a substantial increase in both humoral and CMI responses. These monkeys had developed protective CTL responses and were protected against challenge with SIV/HIV chimeric virus (Letvin *et al.*, 1997).

Another approach to enhancing DNA vaccines is to combine DNA vaccination with live virus vector immunization. Mice were immunized with a DNA vaccine expressing malarial circumsporozite protein and then inoculated with vaccinia virus expressing the same protein. Mice receiving the DNA vaccine developed poor to moderate non-protective antibody responses. In contrast, the majority of mice boosted with either virus expressing antigen or the original DNA vaccine were protected from protozoal challenge, with a survival rate reaching 69% (Sedegah *et al.*, 1998).

1.2.2.3 Manipulation of Genetic Sequences and Type of Antigen Expressed

The immunogenicity of antigens within DNA vaccines may be enhanced by genetically altering the expressed gene. Replacement of several codon sequences of g120 from wild type HIV-1 with sequences from different highly expressed human genes resulted in a significant increase in the *in vitro* expression of the mutated (sp 120) when compared to wild type g120 (Andre *et al.*, 1998). Intramuscular injection of plasmid with sp120 gene induced a five and six-fold increase in CTL and antibody levels, respectively.

The selection of the proper antigen for use in DNA vaccines is critical for the development of protective immunity in animals. If non-protective antigens are expressed in DNA vaccines, this could lead to vaccine failure and the subsequent death of challenge animals. As an example, plasmids expressing structural proteins of Japanese encephalitis virus resulted in disease protection against virally challenged mice. These mice developed antibody profiles that vary from Ig2a alone to a more balanced IgG1/IgG2a ratio. In contrast, if these were immunized with plasmid expressing non-structural proteins, mice succumbed to the disease (Chen *et al.*, 1999a). This example highlights the importance of encoding protective antigens in DNA vaccines.

1.2.2.4 Improved Antigen Targeting and Enhanced Delivery to MHC

Immune responses can be improved by designing vaccine delivery systems that target antigens to either target lymph nodes (LN) or antigen-presenting cells (APC). These DNA vaccines are developed as fusion proteins that express antigen and targeting molecules. For example, DNA vaccines encoding L-selectin can target the high endothelial venules (HEV) of LN while expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) targets antigen to APC. When these modified vectors were injected into mice and serum antibody levels were measured, the researchers demonstrated a 75 and 100-fold increase in antigen-specific antibody production in mice treated with L-selectin and CTLA-4 vaccines, respectively (Boyle *et al.*, 1998).

Similarly, targeting antigens to cell organelles involved in antigen processing can enhance immune responses. Proteosomes are involved in the digestion of cytoplasmic proteins and it has been hypothesized that targeting DNA vaccine antigens to these structures will facilitate protein digestion and result in improved antigen-presentation by MHC. Since ubiquitin directs intracellular proteins to proteosomes, Wu & Kipps (1997) constructed a plasmid expressing an ubiquitin-β-galactosidase chimera and injected it into mice. They measured gene expression *in vitro* and the *in vivo* production of antigen-specific antibodies and CTL. Treatment with proteosome inhibitors caused a 150% increase in β-galactosidase expression, confirming that the chimeric protein was indeed targeted to proteosomes for degradation. Antigen-specific CTL activity was 60% greater in mice treated with plasmid expressing the chimeric protein compared to mice immunized with plasmid expressing β-galactosidase alone. It is noteworthy that antigen-specific antibodies were not detected in mice immunized with plasmid expressing chimeric protein but were detected in mice treated with the plasmid expressing β-galactosidase. The researchers hypothesized that rapid intracellular degradation of antigen caused a reduced amount of peptide available for the induction of an antibody response. This method of antigen targeting therefore may be of value for inducing CMI but not humoral immunity.

1.2.3 Delivery Systems for DNA Vaccination

Various methods to enhance DNA vaccine uptake and improve the induction of an immune response have been developed. These methods include the direct intracellular delivery of plasmid DNA (gene gun) and the use of various lipid formulations and microbial carriers to improve mucosal delivery. These techniques will be discussed in the following sections.

1.2.3.1 Gene Gun

The direct transfer of plasmid DNA to cells can potentially enhance cell transfection and facilitate the induction of immune responses. To achieve this goal, DNA can be delivered intracellularly by ballistic delivery (gene gun) of plasmid annealed to gold particles. Delivery of plasmids into cells should reduce the amount of plasmid lost to enzymatic degradation by tissue DNases and thereby increase the amount of plasmid available to induce immunological products. For instance, the gene gun delivery of a plasmid expressing a malarial antigen produced better immunity in mice than intramuscular (im) injection. Ballistic delivery of plasmid was associated with a six-fold increase in antibody production after three treatments and promoted a switch from a predominately IgG1 immune response to a more balanced IgG1/IgG2a antibody response. Furthermore there was a 30% increase in survival following challenge of the gene gun-immunized mice when compared to im immunized mice (Leitner *et al.*, 1997).

Gene gun vaccination has also been used to induce strong immune responses in domestic animals. Ballistic delivery of pLSIAtgD plasmid increased IFN-γ secretion from collected peripheral blood mononuclear cells, increased neutralizing antibody titers and produced strong proliferative responses. After challenge with BHV-1, vaccinated cattle had increased levels of nasal and serum neutralizing antibodies as well as decreased viral shedding relative to controls (Braun *et al.*, 1999). Unfortunately, a direct comparison between gene gun and other DNA vaccine delivery methods was not addressed in this study.

1.2.3.1 Encapsulation of Plasmid DNA

Encapsulation of DNA within polymers or liposomes can improve vaccine delivery to mucosal surfaces (Jones *et al.*, 1997; Klavinskis *et al.*, 1997). The environment within the gut or respiratory tract can limit the efficacy of inducing immune responses following DNA vaccination. The low pH of the stomach, abundant pancreatic enzymes within the

intestine, and the presence of cilia within the trachea can lead to either the rapid breakdown or clearance of plasmids. One method to overcome some of these 'hazards' is to encapsulate the vaccine prior to delivery. Plasmid encapsulated in poly D L-lactide-coglycolide microspheres was given orally to mice: relative to intraperitoneal injected controls, the oral delivery of 50 μg of encapsulated plasmid caused an approximate 3-, 4- and 7-fold increase in serum IgA, IgM and IgG antibody levels, respectively. A 5 to 20-fold increase in IgA levels was also present in stool samples after treatment. Intradermal injection of microparticle DNA complexes similarly induced markedly enhanced antibody response compared to naked DNA (Jones *et al.*, 1997).

Encapsulation of plasmid in liposomes provides another method to help ameliorate plasmid uptake and vaccine efficiency. The ability to design liposomes that vary in composition, size and number of lipid bi-layers makes liposomes an ideal delivery system (Swenson *et al.*, 1988). In mice, when gene expression in nasal epithelium was compared between plasmid DNA-lipid complexes and naked DNA, a 30-fold increase in gene expression was shown in the DNA-lipid treatment group versus naked controls, indicating that liposomes do indeed enhance plasmid uptake (Klavinskis *et al.*, 1997).

The administration of liposome-DNA complexes to animals, however, does not consistently improve the induction of antigen-specific immune responses. As an example, injection of liposomes encapsulating plasmid expressing *Schistosoma japonicum* into mice, failed to produce antigen-specific antibodies. In comparison, injection with unencapsulated plasmid resulted in strong antibody responses that lasted for eight weeks (Zhang *et al.*, 2000). The researchers did not measure CTL and neither treatment reduced parasite egg shedding.

1.2.3.3 Use of Microorganisms to Deliver DNA

Delivery of DNA vaccines can be enhanced with use of attenuated microorganisms to carry plasmid DNA into cells. Using its natural route of entry into

cells, the microbe should enhance plasmid uptake. Bacteria that carry plasmid can be delivered to the gut, enter through M cells and be picked up by APC that reside in Peyer's patches. In this manner a vaccine can be delivered in a relatively specific fashion to immunocompetent cells. Mice were orally immunized with a strain of attenuated salmonella, containing a plasmid that expressed a glycoprotein of *L. monocytogenes*. After a single oral immunization, all mice developed strong CTL and proliferative responses and had elevated levels of antigen-specific antibody. In a challenge study, 60% of the mice survived following immunization with the attenuated salmonella vector as compared to null vector controls (Darji *et al.*, 1997). This suggests that microbial delivery may be an effective method to develop mucosal immunity.

Alphaviruses are single stranded RNA viruses whose mechanism of intracellular replication has the potential to produce vast quantities of mRNA. Exploiting this phenomenon, Hariharan *et al.* (1998) engineered a new plasmid expressing gB of herpes simplex virus-1 (HSV-1) and compared the alphavirus modified plasmid to a conventional DNA vaccine containing a HCMV promoter. A single treatment with 10 ng of the modified plasmid produced responses that were equivalent to immunization with 10 μg of conventional DNA vaccine. Alphavirus DNA vectors are, therefore, potent and effective inducers of immune responses.

As our understanding of the potential uses for DNA vaccines expands, methods to further optimize vaccination efficacy are needed. Improvement in plasmid construct, the types of antigens expressed and vaccine formulation can enhance immune responses following DNA vaccination.

1.2.4 Routes of Plasmid Delivery

Plasmid delivery has been achieved by a variety of routes to elicit long lasting and often protective immunity. These routes of delivery include intramuscular (im), subcutaneous (sc), intravenous (iv), intraperitoneal (ip), intradermal (id), and topical. To

obtain mucosal immunity, routes of delivery include oral, intranasal, vaginal, and ano-rectal. Each of these delivery routes has been investigated in the effort to improve immune responses either systemically or at mucosal surfaces.

1.2.4.1 Intramuscular Delivery

Original reports regarding gene expression in tissue after DNA vaccination (Wolff *et al.*, 1990) and disease protection (Ulmer *et al.*, 1993) were made after plasmid was injected into the quadriceps muscles of mice. These experiments demonstrated that DNA immunization was possible and that im injection was a potential route of delivery. Davis *et al. (*1994) showed that pre-injection with hypertonic sucrose induced muscle inflammation and low levels of muscle necrosis and that pre-treatment with irritants, five to 10 days prior to DNA injection, caused a 10-fold increase in gene expression and antibody production. They proposed that regenerating muscle acquires more plasmid than normal muscle and thereby generates greater levels of expression.

There is great potential for implementing DNA vaccines in the livestock industry. The method of vaccine delivery is an important consideration for food-producing animals and certain delivery methods such as im injection might not be acceptable in the future. As an example, im injection of DNA vaccines can potentially cause significant muscle necrosis at the injection site and damage could reduce meat quality at slaughter. Therefore, other methods of vaccine delivery that do not compromise vaccine efficiency should be developed for livestock.

1.2.4.2 Subcutaneous, Intravenous and Intraperitoneal Delivery

Other methods of vaccine delivery have been explored due to limited success of im injection. The efficacy of plasmid-based vaccination for the induction of immune responses against varicella zoster was tested by injecting mice im and sc with a plasmid expressing glycoprotein E. Both methods of delivery induced strong and comparable antibody responses that peaked at week 4 and remained elevated up to 12 weeks post-

treatment (Hasan *et al.*, 2000). This study proved that sc and im routes of delivery were equally effective for the induction of an immune response.

Intravenous delivery of DNA vaccines is another approach that can be used to induce immune responses. The iv injection of plasmid expressing HA of influenza induced antigen-specific antibodies and protection during a mouse challenge study with influenza virus (Fynan *et al.*, 1993). This study demonstrated markedly elevated levels of serum IgG antibodies and a small increase in IgA levels post-plasmid treatment. The DNA vaccine induced protective immunity, as 95% of virus challenged animals survived.

Intraperitoneal injection of plasmid DNA may be the least effective delivery method to induce protective immunity. In both mice and chickens, ip injection of plasmid expressing influenza HA protein failed to induce protection against influenza viral challenge and all challenged animals succumbed to illness following plasmid injection. In comparison, 95% of the animals given plasmid by either im and iv routes survived (Fynan *et al.*, 1993). It is noteworthy that ip delivery of plasmid can produce a strong antibody response if the plasmid is administered with microspheres (see section 2.8.1).

1.2.4.3 Topical and Intradermal Delivery

Delivery of DNA vaccines to the skin offers several advantages over parenteral delivery methods. Ease of application, the potential release of immune stimulating-cytokines from epithelial cells and the presence of antigen presenting cells such as Langerhans cells are all potential advantages of dermal delivery.

Expression of plasmid-encoded genes *in vivo* has been documented after topical application of naked plasmid DNA. In mice, Fan *et al.* (1999) demonstrated that gene expression could be detected after exposure to naked plasmid DNA. Interestingly, detectable gene expression was limited to hair follicles. The researchers showed that a small percentage of cells surrounding hair follicles were able to take up DNA and express the encoded gene. They speculated that plasmid DNA may enter the skin through

epithelial cells located within pilosebaceous units and proposed that hair follicles in the anagen phase may incorporate more DNA than resting follicles.

Intradermal delivery is an excellent method of plasmid administration as large quantities of DNA can be delivered into a relatively small area that includes large numbers of APC, keratinocytes and dermal macrophages. One of the initial studies demonstrating that intradermal vaccination induced a good level of protection against viral infection was undertaken by Fynan *et al.* (1993); however, the level of protection was not as effective as im injection. With further developments, it has become apparent that id delivery induces immune responses more effectively than delivery by im injection. Raz *et al.* (1994) showed that, in mice, id injection of plasmid expressing influenza HA antigen induced greater antibody and CTL responses than im injection of the same amount of plasmid. The id injection cross-protected mice from challenge with a viral strain that differed from the virus antigen used in the DNA vaccine. Unfortunately, a challenge study in mice comparing levels of protection following im and id infection was not completed. Therefore, it remains to be determined which form of delivery induces optimal protection.

Similar observations have been made when comparing id and im DNA vaccinations in cattle. In calves, intradermal injection of plasmid expressing the secreted form of BHV-1 glycoprotein D (tgD) induced higher neutralizing antibody titers when compared to im injection. The intradermal injection of the DNA vaccine also improved clinical scores of calves challenged with BHV-1. These calves were less febrile, had better weight gain and less viral shedding than controls (van Drunen Littel-van den Hurk *et al.*, 1998). Unfortunately, no comparable challenge studies have been done in im vaccinated calves. Therefore, while a direct comparison between the efficacy of different delivery methods has not been performed, id vaccinated calves did develop higher antibody titers with fewer injections than im injected calves.

1.2.5 Mucosal Immunity

The development of a strong mucosal immune system can be an important component in host defense. Major pathogens that cause morbidity and mortality in both man and domestic livestock invade the body at mucosal surfaces. As many parenteral vaccines are relatively ineffective at inducing immune responses at mucosal surfaces, it is essential that vaccines be designed to specifically bolster mucosal immunity. Through a common immune system that links the various mucosal surfaces, delivery of vaccines at one mucosal site should induce immune responses at distant mucosal sites. In attempts to develop mucosal immunity, DNA vaccines have been delivered to respiratory, gut and vaginal mucosa.

1.2.5.1 Oral and Intranasal Delivery

As previously mentioned, the development of immunity following oral delivery of DNA vaccines was first described by Jones *et al.* (1997) (see section 2.6.2). This study demonstrated that oral delivery of DNA vaccines could generate antigen-specific antibody responses at distant mucosal sites.

Recently, strong systemic and mucosal responses were observed following a single oral vaccination of fetal sheep. Gerdts *et al.* (2000) demonstrated that delivery of naked DNA into the oral cavity of fetal sheep induced both antigen-specific CMI and humoral responses *in vivo*. It remains to be determined if oral immunization of the fetus can be applied to other species, particularly humans.

Successful intranasal vaccination with naked DNA was first achieved in mice. Intranasal vaccination against influenza virus induced moderate protection with 76% survival rate after viral challenge (Fynan *et al.*, 1993). Since then, researchers have tried to improve mucosal immunity by increasing IgA antibody levels at mucosal surfaces. Using liposome-DNA complexes, both systemic and mucosal immune responses were induced after intranasal administration. In mice, there was a 50% to 80% increase in

CTL activity in cells isolated from the spleen and iliac LN as well as 2 and 60-fold increases in serum IgA and IgG antibody titers, respectively. Four and eight-fold increases in IgA and IgG antibody levels were also detected in vaginal secretions (Klavinskis *et al.*, 1997). These results indicate that intranasal vaccination can induce immunity at various mucosal sites throughout the common mucosal immune system.

Although immunization at nasal mucosal surfaces should elicit immune responses at all mucosal surfaces, this does not always occur following DNA vaccination. Intranasal treatment of mice with lipid complexes of plasmid DNA expressing various membrane proteins of *Chlamydia trachomatis* failed to induce protective immunity. Immunization with plasmids induced modest antibody and CTL responses, but failed to reduce vaginal shedding of the microbe or improve fertility in challenged mice (Pal *et al.*, 1999).

1.2.5.2 Genital and Ano-rectal Delivery

The application of plasmid DNA vaccines to the vaginal and rectal mucosa is potentially convenient method for immunization. Topical application of plasmid DNA to vaginal mucosa and subsequent gene expression was described in mice by Wang *et al.* (1997). To test the adequacy of antigen-specific mucosal immunity, IgA levels were measured in vaginal swabs. This analysis demonstrated an increase in both the amount of IgA secreted and the level of neutralizing antibody present after DNA vaccination.

Delivery of plasmid DNA via the genital tract may offer not only a convenient route for vaccine delivery in domestic animals, but may also induce effective mucosal immunity. To evaluate and compare the efficacy of vaginal plasmid DNA delivery, the plasmid pSLIA-tgD was injected either id or intravaginally (ivn) by gene gun delivery in cattle. Though not significantly different, ivn injected cattle had lower clinical scores than id vaccinated animals following BHV-1 challenge. Furthermore, there was a substantially greater increase in serum antibody titers after ivn injection than that observed for either id injected cattle or non-immunized cattle (Loehr *et al.*, 2000).

Ano-rectal delivery is another potential route for the development of mucosal immunity. Using a gene gun to inoculate the ano-rectal mucosa with VP6 antigen of rotavirus, Chen *et al.* (1999b) detected a two-fold increase in the level of antigen-specific IgG antibody in serum and a moderate increase in intestinal IgA levels. DNA immunization also induced modest levels of protection, with reduced viral shedding, in challenged mice.

As previously indicated, the delivery of DNA to one mucosal surface does not consistently induce immunity at distant mucosal sites. For example, the delivery of plasmid expressing papillomavirus antigen to the rectal mucosa failed to induce either serum antibodies or neutralizing antibodies at the vaginal mucosa. In contrast, vaginal delivery of the same plasmid produced high serum antibody titers and moderate levels of neutralizing antibodies in vaginal secretions (Schreckenberger *et al.*, 2000).

Although there are examples of vaccine failure, in general the delivery of DNA vaccines to mucosal surfaces often generates protective and relatively long-lasting immunity. The delivery of DNA vaccines to mucosal surfaces may have other limitations. Delivery of intranasal vaccines to cattle can be particularly challenging: the necessity for restraint of the head or the expulsion of vaccine in nasal secretions may make this route of delivery impractical. Furthermore, the delivery of DNA vaccines to vaginal or rectal mucosa may require further development of specialized delivery systems.

DNA vaccination has been hailed as the third generation of vaccines (Butts *et al.*, 1998), with the potential to revolutionize conventional vaccine technology. Although DNA vaccination hold great promise for providing immunoprophylaxis against infectious diseases, its efficacy at inducing protective immunity remains inconsistent. I have highlighted several examples of vaccine failure that may be related to either the type of antigen expressed or to the route or method of delivery. Thus, further work is needed to

optimize individual DNA vaccines to ensure consistent induction of protective immunity.

1.2.6 DNA Vaccine Induced Antigen Presentation

For DNA vaccines to induce an immune response, proteins encoded within the plasmid must be expressed, processed and presented to immunologically competent cells. Frequently, the quantity of protein produced after DNA vaccination is miniscule (Gurunathan *et al.*, 2000); therefore, an efficient method for antigen processing and presentation is needed to ensure the induction of strong and protective immune response. Three possible pathways by which antigen presentation can occur following DNA vaccination have been proposed and these are discussed below.

1.2.6.1 Dendritic Cell Activation

One hypothesis is that dendritic cells (DC) take up plasmid and present endogenously expressed antigen in an MHC-restricted fashion. Evidence for DC as the primary antigen-presenting cell has been generated through several separate experiments. In a study by Casares *et al.* (1997), plasmid DNA encoding antigen was injected im into mice and then the draining LN was removed. The LN cells were isolated and then sorted by flow cytometry, into individual cell populations. Purified DC were then cultured with T cell hybridoma cells and this caused significant T cells activation. In comparison, cultures of LN B cells and keratinocytes isolated from the DNA injection site with the same T cells did not induce T cell activation. These data suggested that DC rather than B cells or keratinocytes presented antigen following plasmid injection.

To further identify the cell types involved in antigen-presentation after vaccination, Condon *et al.* (1996) injected into mice a plasmid expressing the gene for green fluorescent protein (GFP) of jellyfish. Using differential interference contrast microscopy, it was shown that cells with a dendritic morphology expressed the protein, but it was not conclusively demonstrated that the cells expressing GFP were indeed DC. Cells such as fibroblasts can take up plasmid and express protein and these cells often display a

dendritic-like morphology (Wolff *et al.*, 1990). It is likely, however, that the majority of GFP expression was produced by dendritic cells with a potentially smaller portion of protein expression in stromal fibroblasts.

1.2.6.2 The Role of Non-Professional Antigen Presenting Cells

As previously described, DNA vaccines injected im are expressed in myocytes (Wolff *et al.*, 1990; Davis *et al.*, 1994) may induce protective immunity (Ulmer *et al.*, 1993). It is difficult to determine, however, the mechanism by which an antigen expressed in a somatic cell can induce an immune response *in vivo*. Unlike APC, muscle cells and other somatic cells appear to lack the co-stimulatory proteins that function as important second signals during the generation of an adaptive immune responses. It is therefore thought, based on evidence from several experiments that antigen presentation is unlikely to occur via somatic cells.

Chimeric crossbred mice lacking MHC-1 expression on APC, were used to demonstrate that myocytes could present antigen when co-stimulatory proteins were expressed on their surface. The myocytes of F1 hybrid mice were co-administered with plasmids encoding the co-stimulatory CD86 protein and an antigen and myocytes expressing CD86 were able to present antigen (Agadjanyan *et al.*, 1999). It is noteworthy that expression of CD86 was verified by immunohistochemistry and myocytes lacking CD86 expression failed to induce an immune response, thus demonstrating the requirement for co-stimulatory molecules.

To further evaluate the role of muscle cells as potential antigen presenters, two groups of mice were injected im with plasmid DNA and the resulting immune responses evaluated. The injection site was excised from the first group of mice within one minute post-inoculation, while the injection site of the second group remained intact. Both groups of mice developed similar humoral responses. This observation suggests that antigen expression in muscle cells was not essential for the induction of a humoral

immune response. In contrast, removal of the overlying skin 24 hours following id plasmid injection abrogated antibody responses. The authors hypothesized that cells residing in the skin were responsible for the induction of antibodies (Torres *et al.*, 1997). They suggested that either direct transfection of Langerhans cells or the activation of keratinocytes with the subsequent enhancement of Langerhans cell function resulted in the induction of an immune response.

Interestingly, unique co-stimulatory molecules have been detected on non-professional antigen presenting cells. Cells such as keratinocytes, synovial fibroblasts and enterocytes express proteins that can function in lymphocyte activation. Stimulation of CD6 which is expressed on both immature and mature cells in mice and on breast carcinoma cell lines, by CD6 ligand can lead to T cell activation. CD6 ligand is an inducible protein that has been detected on skin epithelium. Therefore, it has been suggested that skin could assist in T cell activation along with appropriate professional antigen presenting cells (Wee *et al.*, 1994). Co-stimulatory molecules are also constitutively expressed on keratinocytes surfaces. CD24 (nectadrin), for instance, can be detected on cultured human keratinocytes grown in minimal media. Nectadrin is believed to be homologous to a heat-stable antigen, an antigen that delivers a co-stimulatory signal to T cells leading to T cell activation. It is possible therefore, that nectadrin could also play a role in T cell activation. Further studies are needed however, to determine the function of nectadrin *in vivo* (Redondo *et al.*, 1998).

1.2.6.3 Transfer of Antigen to Antigen-Presenting Cells

The phenomenon of antigen transfer from one cell to another could explain the initial observations that CTL and humoral responses develop after im injections with DNA vaccines. Transfected somatic cells could be the source of expressed antigen that is then acquired by APC to induce an immune response. This idea was delineated by Ulmer *et al.* (1996). Using F1 hybrid mice, from parents with different MHC-restricted

haplotypes, it was shown that somatic cells transferred antigen to professional APC. Myoblasts from F1 hybrid mice that were expressing antigen and were restricted to one of the parents (H^D) were injected into the other parent (H^K). The recipient mouse could generate an effective CTL indicating that antigen expressed on MHC-I of myoblasts was passed to APC as CTL responses were restricted to the MHC haplotype of antigen-presenting cells not that of myoblasts.

1.3 Introduction to Immunostimulatory Oligodeoxyribonucleic Acids

As previously discussed, work in the 1980's demonstrated that bacterial DNA displayed immunostimulatory properties both *in vitro* and *in vivo*. It became apparent that palindromes with CpG central motifs were important in immune stimulation. Sequences causing activation in mice included: AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC and TCGCGA (Yamamoto *et al.*, 1994; Krieg, 1999). DNA sequences lacking a central C or G nucleotide did not stimulate immune responses *in vitro*. Since the 1980's, work has progressed to characterize the immune modulating properties of these short sequences of DNA and it has been shown that CpG motifs flanked by two purines upstream and two pyrimidines downstream were indeed immunostimulatory. This section will focus on the effects of oligodeoxyribonucleic acids (ODN) in the context of CpG hexamers on isolated cell preparations and the *in vivo* enhancement of immune induction.

1.3.1 *In Vitro* Immunostimulation

A variety of cell types including lymphocytes (Krieg *et al.*, 1995), NK cells (Iho *et al.*, 1999), macrophages (Stacey *et al.*, 1996) and DC (Sparwasser *et al.*, 1998) can be activated by immunostimulatory CpG motifs. The cellular responses to GpG motifs include secretion of cytokines and chemokines, enhanced cell proliferation, increased expression of activation markers and costimulatory molecules and enhanced production of oxygen reactive species within cells. Morphologically, cells such as dendritic cells

have an activated appearance with the presence of extended veil-like structures and numerous intracytoplasmic bodies. The effect of immunostimulatory CpG motifs on lymphocytes, macrophages and dendritic cells is discussed below.

1.3.1.1 B cells

The seminal experiment demonstrating direct B cell activation by immunostimulatory CpG motifs was performed by Krieg *et al. (*1995). B cells were isolated from mouse spleens and were incubated with DNA sequences containing defined CpG motifs. Optimal B cell activation was seen in ODN containing CpG preceded by two purines and followed by two pyrimidines. Nuclease-resistant phosphorothioate ODN could activate B cells at lower concentrations than phosphodiesterate ODN, suggesting that ODN with phosphodiester linkages are degraded by DNase. Increased expression of activation markers and increased cytokine production can also occur in B cells following treatment. Spleen cells isolated from mice previously injected with ODN, produced a six-fold increase in IgM antibody titers along with an approximately 50% increase in the level of MHC-II expression on B cells. There was also increased expression of the B cell activation markers B7-2 and Bla-1, but the actual level of expression was not stated. Klinman *et al.* (1996) verified the previous work by Krieg *et al.* (1995) and confirmed that ODN treatment induced B cells to secrete more IL-6 and IL-12 and produce more IgM antibody.

Immunostimulatory oligodeoxyribonucleic acid (ISODN) also inhibits apoptosis in B cells. The addition of ISODN to WEHI 231 B cells, for example, caused an approximate two-log reduction in apoptosis post-treatment. It is believed that ISODN may both down-regulate and up-regulate different proto-oncogenes. Specifically, ISODN can prevent c-myc down-regulation and can promote the up-regulation of bcl2 and bcl-xl. The interactions between these oncogene products are not fully understood, however they be important regulators of either transcription or DNA replication (Yi *et al.*, 1996).

1.3.1.2 T Cells

Unlike B cells, quiescent T cells cannot be stimulated to proliferate in culture by treatment with ISODN. Activated T cells, expressing the CD71 proliferation marker, however, displayed increased cell proliferation after ISODN treatment (Bauer *et al.*, 1999). In contrast to the apparent unresponsiveness of quiescent T cells to ISODN treatment, activated T cells readily secrete cytokines following treatment. For example, CD4 T cells harvested from mouse spleens displayed a 1.5 and 2-fold increase in IFN-γ and IL-6 cytokine production respectively, after treatment with ISODN when compared to unstimulated cells (Klinman *et al.*, 1996). In a second study using antigen-sensitized mice, it was demonstrated that CD4 T cells isolated from spleens and then incubated with ISODN produced 30-fold more in IFN-γ secretion than CD4 T cells isolated from naïve mice (Broide *et al.*, 1998).

Immunostimulatory ODN can also indirectly reduce the secretion of certain cytokines from T cells. In an eosinophil hypersensitivity model, Broide *et al.* (1998) demonstrated an 83%, 76%, and 47% reductions in T cell secretion of IL-5, IL-3 and GM-CSF, respectively, following ISODN treatment. These cytokines are involved in eosinophil production and the researchers suggested that a reduction in eosinophil numbers following ISODN treatment was likely caused by deviation from a Th2 to Th1 type immune response. From this experiment, it was difficult to determine whether T cell activation was caused by a direct ISODN stimulation or by activation of resident dendritic cells followed by the release of mediators that activate T cells. In this experiment, cells isolated from the spleen were incubated with ISODN prior to T cell purification, thus allowing time for the release of cytokines from dendritic cells. It is possible, that ISODN activates T cells both directly and indirectly through the stimulation of local antigen presenting cells.

1.3.1.3 Natural Killer Cells

Natural killer cells may also be activated by ISODN. Treatment of isolated human NK cells with ISODN caused a 4-fold increase in IFN-γ production, independent of TNF-α or IL-12 production. The addition of either IL-12 or IL-2 notably enhanced cytokine production with a further one to eight-fold increase in IFN-γ secretion (Iho *et al.*, 1999). Similar results by Klinman *et al. (*1996) demonstrated a five-fold increase in the number of NK cells secreting IFN-γ following ISODN treatment. Immunostimulatory ODN also enhances activation of NK cells resulting in the expression of activation markers. Exposure of highly purified human NK cells to ISODN caused an increase in CD69 and HLA-ABC expression (Iho *et al.*, 1999).

Adding ISODN to cultured NK cells increased NK lytic activity *in vitro*. Incubation of ISODN with NK cells, from B and T cell depleted spleen cultures, caused a 60% increase in NK cell-induced cell death (Ballas *et al.*, 1996). Interestingly, cell preparations of highly purified NK cells did not respond to ISODN and the researchers suggested that increased NK cell killing is dependent on ISODN activation of accessory cells. Using neutralizing antibodies, Ballas *et al.* (1996) demonstrated that NK cell killing depended on the presence of IL-12, TNF-α and IFN-β and IFN-γ, four cytokines known to be secreted by either macrophages or dendritic cells. The authors believed that release of these cytokines by either macrophages or dendritic cells could be responsible for NK cell activation.

It is difficult to explain the discrepancy between findings of Iho *et al.*,(1999) and Ballas *et al.* (1996). Iho and colleagues used different techniques for isolating NK cells than did Ballas et al. and thereby used a more purified population of NK cells in their experiment. This suggests that NK cells may be directly affected by ISODN, however,

the presence of minute numbers of dendritic cells or macrophages releasing cytokines to activate NK cells can not be excluded.

1.3.1.4 Macrophages and Monocytes

Macrophages play an integral role in the induction of immunity as they link the innate and acquired immune system. In view of the numerous effects of ISODN on lymphocytes, it was perhaps reasonable to assume that ISODN would affect macrophage activity and, in fact, macrophages are activated directly by ISODN to secrete various pro-inflammatory cytokines (Krieg & Wagner, 2000). Incubation of ISODN with mouse macrophages (Stacey *et al.*, 1996), human monocytes (Roman *et al.*, 1997) and isolated peritoneal macrophages from SCID mice (Sparwasser *et al.*, 1997) demonstrated low to substantial elevations in TNF-α secretion. Stimulated peritoneal macrophages had a 30-fold increase in the secretion of TNF-α when compared to unstimulated controls and LPS- treated peritoneal macrophages (Sparwasser *et al.*, 1997). Similarly, ISODN treatment induced the production of IL-1, IL-6 and GM-CSF by mouse macrophages (Sparwasser *et al.*, 1998). To examine the extent of ISODN induction of other cytokines, the mRNA levels for various cytokines expressed by monocytes were measured. The mRNA levels of IFN-γ, IL-12 and IL-18 were all increased after treatment with ISODN, signifying induction of gene expression (Roman *et al.*, 1997).

1.3.1.5 Dendritic Cells

Similar to macrophages, DCs link the innate and acquired immune systems and are activated by ISODN. Direct exposure of DCs to ISODN results in DC maturation and improved antigen presentation (Sparwasser *et al.*, 1998; Hartmann *et al.*, 1999; Krieg & Wagner, 2000). Dendritic cells, isolated from human blood and treated with ISODN displayed enhanced expression of the activation markers MHC-II, ICAM-1, B7-2 (CD86) and CD40. Immunostimulatory ODN treatment of DC also increased the life span of DCs

in culture (Hartmann *et al.*, 1999). Dendritic cell activation by ISODN has also been observed in mice. Following ISODN treatment there is a moderate up regulation of MHC-II, B7-2 (CD86) and CD40 as well as elevated secretion of IL-12, IL-6 and IFN-γ (Sparwasser *et al.*, 1998). The hallmark of DC maturation is the ability to present antigen and activate T cells. Indeed, pre-treatment of DCs with ISODN and incubation with harvested T cells caused a substantial increase in the mixed leukocyte reaction of cultured T cells, signifying T cell activation (Sparwasser *et al.*, 1998). Finally, changes in cell morphology were observed following ISODN treatment. Morphologically, DCs treated with ISODN appeared more mature with increased intracytoplasmic bodies, multi-vesicular structures and sheet-like veiled processes that extended from the cell (Sparwasser *et al.*, 1998; Hartmann *et al.*, 1999).

Direct activation of DCs is fundamental to the integration of innate and acquired immune systems. The ability of ISODN to activate DC is very convincing but refutable. In the experiments of Sparwasser *et al.* (1998) and Hartmann *et al.* (1999), DCs were isolated from a mixed cell population and were enriched to approximately 95-99% purity. It is theoretically possible therefore, that a small percentage of other cells (monocytes or lymphocytes) activated by ISODN were responsible for dendritic cell activation, although it is highly unlikely.

1.3.2 *In Vivo* Effects of Immunostimulatory Oligodeoxyribonucleic Acids

In vitro testing has provided insight into the potential mechanisms and pathways by which ISODN may activate the immune system. *In vitro* data suggest that ISODN should enhance humoral and cell-mediated responses *in vivo.* Indeed, ISODN present as tandem repeats in a plasmid, co-administered with protein antigen, or in plasmids expressing an antigen did improve these immune responses. An early study by Sato *et al.* (1996) examined the effects on antibody responses of two different plasmid constructs, one encoding kanamycin resistance, and the other ampicillin resistance. The

plasmids expressing ampicillin resistance demonstrated a 9-fold improvement in antigen-specific antibody titers and it was believed that the presence of two additional ISODN repeats within the ampicillin gene were responsible for enhanced activity. To clarify this, ISODN sequences were added to the other plasmid expressing kanamycin resistance. The addition of a single ISODN sequence elevated antibody titers by 17-fold and caused a 5-fold increase in IFN-γ production relative to non-modified plasmids (Sato *et al.*, 1996).

Roman *et al.* (1997) characterized the immunomodulatory effects in mice of free ISODN and of ISODN incorporated in plasmid. The addition of a single ISODN sequence to plasmid caused low to moderate improvement in IgG2a antibody titers as compared to mice treated with either alum and plasmid or with plasmid alone. Co-administration of free ISODN with a plasmid vaccine resulted in a marked 17-fold increase in IgG2a antibody production. Cytokine profiles also change in mice after treatment with plasmids containing ISODN, as the IL-4:IFN-γ ratio decreased from 17:1 in control groups to 2.5:1 in plasmid treated groups. Antibody responses and cytokine secretion profiles suggested that both free ISODN and ISODN inserted within plasmids reduced predominantly Th2 type immune response to a more balance Th1 and Th2 type of response.

The co-administration of ISODN and protein antigen can also improve immune responses *in vivo*. Davis *et al.* (1998) injected ISODN with hepatitis B surface antigen (HBSAg) into mice and showed an approximate10-fold increase in antibody titers as compared to mice treated with the HBSAg vaccine formulated with alum. Similarly, mice treated with HBSAg, alum and ISODN responded with an approximately 100-fold increase in antibody titers when compared to mice injected with HBSAg and alum. Interestingly, a more balanced immune response developed in mice with ISODN versus mice treated with alum alone. Alum treatment generated a predominantly IgG1, Th-2 type immune response, while ISODN combined with alum generated a relatively equal

IgG1/IgG2a antibody ratio. The combined treatment of ISODN and alum caused a 35-fold increase in total antibody titers when compared to alum treatment alone. Immunostimulatory ODN combined with alum also improved CTL responses in cells isolated from spleen. Treatment of isolated splenocytes with ISODN and alum induced an approximately 40% increase in CTL activity, while the CTL activity in spleen cells following treatment with alum alone was virtually nil.

In conclusion, ISODN either as a free form or inserted into plasmids, induce a variety of cellular responses *in vitro* and enhanced acquired immune responses *in vivo*. Thus, ISODN are being investigated as a potential adjuvant for vaccines.

1.3.3 Immunoneutralizing Oligodeoxyribonucleic Acids

The characterization of ISODN has implied that immune neutralizing-ODN (INODN) may be also present in prokaryotic and eukaryotic DNA and indeed it has been demonstrated that the addition of either eukaryotic or prokaryotic DNA to cells can reduce or eliminate immune activation. Unmethylated mammalian DNA when added to mouse B cells will not elicit cell activation or enhance proliferative responses (Sun *et al.*, 1997). It is believed that the lack of immune stimulation by mammalian DNA is not entirely dependent on the state of methylation but related to some structural component of the DNA. This idea is supported by the observation that the addition of non-CpG ODN reduces immune activation induced by ISODN. In a macrophage cell line, Sparwasser *et al.* (1998) demonstrated that non-CpG ODN compete with ISODN and block intracellular processing. This interference with DNA processing may result in a lack of activity and it seems reasonable that within biological systems, direct competition between non-CpG ODN and CpG ODN might hamper cell stimulation.

Krieg *et al.* (1998) provided the definitive proof that INODN are indeed present within biological systems. In mouse cells, these researchers showed that DNA from different serotypes of adenovirus could prevent immune stimulation. Adenovirus

serotype 2 caused a 4 to 5 fold reduction in IFN-α and IL-6 secretion when compared to cells treated with adenovirus serotype 12. Although both serotypes have approximately the same number of CpG motifs, the sequences with CpG motifs vary. To elucidate further the inhibitory effects of INODN on cytokine production, they inserted ODN from adenovirus serotype 2 into known ISODN sequences. The introduction of these neutralizing CpG motifs into the ISODN caused a 50% reduction in cytokine production and cells that were treated with synthetic INODN produced very little cytokine.

1.3.4 Cellular Mechanisms by which Plasmid DNA and ODN Activate Cells

The cellular mechanisms by which ISODN or plasmid DNA activates cells to induce immune modulation are beginning to be understood. Recent experiments have helped elucidate some of the events and possible mechanisms involved in immune activation and these are discussed below.

1.3.4.1 Cellular Uptake

Leukocyte surface proteins readily bind large segments of foreign DNA in a ligand-receptor-mediated fashion and this binding and subsequent internalization of DNA can be abrogated by proteolytic digestion of these proteins. The scavenger receptor expressed on macrophages and NK cells is one possible surface protein that may be involved in DNA binding (Kimura *et al.*, 1994). Using a competitive binding assay for DNA uptake, Kimura *et al.* (1994) demonstrated that large fragments of DNA are actively taken up by cells and this uptake can be improved by flanking DNA sequences with polyguanine tails. The authors suggested that NK cells and macrophages might use the scavenger receptors to bind and internalize large fragments of DNA.

An inducible protein, Mac-1, located on granulocytes, macrophages and NK cells has been shown to readily bind ODN. Binding of ODN to Mac 1 occurs in a saturable manner and can be readily blocked by monoclonal antibodies. Treatment of

granulocytes with TNF-α increases Mac-1 expression on the cell's surface and improves ODN induced cell activation (Benimetskaya *et al.*, 1997).

Oligodeoxyribonucleotides can be bound by and internalized into a variety of cells such as myeloid cells, fibroblasts and carcinoma cell lines, in a manner that is temperature dependent and saturable. In one study it was shown that ODN bind to a surface protein that varies from 79-90 KD in size and is then transported into the cell by receptor-mediated endocytosis. Additionally, another study using protein annealing agents and inhibitors of endocytosis verified that ODN bound to surface proteins prior to internalization and internalization occurred via endocytosis. Labeling studies revealed that approximately 1.2×10^6 receptors on the surface of a cell can bind to bacterial DNA. Plasmid DNA blocks uptake of ODN, suggesting that plasmid DNA and ODN may bind to the same protein for cellular transport (Yakubov *et al.*, 1989).

Initially it was suggested that ISODN-induced cell activation required only surface binding and not internalization. Free ODN and ODN coupled to sepharose beads induced similar immune responses. The internalization of sepharose beads was not deemed possible and the authors believed that immune stimulation was induced by the binding of ISODN to surface receptors (Liang *et al.*, 1996). This theory was disputed by Manzel & Macfarlane, (1999) who suggested that sepharose beads were indeed internalized and when ISODN were coupled to particles that truly could not be internalized, then cell activation did not occur.

Oligodeoxynucleotides may also be internalized by non-receptor-mediated processes. In HL60 cell lines, ODN were internalized at concentrations exceeding the binding capacity of the receptor. Stein and Cheng (1993) suggested therefore that uptake of ODN occurs by fluid-phase pinocytosis and showed that hepatoma HEP 62 and HEP 2215 cell lines internalize ODN by both fluid-phase endocytosis and absorptive endocytosis.

The method of ODN internalization may therefore depend on the cell type and internalization can either be a receptor-mediated process or a non-specific form of uptake such as fluid-phase pinocytosis or absorptive endocytosis. It is evident that internalizing ODN through different methods helps ensure that neither ODN nor bacterial DNA escape contact with cells involved with immunosurveillance.

1.3.4.2 Pathways of Cell Activation by Oligodeoxyribonucleic Acids

The mechanisms by which ISODN cause cell activation are currently being explored and it is believed that ODN must be internalized to cause immune stimulation. Once taken up by cells, ODN are translocated to endosomes for acidification, an essential step in cell activation by ISODN. Co-administration of ISODN and known inhibitors of endosomal acidification, such as anti-malarial agents, blocked cell activation (Hacker *et al.*, 1998; Macfarlane & Manzel, 1998).

Following acidification in endosomes, ISODN enter the cytoplasm and possibly the nucleus, with a small proportion of ISODN being released from the cell (Tonkinson & Stein, 1994). Immunostimulatory ODN bind to a receptor within the cytoplasm prior to cell activation. This internal receptor has not been conclusively identified but the Toll like receptor-9 (TLR-9) is considered the most likely candidate (Hemmi *et al.*, 2000; Krieg & Wagner, 2000). Within minutes after internalization, TLR-9 links ISODN to the myeloid differentiation marker 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) and leads to subsequent activation of the ER1 JNK1 and P38 stress kinases (Hacker *et al.*, 2000). Binding of ISODN to MyD88 is essential for cell activation, as mice deficient in MyD88 fail to respond to ISODN treatment (Hacker *et al.*, 2000; Krieg & Wagner, 2000).

The activation of JNK1 and P38 kinases causes phosphorylation of the c-JUN and ATF2 and this leads to activation of the transcriptional factor, activating protein-1. Activating protein-1 is involved in the integration of other signal pathways

that are either directly or indirectly involved with the expression of cytokines such as IL-6, TNF-α and IFN-γ (Hacker *et al.*, 1998). In addition to activating the JNK1 and P38 pathways, ISODN can induce expression of NFκB and increase the production of cytokines through oxygen dependent processes. Using a mouse B cell line, Yi *et al.* (1998) demonstrated an approximately 30% increase in the production of intracellular oxygen following ISODN treatment. Adding an anti-oxidant to pre-stimulated B cells followed by treatment with ISODN caused a marked reduction in IL-6 production.

In conclusion, the mechanisms by which ISODN induce cell activation include the internalization and translocation of ISODN to endosomes for acidification, transport of ISODN to the cytoplasm leading to activation of protein kinases and oxygen reactive species that are important in gene transcription and cytokine release. It is apparent that signaling pathways involved in cell activation are complex and could allow for the integration of different signals induced by different ligands (Krieg & Wagner, 2000).

1.3.5 Innate Immunity

A review of innate immunity was made by Medzhitov and Janeway (1997). For decades, most immunologists focused their attention on studying lymphocyte function and the development of acquired immunity. They have generated vast amounts of information on both the molecular and cellular events that arise during the induction of humoral and cell-mediated immunity. During their endeavors, however, they have generally neglected to evaluate the importance of cells associated with the innate immune system on the induction of acquired immunity. Indeed, it has now become apparent that the innate immune system controls events involved with both the initiation and type of immune response that arises after exposure to a pathogen.

The innate immune system is phylogenetically older than the acquired immune system as it is found in all multicellular organisms and uses receptors encoded in germline DNA (Fearon & Locksley, 1996). The conservation of these receptors suggests that the innate immune system recognized and eliminated pathogens prior to the development of the adaptive immune system. To protect against foreign invaders, cells of the innate immune system have developed receptors that recognize structures that are unique to microorganisms and essential for the microbial survival. These receptors also have perfect self-nonself discrimination, a hallmark of innate immunity (Medzhitov & Janeway, 1997). The molecular structures found in microorganisms are known as pathogen-associated molecular patterns (PAMP) and are recognized by either soluble proteins or cell surface receptors known as pattern recognition receptors (PRR) (Table 1.1).

As discussed previously (see section 1.31), ISODN can induce the release of cytokines and chemokines (Krieg *et al.*, 1995; Klinman *et al.*, 1996; Iho *et al.*, 1999; Takeshita *et al.*, 2000), increase expression of costimulatory molecules (Sparwasser *et al.*, 1998; Hartmann *et al.*, 1999) and enhance effector functions (Ballas *et al.*, 1996) of previously untreated cells. The CpG motifs present within ISODN are responsible for many of these cellular responses by directly activating cells associated with innate immunity. CpG motifs are therefore considered to be PAMP that bind to PRR of different cell populations (Krieg & Wagner, 2000).

1.3.5.1 Disease Protection by ISODN Activation of Innate Immunity

Immunostimulatory ODN can induce cell activation *in vitro* and enhance humoral and cell-mediated immunity *in vivo*. Since the activation of cells *in vitro* suggested that CpG motifs were PAMP and activators of innate immunity, CpG motifs should protect against infectious disease in an antigen-independent manner. Recent studies have shown that ISODN, in the absence of conventional vaccines or

Table 1.1 Selected Ligands and Receptor Involved in Innate Immunity.

Receptor	Site of Expression	Ligand	Function
85	Humoral - liver	1) Polysaccharides	1) Activate complement 2) Enhance Pϕ,
SAP	Humoral - liver	1) Microbe cell wall polysaccharides 2) EC matrix proteins	1) Enhance Pϕ, 2) Stabilized EC matrix proteins
LBP	Humoral - liver	1) LPS transfer to CD14 and lipoproteins	1) Enhance sensitizing of cells to LPS
Mannose Receptor	Macrophages Dendritic cells Endothelial cells	Multiple carbohydrates	1) Targeting to cells expressing MHC-II
Scavenger Receptors	Macrophages Endothelial cell HEV	1) Bacterial and yeast cell walls 2) Bacterial DNA	1) Clearance of LPS and microbes 2) Cell activation
LPS Receptor (CD14)	Macrophages Monocytes PMN	1) LPS 2) Other microbial cell wall components	1) Microbe clearance 2) Enhance LPS sensitivity 3) Induce inflammatory cytokine release
Complement Receptors	Macrophages Monocytes B cells Dendritic cells	1) LPS 2) Complement 3) Fibrinogen	1) Activates B cells 2) LPS clearance 3) Complement cleavage
Toll Receptor	Macrophages Monocytes Dendritic cells	1) LPS 2) Bacterial DNA	1) Cytokine production 2) Cell activation

The table modified from Fearon & Locksley, (1996) and Krieg & Wagner, (2000)
Abbreviations: CRP C-reactive protein; SAP serum amyloid protein; LPS lipo-polysaccharide; LPB LPS binding protein; Pϕ phagocytosis; EC extracellular; HEV high endothelial venules; PMN polymorphonuclear leukocyte.

DNA vaccines, can enhance the ability of mice to survive challenge with a variety of pathogens. Mice were injected with 50 μg of ISODN ip and challenged with *Listeria monocytogenes* over a period of 14 days post-injection. All mice treated with ODN containing CpG motifs survived challenge, while mice treated with GpC ODN succumbed to disease. The experiment was then extended for a four-month period and mice were injected with ISODN containing CpG motifs every 14 days. Similar to the observations of the first experiment, only mice receiving ISODN with CpG motifs survived challenge. Protection was relatively short-lived however, as animals died from bacterial challenge one month following the last ISODN injection (Klinman *et al.*, 1999). The authors believed that protection was mediated by increased secretion of IL-6 and IFN-γ by various cell types and that CpG motifs lowered the threshold necessary to induce cell activation.

In another study, it was shown that ODN with CpG motifs protect mice against infectious peritonitis. Using the colon ascendens stent peritonitis model, Weighardt *et al.* (2000) demonstrated that mice treated with ODN containing CpG motifs had improved survivability and a diminished bacterial load in the peritoneal cavity when compared to GpC ODN controls. They showed enhanced systemic levels of IL-10 and IL-18 had a 4-fold increase in the number of neutrophils present within the abdominal cavity. There was also increased expression of cell receptors such as Mac-1 and FCRγ on neutrophils as well as enhanced effector function that included both increased phagocytosis of bacteria and increased respiratory burst activity. The authors believe that the rapidity of response and improved activation of neutrophils by ISODN with CpG motifs indicate that CpG motifs may directly activate an innate rather than acquired immune response. These examples show that CpG motifs may indeed offer a degree of protection against various bacterial diseases.

Although the use of either bacterial DNA or ISODN for protection against infectious disease has potential clinical application, there are some limitations to this type of treatment. Firstly, treatment with bacterial DNA or ISODN is only effective when administered prior to challenge with the microorganism. As an example, Elkins *et al.* (1999), demonstrated that protection against bacterial challenge occurred when either bacterial DNA or ISODN were given to mice 3-14 days prior to exposure with *Listeria monocytogenes*. If mice were both treated with bacterial DNA or ISODN and exposed to bacteria on the same day, the mice succumbed to disease. Secondly, bacterial DNA or ISODN treatment was only protective against challenge with intracellular organisms: mice infected with *Y. enterocolitica* failed to survive the challenge (Elkins *et al.*, 1999).

1.3.6 Safety Issues

Identifying potential health hazards throughout the development of new biotechnologies is of utmost importance. The use of plasmid DNA and ISODN in people and animals is of no exception, with the potential development of at least seven types of adverse reactions as well as perhaps those yet unforeseen. Firstly, plasmid DNA could randomly integrate into the host genome and lead to the inadvertent activation of proto-oncogenes, inactivation of tumor suppresser genes or inactivation of genes involved in regulation of cell processes. Secondly, DNA vaccines might alter the 'appearance' of the host cell and thereby initiate an autoimmune state within the host. Thirdly, plasmid vaccines might induce antigen tolerance or disease enhancement within the vaccine recipient. Fourthly, immunostimulation and the release of cytokines might cause local or systemic inflammatory responses. Fifthly, deviation of immune responses and a subsequent inability to respond appropriately to infection could occur. Sixthly, long-term exposure to antigen may be detrimental or lead to disease, and finally, plasmid used in animals might be transferred to people following meat consumption. Each of these

potential risks will be further discussed in detail with an attempt to evaluate real or perceived risk of exposure to either plasmid DNA or ODN.

1.3.6.1 Integration of Plasmid into the Host Genome

Plasmid DNA can persist in tissue for many months post-vaccination. In general, intact plasmid or plasmid fragments are thought to be localized epichromosomally and do not covalently integrate into the host's genome. Plasmid DNA was injected iv into mice and tissue samples were subsequently collected at various time points. Tissue assessment for plasmid remnants by polymerase chain reaction (PCR) amplification, indicated that the majority of plasmid DNA underwent degradation within minutes of injection, but fragments of DNA remained present within muscle for up to six months. The amount of DNA present within the nucleus was exceedingly small at roughly 0.012 copies of plasmid per genome (Lew *et al.*, 1995). It was not determined, however, whether the plasmid fragments were incorporated into the host genome.

To prove conclusively that plasmid DNA does not incorporate into the host's genome, it must be demonstrated that neither contamination with other plasmids has occurred nor has foreign DNA annealed to the host's genome. Using PCR to detect plasmid DNA can be problematic, as contaminating DNA will inadvertently be amplified to give a false positive result. To prevent false positives from arising, Nichols *et al.* (1995) used stringent isolation techniques and single sided PCR to analyze for plasmid content after treatment. The researchers analyzed muscle tissue at 1, 2, 3, 6, 12 and 18 weeks following im injection and confirmed that plasmid DNA did not incorporate into the host genome but remained epichromosomal. They estimated that approximately 7.5 copies of plasmid per 1 μg of genomic DNA was present in tissue after DNA vaccination. Using risk analysis to define a 'worst case scenario', the researchers assumed that if all 7.5 copies of plasmid incorporated into genomic DNA, the incorporation frequency of plasmid would still be 1000-fold lower than the natural rate of spontaneous mutation within the

genome. This analysis suggests that if plasmid DNA does incorporate into the genomic DNA, the effects would likely be inconsequential.

A single study exists, however, demonstrating that foreign DNA could incorporate into the host genome. Schubbert *et al.* (1997) delivered phage DNA orally and then detected the presence of the phage DNA within the genomic DNA of splenic B cells, T cells, and macrophages and in gut epithelium of treated mice. To prove that the DNA was indeed of phage origin, they cloned DNA segments from mouse tissue containing phage DNA and then hybridized it to native mouse genomic DNA. They found that one cloned DNA segment hybridized to the mouse IgE gene while two other cloned segments hybridized to distant but unidentified sites of the mouse genome. The hybridization of plasmid to the IgE gene could lead to either impaired antibody function or malignant transformation of IgE producing B cells, both having potential clinical ramifications.

1.3.6.2 Auto-immune Potential of DNA Vaccines

The potential for evoking auto-immunity by DNA vaccination poses a concern and could limit the use of DNA vaccines in a clinical setting. The ability to generate antibodies to bacterial DNA has been known for several decades. Healthy people and patients with systemic lupus erythematosus (SLE) were tested for antibody binding to mammalian and prokaryotic DNA. Serum from healthy people bound strongly to DNA from Gram-positive organisms but did not bind to calf thymus DNA. In comparison, serum from SLE patients bound to calf thymus DNA as well as to DNA from Gram positive and Gram negative organisms (Karounos *et al.*, 1988).

Although the development of cross-reactive antibodies to mammalian DNA after bacterial DNA injection is possible, definitive proof for the induction of autoantibodies by bacterial DNA has not been demonstrated. To address this issue, Gilkeson *et al.* (1993;1996) injected bacterial DNA into both BALB/c and New Zealand black/white (NZB/NZW) mice and demonstrated the development of immune mediated

glomerulonephritis. Mice treated with bacterial DNA administered with bovine serum albumin (BSA) in Freund's adjuvant, had high anti-DNA antibody titers and enhanced proliferation of glomerular tufts with increased deposition of IgM, IgG and C_3 of complement within the mesangium. Unfortunately, treatment of mice with bacterial DNA alone was not included and therefore it is difficult to determine from this study if bacterial DNA alone can induce the production of autoantibodies.

Another study demonstrated that injection of DNA produced antibodies that cross-reacted with mammalian DNA. Deoxyribonucleic acid from *E. coli* was injected into mice and serum was collected. Antibodies in the collected serum bound to calf thymus DNA with approximately 50% of the efficacy of antibody binding to *E. coli* DNA. This observation supported the conclusion that the antibodies were cross-reactive (Gilkeson *et al.*, 1998).

To determine if changes in cytokine profiles occur in mice following injection with bacterial DNA, NZB/NZW mice were injected with DNA from *E. coli.* It was shown that a substantial change in the ratio of cells secreting IFN-γ and IL-4 occurred in isolated spleen cells. Gilkeson *et al.* (1998) demonstrated a 10 to 25-fold increase in the ratio of IFN-γ: IL-4 secreting cells, suggesting that bacterial DNA induced a Th1-like immune state of activation and thereby could reduce the potential to develop SLE. It is believed that SLE manifest as a predominantly Th2 immune response with elevated levels of IL-4.

The presence of bacterial DNA within vertebrates may induce a cell-mediated form of auto-immunity. The transfer of activated antigen-specific T cells from mice susceptible to experimental allergic encephalomyelitis (EAE) to normal mice can induce disease within the recipient. Thymocytes from EAE mice were primed with antigen and exposed to either ISODN or a non-stimulating form of ODN and then injected into untreated mice. Mice receiving ISODN treated T cells developed moderate to severe

EAE, while mice receiving T cells treated with non-stimulating ODN remained asymptomatic. The authors speculated that activation of T cells by ISODN resulted in the induction of IL-12 and a subsequent increase in IFN-γ levels. It is believed that exposure to bacterial DNA during an infection could perhaps inadvertently lead to a Th1 type autoimmune state (Segal *et al.*, 1997).

1.3.6.3 Potential for Immunological Tolerance

The development of immunological tolerance can be potentially life-threatening, in particular if an individual is unable to recognize a pathogen as foreign and mount an appropriate protective immune response. The development of immunological tolerance, however, is unlikely to occur in adults. As previously described, administration of low doses of plasmid expressing antigen can generate long-term immunity and protect against disease (Ulmer *et al.*, 1993; Deck *et al.*, 1997). Vaccination with DNA in the neonate has also induced both strong humoral and CMI (Butts *et al.*, 1998). There remains, however, the potential to induce immunological tolerance in the neonate after vaccination (Silverstein & Segal, 1975) and indeed it has been documented that mice can become tolerant to antigen following DNA vaccination (Mor *et al.*, 1996). Mice between two and five days of age failed to generate antigen-specific antibodies or CTL responses and failed to secrete IL-4 and IFN-γ from splenocytes following an injection of plasmid DNA expressing the circumsporozite protein (CSP) of *Plasmodium yoelii.* No improvement in immune responses was detected after a vaccine boost at six week of age either. Interestingly, neonates exposed to antigen co-administered with the DNA vaccine developed strong humoral and CMI indicating that delivery of plasmid with protein may prevent the development of immune tolerance.

1.3.6.4 Enhancement of Disease

A potential risk of DNA vaccination is the failure to develop protective immunity and to enhance the progression of disease. This was demonstrated in a feline immunodeficiency virus (FIV) vaccine study, in which cats were injected with DNA expressing different envelope proteins and then exposed to virus. Cats immunized with the DNA vaccine developed poor antibody titers and demonstrated markedly elevated virus titers that began one week earlier than control groups and remained elevated for the duration of the experiment (4 weeks). There was a direct correlation between vaccine induced antibody titers and elevated virus titers, as increased antibody levels paralleled the increase in viral loads (Richardson *et al.*, 1997). Unfortunately, immunization with viral protein alone was not performed in this experiment, as this was a necessary control to determine whether an inappropriate vaccine antigen had been selected for protection against disease. It is possible that DNA vaccines expressing a different viral antigen may have been successful in disease prevention. From the experiment, however, the data convincingly shows that viral multiplication was accelerated following the injection of plasmids expressing viral antigens and that DNA vaccines may inadvertently enhance some diseases.

1.3.6.5 Development of Unintentional Local and Systemic Inflammation

From previous discussions, it is apparent that the adjuvanticity of ISODN is partly dependent on the production and release of cytokines. It has been suggested that a fine balance between adjuvanticity and toxicity is always present and the absolute safety of the adjuvant is never guaranteed (Gupta *et al.*, 1993). Therefore, the potential exists for an ISODN adjuvant to cause both local and systemic adverse responses. Administration of bacterial DNA or ISODN into joints (Deng *et al.*, 1999) or lower airways (Schwartz *et al.*, 1997) in mice caused a severe inflammatory reaction. Comparing calf thymus DNA to bacterial DNA or ISODN, Schwartz *et al.* (1997) demonstrated a 4-fold increase in the

numbers of neutrophils, production of macrophage inhibitory protein-2 and a 2-fold increase in TNF-α levels within lung lavages from animals treated with bacterial DNA or ISODN. Similarly, Deng *et al.* (1999) showed that the inflammatory lesions produced by intra-articular injections of bacterial DNA or ISODN were dependent upon the presence of macrophages and elevated expression of TNF-α.

To ensure that the inflammation was due to treatment with either bacterial DNA or ISODN and not LPS contamination, mice were either given LPS and used as controls (Schwartz *et al.*, 1997) or a specialized mouse strain (C3H/Hej) that is unresponsive to LPS was used in certain experiments (Deng *et al.*, 1999). In these studies, it was shown that inflammation was indeed caused by exposure to bacterial DNA or ISODN and not by LPS. Treatment with quantities of LPS that equaled the amount of LPS present in bacterial DNA preparations did not induce detectable inflammation within the airways (Schwartz *et al.*, 1997).

Most DNA vaccine studies demonstrated minimal tissue damage following im injection. In a single study, however, injection of plasmid DNA into mice either previously exposed to virus or vaccinated with DNA expressing a viral antigen, caused severe myositis that persisted for at least 4-days post-exposure. The inflammatory lesions were dependent on the presence of CTL and on the immune status of the mice prior to treatment. The authors speculated that inflammation may have been caused by expression of virus antigen within muscle cells (Yokoyama *et al.*, 1997).

Adverse systemic effects after ISODN treatment have been documented as well. Intraperitoneal injection of ISODN in mice can cause substantial elevations of TNF-α in blood. In β-D-galactosamine (β-GalN) sensitized mice for example, treatment with 2.5-5 nmol (15-30 μg) of ISODN can induce massive liver cell apoptosis and it is believed to be related to elevated levels of serum TNF-α (Sparwasser *et al.*, 1997). It is noteworthy that

the amount of ISODN used in the experiment is well within ranges used in other mouse experiments (Klinman *et al.*, 1997; Davis *et al.*, 1998; Lipford *et al.*, 2000). It is likely that sensitization to β-GalN caused mice to release more TNF- α systemically and thereby cause liver injury. It is evident from the study, however, that massive release of TNF-α into circulation following ISODN treatment is detrimental and an undesirable side effect.

Studies have recently been performed to evaluate the long-term effects of multiple ISODN injections in mice. Evidence demonstrated convincingly that ISODN does not cause unwanted side effects following multiple treatments (Klinman *et al.*, 1999). In this experiment, previously untreated mice were injected weekly with 50 μg of ISODN ip over a four-month duration. All mice survived and none of them displayed either clinical signs of illness, or developed inflammatory lesions within sampled tissue. The lack of mortality and tissue injury following ISODN injection differs from observations seen by Sparwasser *et al.* (1997). It is evident therefore, that the immune status of the animal prior to ISODN treatments, as seen in the experiment by Sparwasser *et al.* (1997), can greatly influence the development of adverse reactions.

1.3.6.6 Long-term Expression of Antigen

Although long-term expression of antigen in tissue has obvious advantages, it may pose a potential hazard as well. It has been suggested that the development of antibodies specific for one strain of influenza virus may reduce the capacity of the immune system to protect against a different strain. Newman *et al.* (1993) vaccinated people with two different influenza vaccines and showed that robust antibody responses were strain specific. Only one group of vaccinated recipients however, developed strong cross-reactive antibodies (Newman *et al.*, 1993). The researchers suggested that prior exposure to a wild strain of influenza and the persistence of viral antigen prevented the development of cross-reactive antibodies in one of the vaccinated groups. This concern

may be unwarranted as DNA vaccines can induce a strong cell-mediated response that cross protects against different viral strains (Ulmer *et al.*, 1993).

Persistent long-term exposure to foreign DNA within cells might also be potentially hazardous. Several chronic diseases have been associated with the presence of foreign viral RNA within tissues. For example, a study examining muscle biopsies from patients with chronic fatigue syndrome demonstrated that 50% of all affected patients had fragments of enterovirus RNA present within cells (Gow *et al.*, 1991). It is difficult to compare this investigation with DNA vaccine studies as the quantity of RNA present in cells was not determined. The long-term effects of plasmid DNA incorporated into cells are unknown. It is possible that the long-term presence of plasmid in cells is hazardous, but it will take decades to determine this, as the use of DNA vaccines in clinical trials has just begun.

1.3.6.7 Transfer of Foreign DNA Through Meat Consumption

Use of DNA vaccines in food producing animals raises safety concerns distinct from the use of DNA vaccines in people. The presence of foreign DNA in meat after im injection and the subsequent ingestion of treated meat might lead to the inadvertent transfer of plasmid DNA to people. To address this concern, Babiuk *et al. (*1998) injected calves im with plasmid and then excised the injection site three weeks later. The excised muscle was fed to rats and various rat tissues were examined for plasmid remnants by PCR amplification. Fragments of plasmid were not detected in any tissue suggesting that transfer of foreign DNA to the consumers will likely not occur. Furthermore, methods of vaccine delivery such as id injection and ballistic (gene gun) delivery can greatly reduce the amount of plasmid used for DNA vaccination and thereby further reduce any potential for the transfer of plasmid from livestock to people.

1.3.7 Oligodeoxyribonucleotides and the T-helper Paradigm

Understanding the categorization of T helper cells is important for determining the effects of DNA vaccines and ISODN on T cell activation. For a review on T helper subclasses see Romagnani (2000). Briefly, T helper cells were initially characterized into type 1 and type 2 T helper cells according to the type of cytokines produced and their influence on the types of antibodies produced by B cells. T helper 1 cells (Th1) produce predominately IFN-γ, IL-2, TNF-α, supported B cell production of opsoninising antibodies (IgG2a) and the development of CTL responses. T helper 2 cells (Th2) produce IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and support antibody responses associated with IgG1 and IgE production. This T helper cell classification has been expanded to include type 0 helper cells that express both Th1 and Th2 cytokines and type 3 helper cells that express high levels of TGF-β. The type of T helper response that develops is affected by cross regulation between various cytokines. The production of IFN-γ for example, greatly inhibits proliferation of Th2 cells while IL-4 inhibits Th1 proliferation. Furthermore, certain cytokines such as IFN-γ and IL-4 are exclusively produced in Th1 and Th2 type immune responses, respectively.

In vitro and *in vivo* experiments suggest that ISODN shift T helper cells towards a Th1 bias. Using CD4 T cells from mice, Zimmermann *et al.* (1998) demonstrated that unstimulated T cells secrete IL-4:IFN-γ at a ratio of approximately 10:1. If these cells were treated with either a moderate or high concentration of ISODN, the IL-4:IFN-γ ratio changed to approximately 1:24 and 1:60 respectively. Furthermore, ISODN treatment caused a greater reduction in the IL-4:IFN-γ ratio than treatment with anti-IL-4 alone. To determine if ISODN caused a bias in Th2 and Th1 cell activation *in vivo*, the researchers examined changes in the immune function of BALB/C mice following challenge with *Leishmania major*. Mice treated with ISODN survived challenge while untreated mice

died eight weeks later. Examination of isolated T cells from both experimental groups revealed that untreated mice produced high amounts of IL-4 and no IFN-γ. In contrast, ISODN treated mice did not produce IL-4 but produced large amounts of IFN-γ. Treated mice also had reduced production of IgG1 and increased production of IgG2a and IgG2b antibodies. Zimmermann *et al. (*1998) also examined the levels of ILR_{12B2} (IL-12 receptor) mRNA expression. The ILR_{12B2} expression was increased in Th1 cells and abolished in Th2 cells, and it was demonstrated that ISODN treated mice strongly expressed ILR_{12b2} while expression of the mRNA was undetectable in untreated mice. These results indeed verified that in mice treated with ISODN, there was a clear shift in T helper cell development with a Th1 bias.

1.3.8 Conclusion for the DNA Vaccination Section

Plasmid DNA and ISODN have demonstrated remarkable biological activity *in vitro* and *in vivo*. The potential use of antigen-encoding plasmids for vaccines and ISODN as adjuvants may revolutionize immunoprophylaxis and gene therapy in the future. Currently, we are beginning to achieve a greater understanding of mechanisms involved in bacterial DNA immune stimulation. As research continues in the area of DNA immunization and immunostimulation, improvements in immune protection should occur, hastening the implementation of DNA vaccines and ISODN into clinical trials.

1.4 Introduction to Lymph Nodes

Lymphoid tissues are structures that play an integral role in immune surveillance and lymphocyte development. These exist either as diffusely dispersed tissue as seen in the alimentary and respiratory tracts or as discrete localized structures such as thymus, spleen and lymph nodes (LN). As LN act as points of immune surveillance, the induction of immune responses and are pivotal in

lymphocyte movement between blood and lymph, this section will examine the structure-function relationships of LN.

1.4.1 The General Structure of Lymph Nodes

A detailed review of LN morphology is given by Junqueira (1986). Briefly, LN are encapsulated structures containing a subcapsular sinus, cortex and medulla. The cortex consists of both a superficial and a deep (paracortex) compartment. The medulla is composed of cords and sinuses. Lymph nodes are also highly vascularized and well innervated with arteries and nerves entering the LN and the vein exiting the hilar region of the LN. Lymph enters the LN via the afferent lymphatics connecting to the capsular surface of the LN and lymph exits through valved efferent lymphatics at the hilum. The internal framework of the LN is composed of a fine trabecular meshwork of reticular fibers that acts as scaffolding to maintain structural integrity (Wheater, 1987).

1.4.1.1 Cortex

The cortex is one of two major regions of the LN and is subdivided into superficial cortex and the deeper paracortex. Between the superficial cortex and the capsule lies the subcapsular sinus. The spherical lymphoid follicles (primary follicles) of the superficial cortex contain small dark staining non-reactive B cells admixed with few dendritic cells (van der Valk & Meijer, 1987). The paracortex is predominantly composed of T cells and small numbers of dendritic cells. This region also contains specialized high endothelial venules (HEV), structures associated with lymphocyte migration from the blood to LN.

1.4.1.1.1 Primary Follicle and Germinal Centers

Germinal centers (GC), also known as secondary follicles, arise from primary follicles following stimulation with antigen. Stimulation with foreign antigen is a prerequisite for GC development, as GC are not detected in germ free animals (Kim

& Watson, 1971). Surrounding the GC is the mantle zone, composed of cell populations found in primary follicles. Monoclonal antibody studies in man verified that the cells within the mantle zone are small B cells that express sIgM, IgD, CD23, CD39 and CD44 antigens (MacLennan *et al.*, 1992; Lagresle *et al.*, 1993) and produce alkaline phosphatase (van der Valk & Meijer, 1987).

1.4.1.1.2 Lymphoid Cells

Lymphoid cells of the GC are categorized into centroblasts, centrocytes and lymphoblasts. Centroblasts are actively dividing cells and appear as large round cells, with sparse basophilic cytoplasm and express little to no surface immunoglobulin (sIg) (MacLennan *et al.*, 1991). Nuclei are vesicular with prominent nucleoli and mitotic figures are abundant (MacLennan *et al.*, 1991). Centrocytes may be either small or medium sized and develop from centroblasts (Nieuwenhuis & Opstelten, 1984). Medium sized centrocytes have large nuclei with fine stippled chromatin, while the nuclei of small centrocytes have condensed chromatin and clefts. The cytoplasm in both cell types is sparse (Nieuwenhuis & Opstelten, 1984). B lymphoblasts, less common than either centroblasts or centrocytes, have medium sized nuclei with in indistinct nucleoli and scant to moderate amounts of dark basophilic cytoplasm (van der Valk & Meijer, 1987). Other lymphoid cells occurring with lower frequency within GC include NK cells, T cells and plasma cells (Namikawa *et al.*, 1987; Janossy et al., 1989; Seva *et al.*, 1998).

1.4.1.1.3 T Cells

Although T cells are a major population of LN, only smaller numbers of T cells have been identified within GC. In a well-differentiated GC, T cells are located in areas adjacent to the mantle zone (Nieuwenhuis & Opstelten, 1984; Imai & Yamakawa, 1996). In mice, these T lymphocytes are predominately CD4 T cells and these cells are believed to assist in germinal center development (Secord *et al.*,

1996). The CD4 T cells out-number other T cells, such as CD8 T cells, by 12:1 (Rouse *et al.*, 1982).

1.4.1.1.4 Tingible-Body Macrophage

Tingible-body macrophages (TBM) are macrophages with a starry-sky appearance and are present within active GC, appearing several days after antigen exposure. Initially these macrophages were considered to be scavengers of apoptotic cells as they could be identified engulfing dead lymphocytes within either reactive or involuting tissues (Fadok *et al.*, 1992). It is becoming apparent, however, that TBM may also regulate GC development. *In vitro* studies with hybridoma cells demonstrate that these macrophages reduce production of IL-2 by B cells. The authors suggest that TBM may also play a role in the down-regulation GC growth (Smith *et al.*, 1998). Interestingly, the number of macrophages present within GC parallels GC growth, as the number of macrophages remains in relatively constant proportion to the increasing number of B cells during the enlargement of GC.

1.4.1.1.5 Follicular Dendritic Cells

Dendritic cells are categorized into B and T lymphocyte-associated cells. The B cell-associated follicular dendritic cells (FDC) are found within follicles, while T cell-associated dendritic cells are located in non-follicular areas. Initially, Chen *et al.* (1978) coined the term FDC to describe specific cells located in GC that trap antigen complexes. Tew *et al.* (1982) further defined them as cells located within follicles that lacked Thy-1 antigen and sIgM and express C3 and Fc receptors. Presently, FDC are categorized by the following: 1) their location within a GC, 2) fulfilling rather stringent immunohistochemical and ultrastructural criteria, 3) their ability to trap and retain antigen for prolonged periods and 4) association with B cells. Accordingly, seven subtypes of FDCs have been identified and the undifferentiated and differentiated forms of these cells are located within the dark and light zones of GC

respectively (Imai & Yamakawa, 1996). Follicular dendritic cells have a plethora of functions within GC. They function as part of GC scaffolding and are tightly associated with the reticular fibers and cytoskeleton proteins of reticular cells (Gloghini & Carbone, 1990; Ogata *et al.*, 1996). Antigen trapped on FDC is presented to B cells as immune complex coated bodies (iccosomes) and FDC help activate B cells to transform into plasma cells (van Rooijen, 1990). Follicular dendritic cells can also prevent induction of apoptosis in B-lymphocytes. Using isolated tonsillar lymphocytes and dendritic cells, Lindhout *et al.* (1993) demonstrated that cells forming FDC-B cell clusters survive in culture for long periods while B cells not associated with FDC rapidly undergo apoptosis. This was a T cell independent process, as T cells were not detected within these clusters.

1.4.1.1.6 The Anatomical Structure and Development of Germinal Center

Germinal centers are the site of clonal expansion and differentiation of B lymphocytes (Kroese *et al.*, 1987; Liu *et al.*, 1989). Germinal center development has been extensively studied in human tonsillar tissue and based on light microscopy of hematoxylin and eosin stained LN sections, the GC have been divided into four relatively distinct anatomical regions (van der Valk & Meijer, 1987). These are the dark, basal light, apical light and outer zones. The area that lies closest to the medulla is the dark zone and this region is surrounded by a pale area composed of two zones, the basal light and the apical light, respectively. Surrounding the entire GC is the outer zone and this zone is the most peripheral area of a GC. The mantle zone surrounds the outer zone but is not considered part of the GC.

1.4.1.1.7 Dark Zone

The dark zone is the main site of GC cell proliferation and contains numerous centroblasts that express various activation markers. Using immunofluoresence in

human tonsillar tissue, Hardie *et al.* (1993) demonstrated that the dark zone contains the highest population of cells undergoing mitosis and expressing markers for B lymphocyte activation. Within the dark zone, there is a rapid transformation of centroblasts into centrocytes. The cell cycle for centroblasts lasts approximately seven hours and therefore, within a 24-hour period the *de novo* production of centrocytes can be substantial. In the dark zone, re-arrangement of IgV genes occurs within centroblasts through somatic mutations (MacLennan *et al.*, 1992). This takes place within the first few days following antigenic stimulation with a mutation rate that exceeds pre B-cells by approximately10 fold (Berek, 1992). Unlike other zones, TBM and FDC numbers are low within the dark zone suggesting that interactions with B cells are minimal. Indeed, few FDC-B cell clusters are found within this area (Kasajima *et al.*, 1986; Imai *et al.*, 1998).

1.4.1.1.8 Basal Light Zone

The basal light zone lies adjacent to the dark zone, contains both centroblasts and centrocytes and the highest number of apoptotic cells of all zones in a GC. The expression of cell markers, high cell turn over rate and presence of FDC suggests that the basal light zone is an important area for the regulation of B cell maturation. The importance of expression of cell markers on centrocytes is highlighted by Liu *et al.* (1989). Studies on isolated human tonsillar B cells showed that GC centrocytes not expressing CD39 or IgD died rapidly by apoptosis. In contrast cells expressing these molecules survived for longer periods. The authors suggested that this form of regulation would ensure that only centrocytes with high antigen affinity would survive to give rise to memory B-cells. Interaction with FDC in the basal light zone is likely an important regulatory event to ensure only antigen-specific stimulated B-lymphocytes expand and differentiate. Indeed, within this zone, FDC are well organized with numerous intercellular connections and they

express activation markers including cell adhesion molecules (Imai & Yamakawa, 1996). This is further exemplified by the numerous tight FDC-B lymphocyte clusters found in this zone.

1.4.1.1.9 Apical Light Zone

The apical light zone contains mainly medium sized centrocytes that display little mitotic activity and undergo little apoptosis. In this area, it is believed that centrocytes differentiate into memory B-cells and plasma cells, enabled by free antigen or antigen presented as iccosomes (van Rooijen, 1990). Follicular dendritic cells are in the highest concentration in the apical light zone, and these cells are activated as demonstrated by the enhanced expression of activation markers. Nematode challenged mice demonstrated marked increases in the expression of the complement receptor (FC,RII) receptor on FDCs located within this area (Maeda *et al.*, 1992). Similar to the basal light zone, this zone has many tight FDC-B cell clusters and these are likely important for FDC and B cell communication. Major isotype class switching also occurs within the apical light zone. The small numbers of T cells which have been identified in this zone may facilitate this by expressing CD40-ligand and secreting IL-4 and IL-10, key elements required for class switching (Liu & Arpin, 1997).

1.4.1.1.10 Outer Zone

A heterogeneous population of cells varying from centroblasts, centrocytes and plasma cells is found in the outer zone (Imai *et al.*, 1998). Occasional T cells, mainly CD4 T cells, are identified at the dark zone-mantle interface (Rouse *et al.*, 1982). Interestingly, in contrast to the tight FDC-B cell clusters detected within the basal and apical light zones, the FDC in the most basal area of the outer zone do not form tight clusters with B cells (Imai & Yamakawa, 1996). The reason for this

altered FDC-B cell relationship within the outer zone compartment remains unknown.

1.4.1.2 Paracortex

The paracortex is the cortical area surrounding follicles that extends to the medulla. It is composed of loosely arranged cells and consists of predominately T cells with rare interdigitating dendritic cells (IDC) (Junqueira, 1986; Wheater, 1987) and an occasional B-cell (Seva *et al.*, 1998). Specialized high endothelial venules (HEV) are present and facilitate the migration of lymphocytes from blood to LN parenchyma.

1.4.1.2.1 T Cells

The majority of T lymphocytes within the paracortex are small cells with dark staining nuclei and scant cytoplasm. Present in fewer numbers are larger T-immunoblasts that resemble centroblasts found in the GC dark zone (van der Valk & Meijer, 1987). All subpopulations of T cells have been identified in the paracortex. While located throughout the region, the most numerous, the CD4 T cells, preferentially aggregate around the GC. CD4 T cells are believed to interact directly with B cells at the follicular periphery and assist in B cell differentiation (Liu & Arpin, 1997) as well as in GC development (Garside *et al.*, 1998). CD4 T cells also form aggregates in tissue similar to FDC-B lymphocyte cluster. Comprised of both newly activated and memory T lymphocytes (Janossy *et al.*, 1989; Seva *et al.*, 1998), these aggregates may facilitate communication between cells and assist in the clonal expansion of T cells. Randomly scattered throughout the paracortex are CD8 T cells, which represent the second largest subpopulation of T cells in the LN. In the LN of the adult ruminant, for example, CD4 T cells out-number CD8 T lymphocytes by approximately 2 fold (Seva *et al.*, 1998).

Rarely found within the paracortex are γδTCR T cells. Using immuno-histochemistry and morphometric analysis, Seva *et al.* (1998) found only occasional clusters of γδ T cells within the paracortex. These cells were detected at a frequency of 1 cell per 1500 mm^2 of total LN area, which represents approximately 9% of the total T cell population.

1.4.1.2.2 Interdigitating Dendritic Cells

A population of cells, known as interdigitating dendritic cells (IDC), forms a network of intercellular connections within the paracortex. These cells are similar to FDC in GC (Imai *et al.*, 1998) and have been shown to present antigen and induce activation of paracortical CD4 T lymphocytes. Ingulli *et al.* (1997) demonstrated with confocal microscopy, that within the paracortex, labeled CD4 T cells cluster around IDC. This aggregation occurs only after antigen stimulation, indicating that both CD4 T cells and IDC were activated and developed a cognate immune interaction.

1.4.1.2.3 Minor Cell Populations within the Paracortex

B cells are found in small numbers within the paracortex. Immunohistochemistry was used to show that B cells migrate from HEV to follicles (Blaschke *et al.*, 1995). It is believed that B cells represent migratory cells and do not permanently reside within the paracortex.

1.4.1.3 Medulla

The medulla is situated between the paracortex and hilum and contains two prominent structures; the medullary cords and the medullary sinuses. Medullary cords are packed with cells and extend as linear rays towards the hilum. Medullary sinuses are spaces that separate medullary cords, extend to the hilum and communicate with efferent lymphatic vessels.

1.4.1.3.1 Medullary Cords

Medullary cords are composed predominantly of B cells and plasma cells with smaller numbers of T cells, macrophages, mast cells and granulocytes (Junqueira, 1986; Wheater, 1987; Welsch *et al.*, 1997). Within the cords of most species a 'plasma cell reaction' occurs, producing newly formed plasma cells, memory B-lymphocytes and increasing antibody production (van der Valk & Meijer, 1987). Straus (1981) used immunohistochemistry and histological criteria to create a rough classification system describing the variety of lymphocytes found in medullary cords. The author characterized four cell types present in cords after antigen exposure and suggested that some cell types may represent various stages of cell progression. Typical lymphocytes were small and round, with a thin rim of scant cytoplasm. Protoplasm cells are precursors to plasma cells and are either small, with small eccentric nuclei or large blast- like cells with large nuclei. Mature plasma cells are characterized by intense antibody staining, eccentric nuclei and reduced nuclear to cytoplasm ratio. The majority of immunoglobulins produced by B lymphocytes within medullary cords are IgG isotypes. This observation was further highlighted by Sato *et al.* (1990). Following oral gavage with live anaerobic bacteria, numerous IgG producing cells with little production of IgA and IgM were detected in medullary cords of LN draining the forestomachs.

Macrophages within medullary cords of LN are closely associated with both B and T cells and may function in lymphocyte cell recruitment or facilitate lymphocyte movement within the medulla. Medullary macrophages for example, express a sialoadhesion molecule that enables them to associate with lymphocytes. The binding affinity of these macrophages to lymphocytes is much stronger than lymphocyte binding to other macrophages such as peritoneal macrophages and in

some cases stronger than lymphocyte binding to antigen presenting dendritic cells (van den Berg *et al.*, 1992).

Mast cells and eosinophils found in medullary cords generally occur grouped together or in association with plasma cells. The presence of mast cells has been associated with lymphocyte activation in man and may explain their relationship with plasma cells (Battezzati & Donini, 1973). Mast cells may also ingest lipid that passes through the sinuses, as heparin that is present in mast cell granules is considered a lipid clearing factor and may be responsible for lipid uptake (Welsch *et al.*, 1997). Eosinophils are likely attracted to these same areas where mast cells are present by mast cells releasing eosinophil chemotatic factor (Cotran *et al.*, 1999).

1.4.1.3.2 Medullary Sinuses

Medullary sinuses are channels that carry lymph from the cortex to the hilum. These are not merely empty spaces but contain various cell types that include lymphocytes and macrophages. Macrophages within this area are similar to macrophages present in medullary cords and display many of the same morphological features and have the same enzyme activities (van der Valk & Meijer, 1987). As omnivorous phagocytes, they display undulating membranes and cellular projections and contain numerous lysosomes and phagosomes. These cells do not normally present antigen and do not constitutively express Ia antigen in mice (Fossum & Ford, 1985).

1.4.1.4 Lymph Node Framework

Lymph nodes maintain their three-dimensional structural integrity using complex arrangements of extracellular proteins to form reticular fibers (Castanos-Velez *et al.*, 1995). Reticular fibers interconnect to form an intricate network for cellular communications that varies between LN compartments. Ushiki *et al.* (1995), demonstrated that reticular fibers extend in a perpendicular fashion from the capsule

to hilum and the thickness of reticular fiber bundles varied at different LN locations. For example, reticular fibers surrounding primary follicles or germinal centers located within the paracortex, were more compressed than those within the medullary cords.

The reticular fibers are enclosed by fibroblastic reticular cells (FRC) with the basal lamella of the reticular cell often investing the fiber (Ushiki *et al.*, 1995). Initially, reticular fibers were thought to be composed of Type III collagen (Fossum & Ford, 1985). Recently, however, researchers have demonstrated that Type I and IV collagen are also present and the proportions of collagen found within the fibers vary between LN compartments (Castanos-Velez *et al.*, 1995). Fibroblastic reticular cells contain other proteins, including myofilaments, enabling the LN to expand and contract after stimulation (Tykocinski *et al.*, 1983).

1.4.1.5 Lymph Node Vasculature

Lymph node vasculature is divided into two groups: blood vessels and lymphatic vessels. These groups will be discussed in the following sections.

1.4.1.5.1 Blood Vessels

Blood vessels supply the LN with nutrients and oxygen and remove metabolic waste products. Blood to the LN arrives via one or two arteries that enter the LN at the hilum (Herman *et al.*, 1972; Anderson & Anderson, 1975). A complex vasculature system exists within the LN and the LN contains numerous arteriovenous connections and innervated venous sphincters. After the arteries enter the LN they continue longitudinally either branching to form cortical capillaries or entering the medulla to form a capillary network. Follicles and GC within the cortex have different microcirculatory blood flow patterns. Follicles are supplied by small arterioles that enter the follicle directly while GC receives blood from plexuses of arterioles and capillaries arising from branches of longitudinal arteries. Blood from venous plexuses drains to larger vessels by a centripetal drainage pattern.

Venous blood is then returned into a single vein to exit the LN at the hilum (Herman *et al.*, 1972; Anderson & Anderson, 1975). Arteriovenous communications are seen throughout the cortex and medulla. These structures are innervated by unmyelinated fibers and as a result blood flow is influenced by sympathetic stimulation.

1.4.1.5.1.1 High Endothelial Venules

High endothelial venules (HEV), also known as post-capillary venules, are highly specialized structures involved in the emigration of lymphocytes from circulation into the LN. They are located predominately within the paracortex with a few present as 'fine' vessels below the subcapsular sinus (Sainte-Marie & Peng, 1996). Histologically, HEV are distinct from other vessels found in LN and other tissues. Unusually tall columnar endothelial cells, with abundant cytoplasm and a prominent nucleus are characteristic features of HEV. The pleomorphic capability of the cells enables them to flatten and elongate, thereby, resembling squamous cells (Sainte-Marie, 1966). The endothelial cell morphology of HEV appears to be influenced by the local tissue environment. Occlusion of afferent lymphatic vessels for example, will change HEV from tall columnar endothelial cells into flat elongated cells that have a reduced metabolic activity and morphologically resemble normal endothelium (Hendriks *et al.*, 1987).

Lymphocytes can readily bind to HEV and be extracted from blood. It has been suggested that approximately 85-90% of all lymphocytes found in efferent lymph are blood lymphocytes that have entered the LN (Young, 1999). Anderson & Anderson (1976) used serial sections of rat LN to show that approximately 76% of lymphocytes found in deep paracortical vessels were in contact with HEV. These lymphocytes had microvillous projections that attached to 'pits' randomly located on endothelial surfaces. Furthermore, numerous lymphocytes were identified migrating

across HEV at cell junctions, with a small number of lymphocytes being engulfed by endothelial cells. Approximately 25% of all lymphocytes that flow through a resting LN pass through the HEV into LN parenchyma (Girard & Springer, 1995).

The method by which lymphocytes bind and cross HEV involves four stages; tethering, triggering, strong activation and migration (Anderson & Shaw, 1993). Briefly, tethering is the loose attachment of lymphocytes to endothelial cells and likely involves the interaction between L-selectins of lymphocytes binding to proteoglycans on endothelial cell surfaces. Triggering involves the binding of several ligands between lymphocytes and endothelium causing the rapid adhesion of these and other protein receptors. Once triggered, the binding allows for strong activation and strong adherence between lymphocytes and endothelium causing the adherence of tumbling lymphocytes. Finally, the lymphocyte migrates across the endothelium, predominantly through intercellular spaces.

On the luminal surface of HEV endothelium is a negatively charged glycocalyx. The glycocalyx is rich in sialic carbohydrates and O-sulfated proteoglycans (Van Damme *et al.*, 1994) which may play an important role in the immobilization of pro-adhesion molecules. These molecules, which may regulate lymphocyte trafficking include various cytokines and chemokines (Table 1.2). Interestingly, venules displaying the same morphological features as HEV in rodents and man have not been identified in ruminants (Harp *et al.*, 1990). The post-capillary venules in sheep are lined by low to medium sized endothelial cells, but are functionally similar to HEV found in other species. Using fluorescent-labeled cells, Harp *et al.* (1990) demonstrated similar efficacy in lymphocyte migration across HEV in mice and across post-capillary venules in sheep.

Table 1.2. Chemokine ligands and chemokine receptors associated with lymphocyte migration.

Biological Activity	Chemokine Receptor	Chemokine Ligands
Migration of naïve T-cells to lymph nodes and Peyer's patches	CCR7	SLC, ELC
Migration of naïve T-cells within lymphoid tissue	CXCR4	SDF-1α
Migration of memory T-cells to lymph nodes	CCR7	SLC, ELC
Migration of memory T-cells to gut	CCR9	TECK
Migration of memory T-cells to sites of inflammation	CCR2 CCR5	MCP-1,3,4 RANTES, MIP-1α,1β
Migration of Th1 effector T-cells	CCR2 CCR5 CXCR3	MCP-1,3,4 RANTES, MIP-1α,1β IP-10, Mig, 1-TAC
Migration of Th2 effector T-cells	CCR3 CCR4 CXCR4	Eotaxin-1,2, RANTES,MCP-2,3, HCC TARC, MDC-1 SDF-1α
Migration of B-cells	CCR7 CXCR4 CXCR5	SLC SDF-1α BLC

Table modified from von Andrian & MacKay, (2000).
Abbreviations: SLC secondary lymphoid tissue chemokine; ELC Epstein-Barr-virus-induced gene 1 ligand chemokine; SDF-1α stroma-derived factor-1α, TECK thymus-expressed chemokine; MCP monocyte chemoattractant protein; RANTES regulated on activation normal T-cell expressed and secreted; MIP macrophage inflammatory protein protein; HCC human C-C chemokine; IP-10 inducible protein of 10 kd; Mig monokine induced by INF-γ; 1-TAC interferon inducible T cell α-chemoattractant; TARC thymus-and activation-regulated chemokine; MDC-1 macrophage-derived chemokine-1; BLC B-lymphocyte chemoattractant.

1.4.1.5.2 Lymphatic Vessels

Extranodal vessels transporting lymph are categorized into either afferent or efferent lymphatics. Afferent lymphatic vessels collect extracellular fluid and cells from tissue and transport the lymph to the LN. Efferent lymphatics, in contrast, collect lymph from LN and through connections with other lymphatic vessels return lymph to the thoracic duct. Lymphatic vessels are valved with the orientation of valves in the direction of lymph flow (Wheater, 1987). Lymphatic vessels are lined with a flat endothelium similar to that found in blood vessels. Borron & Hay (1994) demonstrated that lymphatic endothelium has similar morphology, metabolic activity, surface marker expression and capacity to bind lymphocytes as does vascular endothelium. Besides these similarities between vascular and lymphatic endothelium, it is postulated that endothelium of efferent vessels may also regulate cell migration. This was first proposed by Binns & Licence (1988), using lymphocytes from suckling pigs and fetal lambs. Lymphocytes collected from pigs were injected iv into sheep and these lymphocytes exited the LN through efferent lymphatics. Conversely, sheep lymphocytes administered iv to pigs left the LN through HEV. These results indicate that signals required for lymphocyte migration through a LN is recipient dependent and may be controlled by both the vascular endothelium of HEV and lymphatic endothelium.

1.4.1.6 Innervation of Lymphoid Tissue

Adrenergic receptors and noradrenergic nerve fibers have been identified in LN. Noradrenergic fibers and $\alpha 2$ and β adrenergic receptors are found in high concentration in medullary cords and within the paracortex (Felten *et al.*, 1985; Fernandez-Lopez *et al.*, 1994a; Fernandez-Lopez & Pazos, 1994). Felten *et al.* (1985) demonstrated that nerve fibers innervating blood vessels terminate as plexuses within LN parenchyma and suggested that nerve stimulation could alter

both blood flow to various compartments and influence lymphocyte function by flooding the microenvironment with catecholamines. Parasympathetic fibers have not been found in LN; however, the occasional presence of acetylcholinesterases along nerve fibers may indicate muscarinic innervation (Felten *et al.*, 1985).

1.4.1.7 Effects of ISODN on Lymph Node Cellularity

As previously described, bacterial DNA (1.2 section) and ISODN (section 1.3) can stimulate cells and play an integral role in both the development and maintenance of a variety of immune responses. It is intuitive that with the induction of an immune response, structural changes in the draining LN should occur as well. Until recently, such effects of either bacterial DNA or ISODN on the draining LN have not been examined and only one study, in mice reported relatively short-term changes in the draining LN after ISODN treatment. Injection of 5 nmol (30 ug) of ISODN into the footpad of mice cause an increase in cell numbers isolated from a draining LN. It was observed that cell numbers began to rise within the first 24 hours following treatment, with the number peaking by the tenth day post-treatment. A maximal 88-fold increase in cell number was demonstrated, with substantial yet varied increases in lymphocytes, dendritic cells and granulocytes throughout the experiment (Lipford *et al.*, 2000). This study unfortunately, did not describe morphologic changes within the lymph node after treatment, so the effects of ISODN on cortical and medullary structures are unknown.

1.4.1.8 Conclusion for the Lymph Node Section

Lymph nodes are relatively complex structures that contain diverse cell populations important for immune function. The structure of LN allows for optimal cellular interactions and ensures proper processing of foreign antigen. As LN are dynamic tissues capable of major structural change after stimulation, exposing a LN to plasmid

DNA should enable us to determine the capacity of DNA to induce either short or long-term changes in LN structure.

1.5 Introduction to Cell Trafficking

Lymph is an extracellular fluid categorized on composition and location. Interstitial fluid (ICF), also known as tissue lymph, occupies intercellular spaces and is in direct contact with the extracellular matrix. Interstitial fluid contains few cells and low protein concentrations (Battezzati & Donini, 1973). Lymph is divided into three groups; peripheral, intermediate and central lymph. Peripheral lymph (afferent lymph) is located between the tissue and the draining LN. Intermediate lymph (efferent lymph) is fluid that has crossed lymphoid tissue but has not yet reached the main lymphatic trunks; and central lymph is lymph found in the main lymphatic trunk prior to reaching the venous circulation (Battezzati & Donini, 1973).

1.5.1 Non-Cellular Lymph

Lymph consists of both cellular and non-cellular components. The non-cellular component of lymph contains all protein fractions found in blood (Battezzati & Donini, 1973), but generally in lower concentrations (Schmid-Schonbein, 1990). In sheep for instance, the protein content of lymph from the mediastinal LN was 60% of that found in blood (Spencer & Hall, 1984). Interestingly, not all proteins have reduced levels in lymph when compared to blood. Spencer & Hall (1984) demonstrated that IgA levels were markedly elevated in lymph collected from the thoracic duct compared to IgA levels in blood. Lymph can contain biologically active molecules such as cytokines as well. In people, Yawalkar *et al.* (1998) demonstrated that following ultraviolet light exposure, afferent lymph had increased concentrations of IL-6 and IL-8. Similarly, treatment of sheep with antigen causes a significant rise in IL-2 levels within efferent lymph (Bujdoso *et al.*, 1990). Concentrations of electrolytes and other small molecules in lymph differ from blood.

Calcium, phosphorous and potassium levels for example, are slightly lower in lymph, while nitrogen and glucose have comparable values (Battezzati & Donini, 1973). Lipids are also present in lymph and the amount of lipid present is dependant on diet. In people for instance, it was shown that consumption of a meal will cause a substantial rise in the cholesterol and triglyceride content of intestinal lymph (Battezzati & Donini, 1973).

1.5.2 Cellular Lymph

Cell populations found in lymph vary considerably between the different compartments (Young, 1999). For the purpose of this discussion, only afferent and efferent lymph will be discussed in detail.

1.5.2.1 Afferent Lymph

Afferent lymph is composed of erythrocytes, granulocytes (Battezzati & Donini, 1973), lymphocytes (Hall & Morris, 1965; Hall, 1967; Smith *et al.*, 1970b) and dendritic cells (Bujdoso *et al.*, 1989). Afferent lymph flow rates are approximately 1 x 10^6 cells/hour/vessel and in general the lymph contains 85-95% lymphocytes and 10-15% macrophages and dendritic-like cells (Issekutz *et al.*, 1981; Young, 1999). The majority of lymphocytes are T cells, representing 80% of the entire lymphocyte population. The subpopulations of T cells correspond to 40-55% CD4 T cells, 13-15% CD8 T cells and 11-30% $\gamma\delta$TCR T cells. These T cells can express high levels of activation markers as well: for instance, the proportion of CD4 and CD8 cells expressing IL2α receptor (CD25), L selectin and MHCII are 36%, 67%, 89% and 19%, 75%, 80% respectively (Haig *et al.*, 1999). B cells represent the remaining 15-20% of lymphocyte populations found in afferent lymph (Haig *et al.*, 1999; Young, 1999).

The number of cells that traffic through an afferent vessel varies considerably and is dependent on local tissue reactions. In sheep, Smith *et al.* (1970a) demonstrated that resting afferent lymph from the lower leg flowed at approximately 9×10^6 cells/hour and contained 80-90% small lymphocytes, 2-5% lymphoblasts and 5-10% macrophage and macrophage-like cells with few granulocytes. After treatment with killed influenza virus and Freund's incomplete adjuvant, there was a marked change in cell number and phenotype. Within the first 24-48 hours, 70-90% of afferent cells were granulocytes. After 96 hours, however, the majority of cells were small lymphocytes, lymphoblasts and plasma cells with few numbers of macrophages. These cell populations remained relatively constant for the duration of the experiment. It is noteworthy, that as a granuloma developed at the injection site, there was a gradual increase in cell flow. A peak flow of 60×10^6 cells/hour occurred between Day 20 and Day 40-post treatment.

An experiment by Cahill *et al.* (1976) highlights the effect of short-term antigen exposure on cell traffic. Unlike the previous experiment, Cahill's group injected sheep with killed influenza virus in the absence of Freund's incomplete adjuvant. They demonstrated a rapid but brief increase in afferent cell flow that returned to pre-stimulation levels by Day 4. Smith *et al.* (1970a) reported similar findings, that treatment with Freund's adjuvant and killed virus induced a rapid influx of neutrophils two hours following treatment and a decline in neutrophil number that then occurred within the first 18 hours after treatment. Interestingly, the number of small lymphocytes remained constant throughout the experiment with a slight but transient increase in macrophage numbers. Unfortunately, neither experiment examined changes in the various lymphocyte subpopulations after treatment.

A more recent study examined the effects of a parasite-induced, acute dermatitis on lymphocyte populations of afferent lymph. A 4-fold increase in both

CD 4 and γδTCR T cells was seen in afferent lymph 30 hours post-treatment. Both lymphocyte populations appeared to be activated, with strong expression of the IL-2 receptor (CD25). It is noteworthy that not all cell populations were affected by the treatment as CD8 T cells, CD45R B cells and $CD1^+$ dendritic cells remained relatively unchanged (Nash *et al.*, 1996).

Not all treatment modalities will cause changes in cell populations of afferent lymph, even in the presence of enhanced cell flow. For example, short-term treatment of skin with ultraviolet light can cause a three-fold increase in cell flow in people. Analysis of the collected cells, however, indicated that there was no appreciable change in the frequency of cell populations. The CD4 and CD8 T cells and the CD1a dendritic cells remained at constant levels over the 7-day trial (Yawalkar *et al.*, 1998).

From the above described experiments, it is evident that cell flow and cell populations in afferent lymph can vary greatly. Differences in the treatment regimes, including type of antigen, duration of exposure and route of administration, result in markedly different responses. It is difficult, therefore, to make accurate predictions of the changes that may occur in afferent lymph following a specific treatment. In general, however, treatment with an antigen causes increased cell trafficking with variable changes in the numbers for individual cell populations.

1.5.2.2 Efferent Lymph

There are significant differences between the population of cells seen in efferent and afferent lymph as efferent lymph is almost entirely composed of lymphocytes. Lymphocytes in efferent lymph consist of approximately 75% T cells and 25% B cells. The CD4, CD8 and γδ T cells represent approximately 40-43%, 14-15% and 0-10% of total cell populations in efferent lymph, respectively (Haig *et*

al., 1999; Young, 1999). The T cells present in efferent lymph also have a reduced expression of activation markers relative to T cells in afferent lymph. The CD4 and CD8 T cells expressing IL-2α receptor (CD25), L selectin and MHCII are 5%, 35%, 30% and 1%, 64% and 38% respectively (Haig *et al.*, 1999).

1.5.2.2.1 Kinetics of Cell Flow in Efferent Lymph

The movement of lymphocytes through an antigen stimulated LN is well studied. The magnitude and duration of cell flow and changes in phenotype of cells emigrating from LN are quite variable and vary with the antigen administered. In general, efferent lymph has a cell flow rate of approximately 30 x 10^6 cells/hour/gram of LN and changes occur in three distinct phases following antigen administration (Young, 1999).

A remarkable drop in cell flow in efferent lymph after antigen exposure characterizes Phase 1. Using cannulated efferent lymphatic vessels in sheep, Cahill *et al.* (1976) demonstrated that the magnitude and duration of cell flow 'shut down' not only differed between antigens but also differed between mitogens as well. Injection with bacteriophage for instance, resulted in a nearly acelluar lymph that lasted for approximately 12 hours post-treatment. In contrast, injection of horse red blood cells caused a drop in cell output that was less than 10% of pre-stimulation values. A similar disparity in cell flow 'shut down' was demonstrated after treatment with two B-cell mitogens. Administration of lipopolysaccharide (LPS) caused an almost complete cessation in cell traffic that lasted for 18 hours. In contrast, injection of pokeweed mitogen (PWM) had little effect. In all treatments, the numbers of cells in efferent lymph were near pre-treatment levels by the 6th day post-treatment

The apparent shutdown in cell traffic seen in Phase 1 is probably not due to reduced cell movement from the blood to LN, but rather, to an enhanced retention of cells within the LN. Using radiolabelled cells, researchers determined that emigration of cells from blood to LN was equal in both antigen treated and untreated LN. There was however, a marked reduction in cell numbers leaving the LN within efferent lymph of the antigen treated LN (Cahill *et al.*, 1976; Issekutz *et al.*, 1981).

In general, Phase 2 is characterized by a large increase in cell flow that reaches maximal levels within 24 hours after treatment (Young, 1999). The duration and magnitude of this response, however, is greatly dependent on the type of antigen administered and the duration of antigenic exposure. For instance, infusion of allogeneic lymphocytes in sheep caused a 7-fold (35×10^7 cells/hour) increase in cell flow that peaked approximately 50 hours post-treatment and returned to pre-stimulation values by Day 6 (Cahill *et al.*, 1976). In comparison, Pedersen *et al.* (1975) demonstrated a 7-fold rise (35×10^7 cell/hour) in cell flow following renal allograft transplantation, with cell flow being maximal 80 hours post-treatment and remaining markedly elevated for more than 10 days. These two experiments illustrate the importance of antigen form and administration on cell flow. The long duration of cell flow in the experiment by Pedersen *et al.* (1975) is likely caused by the prolonged presence of antigen and the later onset of maximal flow may indicate that foreign renal tissue is not as effective as foreign lymphocytes at inducing events leading to increases in cell trafficking.

Efferent cell flow is markedly affected by treatment with mitogens as well. In sheep, Cahill *et al.* (1976) demonstrated that LPS induced increased cell flow to a maximum of 25×10^7 cell/hour 48 hours following treatment and flow rates then returned to pre-stimulation levels by Day 6. In contrast, PWM caused a maximal rise

in cell flow 24 hours post-treatment that was equivalent to the cell flow rate observed following treatment with LPS. Cell flow continued to rise and was greatest by the third day. Unlike LPS treated animals, cell flow in PWM treated sheep did not return to baseline levels by the 6^{th} day.

In phase 2, the increased numbers of lymphocytes in efferent lymph is caused by an increased recruitment of lymphocytes from blood. In sheep, Hay & Hobbs, (1977) demonstrated that stimulation of LN with allogeneic lymphocytes could cause a four-fold increase in blood flow. A four-fold increase in blood flow corresponded to a relatively equivalent four-fold rise in the numbers of efferent lymphocytes. The researchers also determined that in a stimulated LN, 25% of all lymphocytes that flow through the LN exited in efferent lymph, while the remaining 75% of lymphocytes stayed within blood.

Generally, phase 3 is characterized by the appearance of blast cells and activated lymphocytes and lasts for 2-3 days prior to returning to baseline levels (Young, 1999). Similar to the first two phases, Phase 3 is affected by the type of antigen treatment. Injection of allogeneic lymphocytes for instance, caused an elevation of the proportion of blast cells beginning on Day 5 post-treatment and subsiding by Day 7. The blast cells represented approximately 20% of all efferent lymphocytes (Hay & Hobbs, 1977). This differs greatly from transplantation of skin graphs, where the presence of blast cells in lymph begins on Day 5, peaks at Day 15 and returns to pre-treatment levels at Day 21 (Hall, 1967).

The increased number of blast cells in lymph usually preceded an increased release of activated cells as well. Salmonella antigen was injected subcutaneously in sheep and efferent lymph cells were collected. Twenty-four hours post-injection, there was an increase in blast cells and 48 hours after injection there was a

significant increase in activated lymphocytes producing antibody. Using a plaque-forming assay, the researcher demonstrated an approximate 20-fold increase in antibody production that continued throughout the trial (Hay *et al.*, 1972).

1.5.2.2.2 Cell Phenotypes within Efferent Lymph

Cell populations found in efferent lymph can be greatly affected by antigen treatment. As an example, Haig *et al.* (1996) treated sheep with *orf* virus and measured the phenotypes of lymphocytes in efferent lymph. They demonstrated a biphasic response that was characterized by increased numbers of memory CD4 T cells with fewer numbers of memory CD8 T cells. Naïve CD4 and CD8 T cells were lower in number as compared to their memory counterparts and $\gamma\delta$ T cells were rarely present. In contrast, lymph collected from sheep treated with *Toxoplasma gondii* demonstrated an initial increase in both CD4 and CD8 T cells in the early stages of infection; however, as the infection progressed, CD8 T cells became the dominant T cell population (Innes *et al.*, 1995).

The effect of antigen treatment on B cell populations was demonstrated by Gohin *et al.* (1997). Efferent lymph collected from sheep after administration of *Salmonella abortusovis* demonstrated an increase in both B and T cells in the early stages following treatment. At the peak response, however, T cell populations diminished while B cells increased markedly with all lymphoblasts being activated B cells (Gohin *et al.*, 1997).

1.5.3 Lymphocyte Recirculation

The recirculation of lymphocytes between different immune compartments is an important component of the immune system. Usually small lymphocytes migrate from blood to the LN and back to lymph, allowing for continual immunological

surveillance (Gupta *et al.*, 1998). Lymphocyte recirculation, therefore, is a selective process that affects both B and T cells and can be tissue specific.

Good examples of tissue specific lymphocyte recirculation were demonstrated by Abernethy *et al.* (1991) and Mackay *et al.* (1996). The former group collected intestinal lymphocytes from efferent lymph, labeled them with a fluorochrome marker and then injected cells back into venous blood. The researchers demonstrated a 4-5-fold increase in labeled cells collected in both afferent and efferent intestinal lymph as compared to prescapular afferent lymph. The latter group, using two fluorochromes, further demonstrated tissue specific recirculation. Cells collected from intestinal efferent lymph were labeled with a green marker, while cells collected from prescapular efferent lymph were labeled with a red marker. Cells were then returned to the venous circulation and recollected from both intestinal and prescapular lymph. Mackay *et al.* (1996) demonstrated a two fold enrichment in green cells collected from intestine and in red cells collected from skin. These observations revealed tissue-specific homing of lymphocyte populations.

Interestingly, not all lymphocytes will recirculate. Two distinct populations of B cells have been identified in sheep based on expression of CD11b and CD21. The $CD11b^+$ B cells are found only in blood and the marginal zone of the spleen. $CD21^+$ B cells are found in blood and the primary follicles of spleen, Peyer's patches and LN. When peripheral blood B cells were labeled and reinjected back into circulation, only $CD21^+$ B cells were collected from efferent lymph, and $CD11b^+$ B cells were absent in lymph at all times after injection (Gupta *et al.*, 1998).

Lymphocyte subpopulations emigrate from blood to efferent lymph at different rates and in different frequencies. The resting LN for instance, will extract CD4 and $\gamma\delta$ T cells at the same rate, and this rate is significantly faster than the rate

at which CD8 T cells or B cells are extracted. Phenotype analysis indicated that the resting LN preferentially extracts more CD4 T cells than other lymphocyte subpopulations. For example, CD4 T cells represent approximately 70% migrating lymphocytes, while low numbers of CD8 T cells and B cells and rare γδ T cells make up the remaining lymphocytes (Witherden *et al.*, 1990). These findings are similar to Abernethy *et al.* (1991), with approximately 62-72% of CD4 T cells and 5-12% CD8 T cells being extracted from blood into efferent lymph over a 48-hour collection period. Unfortunately, this experiment did not measure extraction rate of γδ T cells.

Lymphocytes can migrate to both inflamed and healthy tissue. Cells collected from afferent vessels draining a subcutaneous granuloma were labeled with ^{111}In-oxime and returned to blood. The numbers of labeled lymphocytes migrating back to the granuloma were 4-5 times greater than the numbers of cell recirculating through efferent lymph (Issekutz *et al.*, 1980).

1.5.4 Non-Antigenic and Non-Mitogenic Mediators

Biologically active compounds other than antigen or mitogens can influence cell movement in efferent lymph. These compounds vary from large protein complexes including complement to small molecules such as cyclic nucleotides and cytokines. Interestingly, not all of these compounds will cause a 'shut down' in cell traffic followed by a period of increased cell flow that occurs after antigen treatment. This section will examine how different compounds influence cell trafficking, highlighting the differences in cell trafficking responses following treatment.

Complement can induce a pause in the migration of cells into the efferent lymph post-treatment. Infusion of complement or complement activating factors, including immune complexes, cobra venom factor and inulin, into afferent lymph caused a rapid and short-lived reduction in cell output. Of interest, a complete

disappearance of B cells in efferent lymph was noted, while the level of T cells remained relatively constant. This observation supports the conclusion that a non-random selection of lymphocyte populations occurred post-treatment (McConnell & Hopkins, 1981).

Mediators of inflammation and non-inflammatory vasoactive compounds can also change cell trafficking after injection. These mediators have distinct effects on the kinetics and magnitude of cell flow. Prostaglandins (PG) for example, cause a reduction in cell flow. Hopkins *et al.* (1981) demonstrated that infusion of PGE_2 into afferent vessels caused an 84% reduction in cell flow that began minutes following treatment and remained reduced for six hours. Similar to the effects of treatment with complement, infusion with PGE_2 caused a complete disappearance of B cells. To determine if PGE_2 was a specific mediator causing a 'shut down' in cell trafficking, inhibitors of prostaglandin synthesis were infused prior to treatment with PGE_2. The researchers demonstrated that aspirin and indomethacin prevented cell flow shut down, while imidazole, a selective inhibitor of thromboxane A_2, had no effect. This suggests that prostaglandins rather than thromboxanes influence cell trafficking.

The direct effect of cytokines on cell trafficking has not been extensively studied. The cytokine IFN-α, however, has been tested in sheep and both intravenous and intradermal injections of IFN-α induce a marked reduction in cell numbers within efferent lymph. For example, injection of nanogram quantities of IFN-α will cause a 10-fold reduction of cell traffic into efferent lymph that lasts for approximately 30 hours. This drop in cell numbers correlates with only a transient increase in numbers of lymphocytes extracted from blood. This observation

supports the conclusion that lymphocytes are retained within the LN over this period (Kalaaji *et al.*, 1988).

Bradykinin is a potent vasoactive compound and a strong mediator of pain impulses. The kinetics and magnitude of cell trafficking responses induced by bradykinin differs significantly from cell trafficking responses following PGE_2 treatment. Infusion of bradykinin caused a 35% reduction in cell flow within the first 20 minutes after treatment. And then cell output increased to 200% above baseline levels for approximately 80 minutes (Moore, 1984). The initial decrease in cell flow was not a consistent finding, as only one-third of the sheep demonstrated reduced cell trafficking.

Treatment with vasoactive intestinal peptide (VIP) illustrated the ability of short-acting molecules to produce a prolonged reduction in cell traffic. Administration of VIP induced a 7-fold decrease in cell output within the first 60 minutes with a return to baseline levels 22 hours post-treatment. Even in the presence of reduced cell trafficking, not all lymphocyte subpopulations were reduced in number. Moore *et al.* (1988) demonstrated that VIP caused a significant reduction in CD8 T cells and B cells but a moderate increase in CD4 T cells.

As mentioned, not all vasoactive compounds cause an initial 'shut down' in cell trafficking after injection. The vasoactive neurotransmitter Substance P for instance, caused a prompt and marked increased in cell migration. Infusion of 50 μg of Substance P into afferent lymphatics causes an increase in cell trafficking 2 hours post-treatment with cell-output peaking by the third and fourth day. At peak periods, cell output was 8 fold greater than pre-stimulation levels (Moore *et al.*, 1989). In a subsequent experiment, Moore *et al.* (1990) demonstrated that increased cell output was associated with elevated numbers of CD4 T cells and a comparable reduction in CD8 T cells and B cells.

Other peptides including bombensin, (met)enkephalin and serotonin caused a rapid increase in cell traffic as well. Moore (1984), demonstrated that bombensin was a potent peptide capable of increasing cell trafficking. The administration of bombensin caused a 2-fold and 6-fold increase in lymphoblasts and small lymphocytes in collected lymph respectively. The increased cell output was rapid with the numbers of lymphoblasts peaking 30 minutes after treatment.

Stimulation of peripheral nerves also causes a short-lived increase in cell traffic that is mediated through the release of catecholamines. Stimulation of the splanchnic nerve will cause an 80% elevation in fluid flow and a 200% increase in cell migration, that return to baseline levels 5 minutes post-treatment. Infusion with norepinephrine produced a similar pattern in cell flow, and this increased flow could be abrogated with an α-adrenergic antagonist. This observation suggested that α-adrenergic stimulation and the subsequent release of norepinephrine enhanced cell trafficking (McGeown, 1993).

It is believed that the effects of several vasoactive peptides are mediated through the release of second messengers. Indirect *in vitro* evidence suggests that vasoactive peptides can increase the levels of cyclic nucleotide compounds in cells. To determine whether cyclic nucleotides can alter cell trafficking, Moore & Lachmann (1982) infused cAMP and cGMP into afferent lymphatic vessels. Treatment with cAMP produced a rapid decrease in cell trafficking that persisted for eight hours. There was also a decrease in the number of small lymphocytes and lymphoblasts and the volume of lymph flow. In contrast, cGMP caused a rapid eight-hour increase in fluid flow and the release of small lymphocytes and lymphoblasts into efferent lymph.

The effects of various mediators on cell movement have been examined in non-cannulated tissue such as skin. Colditz & Watson (1992) demonstrated that

various cytokines and proteins have distinct effects on cell movement through the dermis. They demonstrated that intradermal injections of IFN-γ, TNF-α, IL-8 and zymosan-activated plasma caused a significant influx of lymphocytes into skin that persisted for a 9-hour period. In contrast, treatment with IL-12, leukotriene B_4 (LTB_4), platelet activating factor and phytohemagglutinin did not enhance cell movement. Immunohistochemical analysis of the treated skin demonstrated marked dermal infiltrates of predominately CD4 T cell, with fewer CD8 T cells and rare $\gamma\delta$ T cells.

1.5.5 Movement of Fluid between Tissue and the Vasculature

Extracellular fluid accumulates in the interstitium by a net movement of fluid from blood vessels. Protein rich interstitial fluid flows either into lymphatics through clefts between endothelial cells or via transcellular migration across plasmalemmal vesicles (Dobbins & Rollins, 1970; Leak, 1971). The movement of fluid through the interstitium is dependent both on the hydrostatic pressure of blood and external forces (Aukland & Reed, 1993). The movement of extracellular fluid across blood vessel endothelium occurs by 3 distinct mechanisms: the intrinsic, extrinsic and retrograde lymph pumps. The intrinsic lymph pump consists of oscillatory constriction of smooth muscle around lymphatic vessels. Fluid moves in an anterograde fashion during periods of relaxation allowing, for vessel filling (Schmid-Schonbein, 1990). The extrinsic pump differs from the intrinsic lymph pump in that periodic compression and expansion of the vessel is dependent on surrounding tissue. This mechanism is particularly important for lymph movement in skeletal muscle (Schmid-Schonbein, 1990). Movement of lymph by the retrograde pump occurs as lymph travels along the lymphatic and develops a 'suction' between an open upstream valve and the closed downstream valve. This causes a pressure

gradient across the vessel and surrounding tissue to form and thereby allows extracellular fluid to flow across the lymphatic endothelium (Reddy *et al.*, 1975). This mechanism is believed to facilitate the movement of extracellular fluid into mesenteric vessels.

Transcellular migration of extracellular fluid into endothelial cells plays an important role in uptake of large solutes including ferritin and particulate matter. Transport of fluid occurs in vesicles and this form of transport may play a role in receptor-mediated processes. It is postulated however, that the transcellular movement of extracellular fluid likely does not involve large quantities of fluid moving across the vessel wall into the lumen (Rippe & Haraldsson, 1994) and therefore is of minor importance in lymph flow after antigen challenge.

1.5.6 Conclusion for the Cell Trafficking Section

The movement of lymph between various compartments is a dynamic process. Treatment with antigens, mitogens and other biologically active compounds can cause significant changes in the both the number and phenotype of cells migrating between the afferent lymph, LN and efferent lymph. As the effects of different antigens and other products on cell trafficking have been extensively studied in sheep, the use of the sheep model is ideal for studying the effects of plasmid DNA and immunostimulatory oligodeoxyribonucleotides on cell trafficking in a localized area.

1.6 Summary of the Literature Review

A review of literature in the area of DNA vaccination has shown that plasmid DNA encoding antigen can induce effective and protective immune responses. These responses are affected by route of administration and the presence of

immune modulating products such as encoded cytokines and immunostimulatory CpG motifs within the plasmid. CpG motifs in particular are quite effective at inducing immune responses as small CpG sequences in the form of ODN can protect against disease following challenge with different bacteria. It is well established that products such as antigen and mitogen can alter immune surveillance by changing cell trafficking and altering lymph node structure. These changes are likely important for the dissemination of 'immunological information' and generation of robust immune responses. It is of interest therefore, to determine whether plasmid DNA and immunostimulatory CpG motifs also influence immune surveillance, and thereby help to elucidate mechanisms that may be involved in immune modulation by bacterial DNA products.

CHAPTER 2

THE RESEARCH HYPOTHESIS

Within the last decade, it has become apparent the bacterial DNA has a remarkable capacity to stimulate cells involved in immune induction. The presence of hexameric CpG motifs within bacterial DNA is considered essential for immune activation and indeed it is well documented that CpG motifs are capable of stimulating both innate and adaptive immune responses. As examples, CpG motifs with synthetic oligodeoxyribonucleotides (ODN) can induce the release of cytokines and increase the expression of chemokines and costimulatory molecules (Krieg *et al.*, 1995; Ballas *et al.*, 1996; Yi *et al.*, 1998, Takeshita *et al.*, 2000). These motifs can also enhance humoral and cell-mediated immunity and protect against disease following a challenge with a variety of pathogens. CpG motifs are believed to stimulate immune system by acting as a pathogen-associated molecular pattern (PAMP) and binding to pattern recognition receptors (PRR) present on or within many cells.

Lymphocytes have the unique ability to migrate from blood to tissue and back to blood. This recirculation of cells allows for continuous communication between different immune compartments, facilitating immune surveillance and the dissemination of immunological information throughout the body. Although PAMP can influence the migration of inflammatory cells in tissue, the effects of PAMP cell trafficking are not fully understood. At present, only one PAMP, LPS, has been

shown to affect cell trafficking, and like antigen it causes a shut down of cell flow in efferent lymph.

I hypothesized that immunostimulatory CpG motifs, either in the form of synthetic ODN or encoded within plasmid DNA, function as a PAMP that can alter immune surveillance. Changes in immunosurveillance were monitored by measuring cell trafficking in the efferent lymph of the prescapular lymph node and assessing cellular and structural changes within this lymph node following a single intradermal injection of either plasmid DNA or an immunostimulatory ODN.

The research objectives therefore were divided into three main areas:

1) A long-term lymph node study (30 days) to determine if induced a prolonged change in lymph node size and architecture.

2) A lymphatic cannulation study to measure cell migration through a lymph node following a single intradermal injection of either plasmid DNA or an immunostimulatory ODN.

3) A short-term study (4 days) to determine whether a single intradermal injection of either plasmid DNA or an immunostimulatory ODN induced detectable changes in lymph node architecture and cellular composition. This analysis of lymph node structural changes provided insight into the mechanisms by which CpG motifs might alter cell trafficking

These research objectives were designed to test the hypothesis that a specific PAMP, immunostimulatory CpG motifs present in bacterial DNA, could indeed affect immune surveillance within a sheep lymph node.

CHAPTER 3

PLASMID DESIGN AND *IN VITRO* TRANSFECTION STUDIES

3.1 Introduction

DNA vaccination is a plasmid-based technology developed to induce antigen-specific immunity. To achieve this goal, the first priority was to develop plasmids that induced protective immune responses. Plasmids were designed to enhance gene expression and to improve vaccine adjuvanticity. Strategies aimed at improving gene expression included: the addition of a strong eukaryotic promoter (Boshart *et al.*, 1985); the insertion of intron A (Chapman *et al.*, 1991); and the presence of poly A adenylation sequences (poly [A] tail) (Pfarr *et al.*, 1986). Vaccine adjuvanticity has also been enhanced by incorporating immunostimulatory CpG motifs into plasmids (Sato *et al.*, 1996; Roman *et al.*, 1997). These motifs were added to some plasmids either by inserting tandem repeats of a known immunostimulatory DNA sequence or by using β-lactamase to select plasmids in culture.

One of the objectives of the cannulation experiment (chapter 5) was to determine whether cells migrating from the site of DNA injection expressed protein. To test this, several plasmids were created that expressed reporter genes, namely the wild type green fluorescent protein of jellyfish (GFP) and a variant form of the jellyfish protein, eGFP. Any cells transfected by plasmid and expressing the

encoded GFP protein could then be detected by fluorescence. This would therefore aid in the detection of individual cells that were transfected by plasmid DNA and expressed GFP in collected lymph.

This chapter will describe the creation of the following plasmids: pCAN1, pCAN1-GFP, pCAN1-eGFP, pBISIA and pBISIA-88 and examine the expression efficiency of pCAN1-GFP and pCAN1-eGFP plasmids following transfection of COS 7 cells.

3.2 Methods and Materials

3.2.1 Plasmid Construction and Purification

The plasmid pCAN1 was generated by ligating a fragment of plasmid from pUC19 (Yanisch-Perron *et al.*, 1985) with a fragment from the pSLIA plasmid (Braun *et al.*, 1997). An 1814 base pair fragment containing the origin of replication and β-lactamase was digested from pUC19 with the restriction enzymes *Aat*II and *Afl*III. A 2244 base pair fragment encoding the human cytomegalovirus (HCMV) promoter, intron A and the bovine growth hormone poly (A) region was cut from pSLIA with *EcoR*I and H*ind*III. These two fragments were blunted with T4 DNA polymerase and ligated with T4 DNA ligase. The resulting plasmid pCAN1 incorporates 17 hexameric CpG sequences that potentially display immune stimulating properties.

The plasmid pCAN1-eGFP was created by excising the eGFP gene from peGFP with *Xba*I sites (Clonetec Laboratories Inc., CA, USA) and ligating this fragment into pCAN1 at the *Xba*I site. Linear DNA with 1752 and 3069 bp fragments were created by digesting 2000 μg pCAN1-eGFP with 2500u *Pst*I and 2000u *Ssp*I for 6 hours at 37 °C. The oligodeoxynucleotide (ODN 2135), 5'-TCGTCGTTTG-TCGTTTTGTCGTT-3' with a nuclease-resistant phosphorothioate backbone induced *in vitro* B cell proliferation and IFN-γ production by sheep blood lymphocytes

(Pontarollo *et al.*, 2001) and hence was used in future experiments either alone or as an insert into plasmids. To insert ODN 2135 into plasmids, two copies of ODN 2135 were joined to form 5'-ACTCGTCGTTTGTCGTTTTGTCGTTTCGTCGTTT-GTCGTTTTGTCGTTG-3' and this construct (dimer) along with its complementary strand were annealed and inserted into the *Ava*II site of the pMAS plasmid (Krieg *et al.*, 1998)(pMAS was a gift from H.L. Davis, Loeb Research Institute, ON, Canada). The resulting construct, pBIS-88; contained 22 inserts of ODN 2135, which is equivalent to 88 CpG motifs. To create pMASIA and pBISIA-88, the HCMV intron A was isolated from pCAN1 by digestion with *Eag*I and *Pst*I and then ligated into pMAS and pBIS-88 cut with *Eag*I and *Eco*RV. Plasmid pMASIA has 18 potentially immunostimulatory hexameric sequences. To ensure these plasmids were created correctly and contained proper inserts, all plasmids were digested with restriction enzymes and run on 1% agarose gel. Purified plasmids were grown in *E. coli* strain DH5α (New England Biolabs, ON, Canada) and production and purification of plasmid DNA were performed according to manufacturer's specifications (Giga Kits, Qiagen Inc., On, Canada). All enzymes used for ligations were purchased from Pharmacia Biotech (Baie-Uréfé, QB, Canada) and plasmids were resuspended in saline (1 µg/µl) and stored at -20 °C.

3.2.2 Endotoxin Assay

Endotoxin contamination of plasmid preparations was assessed using the QCL-1000 Limulus Amebocyte Lysate Kit (BioWhittaker Inc., Walkersville, USA) according to manufacturer's specifications. Plasmid preparations containing 400 µg of DNA had endotoxin levels that varied from 2 pg to 500 pg.

3.2.3 Transfection of COS 7 Fibroblasts with Plasmid Expressing GFP and eGFP.

The COS 7 fibroblast cell line was used in transfection experiments and

these cells were grown to confluence in 6 well plates with growth medium (Iscove's Modified Dulbecco's Media, Gibco-BRL, ON, Canada) containing 2.5 % fetal bovine serum (FBS, Gibco-BRL, ON, Canada) and penicillin and streptomycin (Gibco-BRL, ON, Canada). Plasmid DNA was prepared for transfection by adding lipofectamine (Lipofectin Reagent, Gibco-BRL, Canada) to plasmid DNA in Optimem (Opti-MEM, Gibco-BRL, ON, Canada) at a 5:1 ratio (vol/wt), and then incubating for 15 minutes at room temperature. During this incubation period, COS7 cells were washed twice with Optimem at 37°C. The lipofectamine treated plasmids were added to confluent COS 7 cells and incubated at 37°C in 5% CO2 for 2 hours. The cells were then washed with growth medium and incubated at 37°C in 5 % CO2 for the duration of the experiment. Measurements of cell fluorescence were achieved with a FACSscan flow cytometer (Becton Dickinson, CA, USA) and the Cell Quest (Becton Dickinson, CA, USA) program was used for data acquisition and analysis. Photographs of green fluorescing COS 7 fibroblasts were taken with 100 ASA color film (Kodak Eastman, ON. Canada) exposed to ultraviolet light for 30 seconds.

3.2.4 Data Analysis

Comparisons of the values for fluorescence of cells transfected with either pCAN1-GFP or pCAN1-eGFP were performed with a paired Student's t-test. Levels of significance were measured and any *P* values less than 0.05 were considered significant. All numerical values are expressed as mean ± standard error of the mean (SEM).

3.3 Results

3.3.1 Enzymatic Digestion of Plasmid DNA

To ensure that plasmids isolated from bacterial culture were the appropriate constructs, plasmids were digested with various restriction enzymes and run on 1%

agarose gels. This analysis would verify the molecular weight and confirm the presence of either the reporter gene or ODN 2135 inserts. All plasmids were cut at a single restriction site and the size of the plasmid was determined. The molecular weights of pCAN1, PCAN1-GFP, pCAN1-eGFP, pMASIA and pBISIA-88 were 4058, 4731, 4821, 4232 and 4833 base pairs (bp), respectively (Fig. 3.1: Lanes 1, 2, 4, 7and 9). All plasmids except for pCAN1 were cut with two enzymes to confirm that these plasmids contained the appropriate insert. The digests of plasmids pCAN1-GFP and pCAN1-eGFP generated 724 and 4007 and 763 and 4058 bp DNA fragments, respectively (Fig. 3.1: Lanes 3 and 5). The reporter proteins GFP and eGFP correlate to the 724 and 763 bp segments. Similarly, digests of pMASIA and pBISIA-88 produced 1221 and 3011 and 1221 and 3612 bp DNA fragments, respectively (Fig. 3.1: Lanes 8 and 10). The ODN 2135 tandem repeat segment (600 bp) is located within the 3612 DNA fragment of pBISIA-88. Diagrams of the five plasmids are found in the appendix (Appendix: Fig. 1).

3.3.2 Transfection of COS 7 Cells with Plasmid DNA

The results from the plasmid digests suggested that both GFP and eGFP were successfully cloned into the plasmid pCAN1. Gene insertion within the plasmid does not necessarily ensure biological activity, however, as orientation of the insert could be inverted. To assess biological activity, plasmids were transfected into COS 7 cells and protein fluorescence was assessed. The results verified that both GFP (Fig 3.2 and 3.3) and eGFP (Fig 3.3) were ligated with proper orientation into the plasmid pCAN1 as COS 7 cells fluoresced following transfection with both plasmids. Figure 3.3 demonstrates that the amount of fluorescence differed in cells transfected with the two GFP inserts. It also shows that fluorescence was influenced by both the concentration of plasmid and the duration of incubation following transfection. Cells transfected with 2 μg of plasmid pCAN1-GFP and incubated for 48 hours had the

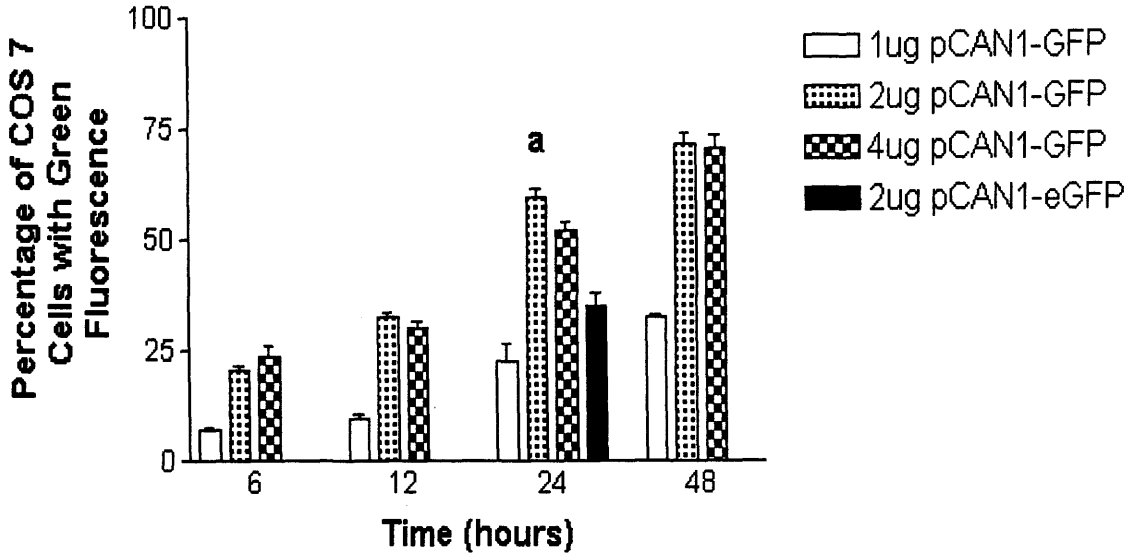

Figure 3.3. The percentage of COS7 cells that fluoresce following transfection with pCAN1-GFP or pCAN1-eGFP. The bar represents mean ± SEM and all samples were run in triplicate.
[a] Significant difference in fluorescence of COS 7 cells transfected with 2 μg of pCAN1-GFP and 2 μg pCAN1-eGFP ($P < 0.05$).

highest level of fluorescence. These results were similar to cells treated with 4 μg of the same plasmid and incubated over 48 hour period. This suggests that gene expression does not improve following transfection with more than 2 μg of plasmid.

Cultured COS 7 cells developed moderate levels of fluorescence following transfection with pCAN1-eGFP. In this experiment, transfection was only examined in cells treated with 2 μg of plasmid and incubated for 24 hours (Fig. 3.3). The number of fluorescent cells following pCAN1-eGFP was approximately 48% of that observed following pCAN1-GFP transfection ($P < 0.05$).

To determine whether the amount of GFP and eGFP expression differed in transfected cells, the mean fluorescence intensity (MFI) was measured in COS 7

cells following transfection. Table 3.1 demonstrates that cells transfected with either 2 μg of pCAN1-GFP or 2 μg of pCAN1-eGFP and incubated for 24 hours had similar MFI. This indicates that transfected cells expressed both proteins with comparable efficiencies. An increase in the MFI of transfected cells was also seen following transfection with increased concentrations of plasmid as well as an increased expression time post-transfection (Table 3.1). Interestingly, there was no variation in the MFI of cells transfected with either 2 μg or 4 μg of pCAN1-GFP when analyzed 48 hours post transfection. This supports the finding that GFP expression is not improved in cells transfected with concentrations of plasmid greater than 2 μg.

Table 3.1 Mean fluorescence intensity of COS 7 cells transfected with pCAN1-GFP or pCAN1-eGFP.

	Post-Transfection (hours)			
Treatment	6	12	24	48
1 μg pCAN1-GFP	306 ± 19.0	548.7 ± 14.7	961.7 ± 108.0	1720.0 ± 55.2
2 μg pCAN1-GFP	481.6 ± 9.4	1189 ± 32.9	2217.6 ± 71.1	2894.3 ± 61.7
4 μg pCAN1-GFP	590.7 ± 30.5	1220.7 ± 109.4	1972.3 ± 48.2	3081.0 ± 159.6
2 μg pCAN1-eGFP	ND	ND	2406.7 ± 119.0	ND

Values represent the mean ± SEM for the fluorescence intensity of CO7 cells transfected with pCAN1-GFP and pCAN1-eGFP (n=3). ND indicates that the data was not determined.

3.4 Discussion

Development of plasmids with both strong immunostimulatory properties and the potential to express high amounts of antigen is important in the development of an effective DNA vaccine. Using this rationale, plasmids were designed that could effectively express antigen and these plasmids were further supplemented with strong immunostimulatory DNA sequences to improve adjuvanticity. Several methods were employed to further improve antigen expression, including the addition of the human cytomegalovirus (HMCV) promoter, intron A and poly [A] tail sequences and the use of ampicillin resistance for plasmid selection. As an example, the HMCV promoter has induced strong antibody responses *in vivo*. Braun *et al.* (1997) compared antibody production in mice after treatment with plasmids containing either Rous sarcoma virus or HMCV promoters. These researchers showed that mice injected either intradermally or intramuscularly with a plasmid containing HCMV promoter, induced rapid production of antigen-specific antibodies that remained elevated for the duration of the experiment. In comparison, mice injected with plasmid containing the Rous sarcoma virus promoter, produced antibody titers that peaked weeks later than mice treated with plasmid incorporating the HCMV promoter.

Plasmids with an intron A sequence also expressed higher levels of antigen when compared to plasmids lacking the intron (Chapman *et al.*, 1991). Expression of a bacterial surface protein was absent in cells treated with plasmids missing intron A. This differed from cells transfected with plasmids containing the intron, as these cells had marked protein expression (Chapman *et al.*, 1991). To further improve gene expression, a poly [A] tail from bovine growth hormone was added to plasmids. Poly [A] tails present at the 3' end of mRNA have been shown to improve mRNA stability and gene expression (Pfarr *et al.*, 1986). Finally, adjuvanticity can be

improved by adding the ampicillin resistance gene to plasmids, as *in vivo* studies showed improvement in immune responses following immunization with plasmids expressing β-lactamase. Sato *et al.* (1996) for example, demonstrated a 9-fold increase in antibody titers in mice injected with plasmids encoding β-lactamase when compared to plasmids encoding kanamycin resistance. It is believed that the presence of two additional immunostimulatory CpG motifs within the ampicillin resistance gene is responsible for the enhanced adjuvanticity.

All plasmids generated have an origin of replication site derived from the self replicating plasmid CoL E1, a plasmid that can generate large copy number in bacterial culture (Clewell, 1972). Incorporating the origin of replication into plasmids ensures the production of large quantities of plasmid.

The gene sequence encoding for the green fluorescent protein of jellyfish (GFP) was added to the plasmid pCAN1 to facilitate the detection of cells transfected with plasmid DNA during cell trafficking studies. The transfection experiments demonstrated that both pCAN1-GFP and pCAN1-eGFP express adequate levels of protein. Interestingly, pCAN1-eGFP transfection was approximately one-half as efficient as pCAN1-GFP transfection (Figure 3.3). It is difficult to explain the transfection differences between the two plasmids. The pCAN1-GFP did not express protein with greater efficacy than pCAN1-eGFP, as the MFI of transfected cells were similar (Table 3.1). Furthermore, the protein sequences of GFP and eGFP are quite similar and eGFP has been engineered to have improved gene expression. The eGFP protein has modified translational initiation sites (Kozak, 1987) and fluorescence is enhanced 100-fold by shifting the light emission spectrum (Cormack *et al.*, 1996). One possible cause for differences in the amount of fluorescence was the potential for variations in test conditions.

Cells were transfected with the two plasmids at different times and changes in test conditions could alter transfection efficiency. Another possible explanation is that pCAN1-GFP is incorporated into cells with greater efficiency than pCAN1-eGFP and this transfection efficiency may be dose-dependent. This possibility is unlikely, however, as both plasmids are similar in size and structure. Nevertheless, a dose-response study with pCAN1-eGFP was not carried out to determine whether transfection of cells with a higher concentration (4μg) of pCAN1-eGFP would have improved transfection efficiency. The transfection experiments, however, verified that both plasmids had adequate gene expression to detect transfected cells and either plasmid could be used in the lymph node or cell trafficking studies.

The plasmid pMASIA was created from pMAS (Krieg *et al.*, 1998) and pMAS was generated from the plasmid pUk21-A2 by removing 36 CpG motifs from at least 15 immunoneutralizing DNA sequences. The plasmid pMAS was shown to generate strong humoral and cell-mediated responses *in vivo*. Mice treated with pMAS for example, demonstrated a 10% and 300% increase in CTL responses and hepatitis B antigen antibody titers respectively, when compared to pUK21-A2 treated mice (Krieg *et al.*, 1998). The plasmid pBISIA-88 was created by inserting 11 tandem repeats of the ODN 2135 dimer into pMASIA. For DNA sequences to be immunostimulatory they must maintain a proper CpG orientation. If these sequences are reversed and have a GpC orientation, the DNA segments are no longer immunostimulatory. Proper orientation of the ODN 2135 insertion into pMASIA was accomplished by cutting both the plasmid and oligodeoxyribo-nucleotides (ODN) with restriction enzymes to form sticky ends. After annealing ODN 2135 to its complementary strand, ODN 2135 was cut with the restriction enzyme *Ava*II to form sticky ends. The resulting duplex was joined end to end with another *Ava*II cut ODN 2135 to form a single large ODN containing 11 tandem

repeats of the ODN 2135 dimer. The plasmid pBIS was also cut with *Ava*II to form sticky ends, and the tandem ODN was ligated into pBIS to create the plasmid pBIS-88. The rationale for inserting tandem repeats of ODN 2135 into plasmid was based on studies that demonstrated improved adjuvanticity in DNA vaccines containing immunostimulatory CpG motifs (Sato *et al.*, 1996; Roman *et al.*, 1997). Plasmids containing known immunostimulatory ODN provided an effective tool to address the role of CpG motifs in modulating cell trafficking through a draining lymph node.

3.5 Summary

To study the effects of plasmid (bacterial) DNA on lymph node morphology and cell trafficking, various plasmids were created. These plasmids included two null vectors lacking inserts (pCAN1 and pMASIA), a plasmid with enhanced immunostimulatory CpG motifs (pBISIA-88) and plasmids containing functional eukaryotic reporter genes (pCAN1-GFP and pCAN1-eGFP).

CHAPTER 4

LONG-TERM EFFECTS OF INTRADERMALLY INJECTED PLASMID DNA ON THE DRAINING LYMPH NODE MORPHOLOGY

4.1 Introduction

Deoxyribonucleic acid (DNA) immunization is a recent innovation in vaccine delivery. Bacterial DNA, usually in the form of double stranded circular plasmids, has been extensively studied and used for DNA immunization. The relative ease and low expense of large scale plasmid production and the simplicity of inserting genes that encode antigens or modulate immune function makes plasmid DNA an ideal candidate for immunization or gene therapy. Plasmid DNA has been administered intramuscularly (Braun *et al.*, 1997; van Drunen Littel-van den Hurk *et al.*, 1998), intravascularly (Kawabata *et al.*, 1995; Lew *et al.*, 1995; Isner *et al.*, 1996) and intradermally (Lew *et al.*, 1995; Braun *et al.*, 1997; van Drunen Littel-van den Hurk *et al.*, 1998) using techniques that varied from syringe and needle to ballistic delivery (Braun *et al.*, 1997; van Drunen Littel-van den Hurk *et al.*, 1998). The method of delivery, location of injection and amount of plasmid DNA administered can influence the immune response to an encoded antigen.

Initially, bacterial plasmid DNA was considered inert and an ideal vehicle for immunization. Within the past decade, however, plasmid DNA has been shown to induce B lymphocyte proliferation (Krieg *et al.*, 1995) activate natural killer cells (Yamamoto *et al.*, 1992; Ballas *et al.*, 1996; Cowdery *et al.*, 1996) and enhance production of cytokines that influence both cell-mediated (Corr *et al.*, 1996; Klinman

et al., 1996; Sato *et al.*, 1996; Roman *et al.*, 1997) and humoral (Klinman *et al.*, 1996; van Drunen Littel-van den Hurk *et al.*, 1998) immune responses. It is believed that bacterial DNA has inherent immunostimulatory properties. Unmethylated cytosine and guanine dimers (CpG), flanked by two purines upstream and two pyrimidines downstream to form a hexameric sequence may be responsible for this phenomenon (Klinman *et al.*, 1997). These CpG motifs occur approximately 16 times more frequently in bacterial DNA than in vertebrate DNA (Krieg *et al.*, 1995). It is postulated that these differences between bacterial and vertebrate DNA aid in the recognition of microbial invasion and influence the capacity of the vertebrate immune system to mount an appropriate response.

To date, the long-term effects of administering plasmid DNA on draining lymph node size or morphology have not been investigated. As DNA vaccines may be widely used in people and food producing animals in the future, the effects of plasmid administration on lymph node size and morphology should be addressed. The objective of this study was to use ultrasonography to follow changes in ovine popliteal lymph nodes following plasmid DNA administration and then to examine lymph nodes for macroscopic and histological changes.

4.2 Materials and Methods

4.2.1 Experimental Design and Lymph Node measurements

Five, 20-week-old male or female Suffolk sheep (Department of Poultry and Animal Science, University of Saskatchewan, Canada), were injected intradermally two centimeters distal to the lateral prominence of the head of the fibula with 400 µg (1µg/µl) of purified pCAN1 plasmid. An equal volume of saline was injected at the same location on the contralateral leg. Ultrasound imagery of lymph nodes was done every seven days for 28 days using a B-mode ultrasound scanner equipped with a 7.5 MHz linear-array transducer (Aloka SSD-500 Instruments, Science and

Medicine Inc., Vancouver, Canada). The wool was shaved and measurements were done transcutaneously over the caudo-lateral aspect of the femorotibial joint in the region of the popliteal fossa. Mineral oil was used as a coupling medium. The transducer was maneuvered to obtain the most complete medial section of the lymph node. The image was frozen and lymph node height and width were measured using the integrated electronic calipers of the ultrasound machine. Area measurements were estimated with the formula of an ellipse (½ height x ½ width x π). Sheep were euthanized 30 days after plasmid DNA injection and popliteal lymph nodes were removed. Adipose tissue was stripped from the capsular surface and lymph node volume and weight were assessed by water displacement and gravimetric measurements, respectively. A midline section through the long axis of each lymph node was fixed in 10% buffered formalin, processed routinely and embedded in paraffin. Histological sections used for morphologic evaluation were stained with hematoxylin and eosin (HE). Tissue sections were also stained with Gordon and Sweet's silver stain for reticular fibers to delineate germinal centers and help visualize changes within the medulla.

Total cortical and medullary areas of the lymph node were determined by image analysis (Northern Eclipse, Empix Imaging Inc., Mississauga, Ont.). The diameter of germinal centers, thickness of medullary cords and distance spanning medullary sinuses were measured with a micrometer at 100x and 400x magnification. The total number of germinal centers was determined by counting all germinal centers per histological section. To ensure an accurate measurement of germinal centers, the radius of each germinal center was taken as the average of two radii measurements. It was assumed that germinal centers were spherical and the area of each germinal center was calculated (area = πr^2). The total germinal

center area was a calculated summation of all germinal center areas per histological section. Measurements for medullary cords and sinuses were taken as the average of measurements of five random locations within the medulla in histological section.

4.22 Data Analysis

Comparisons of the values for plasmid and saline-treated popliteal lymph nodes were performed with a paired Student's t-test. Levels of significance were measured and any *P* values less than 0.05 were considered significant. All numerical values are expressed as mean ± standard error of the mean (SEM).

4.3 Results

4.3.1 Analysis of Popliteal Lymph with Ultrasound and Gravimetric and Volumetric Measurements

Following plasmid injection, lymph node size was measured weekly. Ultrasound measurements demonstrated a progressive increase in lymph node size for both saline and plasmid DNA treatment (Fig.4.1). Lymph nodes were collected on day 30 post-treatment and gravimetric analysis demonstrated a significant increase ($P < 0.05$) in plasmid treated lymph nodes (Table 4.1).

4.3.2 Histological Measurements of Popliteal Lymph Nodes

Histological examination of control and plasmid-exposed lymph nodes revealed no statistically significant changes in total, medullary or cortical lymph node area (Table 4.2). It is noteworthy, that a 40% increase in medullary area was not statistically significant ($P < 0.13$); however, such a large change could have biological importance.

The most prominent histological changes in plasmid-exposed lymph nodes were increased germinal center number and germinal center area, with marked enlargement of medullary cords and sinuses. Figures 4.2a and 4.2b show that both the number (71.4 ± 17.7) and total area (4.0 ± 1.3 mm^2) of germinal centers within

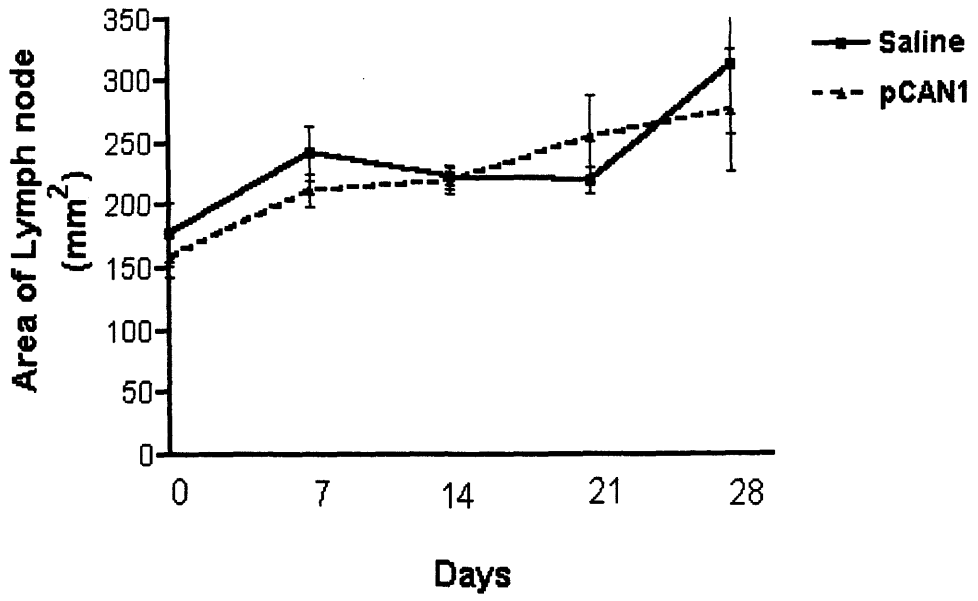

Figure 4.1 Ultrasound measurements of the area of popliteal lymph nodes exposed to 400 µg of plasmid (1µg/µl) or 400 µl of saline over a 28-day period. Bars represent mean ± SEM of values for 5 sheep.

Table 4.1. Gravimetric and volumetric measurements at 30 days post-exposure of popliteal lymph nodes exposed to plasmid DNA.

Lymph Node Measurements	Plasmid DNA[1]	Saline[2]	Percentage Change[3]
Weight (g)	2.8 ± 0.1	2.0 ± 0.6	40*
Volume (ml)	2.2 ± 0.8	1.6 ± 0.6	38
Density (g/ml)	1.5 ± 0.3	1.4 ± 0.1	7

Data represents the mean ± SEM for 5 sheep treated with plasmid or saline.
[1]400 µl (1 µg/µl) of plasmid DNA injected intradermally.
[2]Contralateral lymph node was exposed to 400 µl of saline injected intradermally.
[3]Percentage change represents the mean value of plasmid DNA treated lymph nodes divided by the mean value for saline treated lymph nodes.
* Significant difference between plasmid and saline treated lymph nodes ($P < 0.05$).

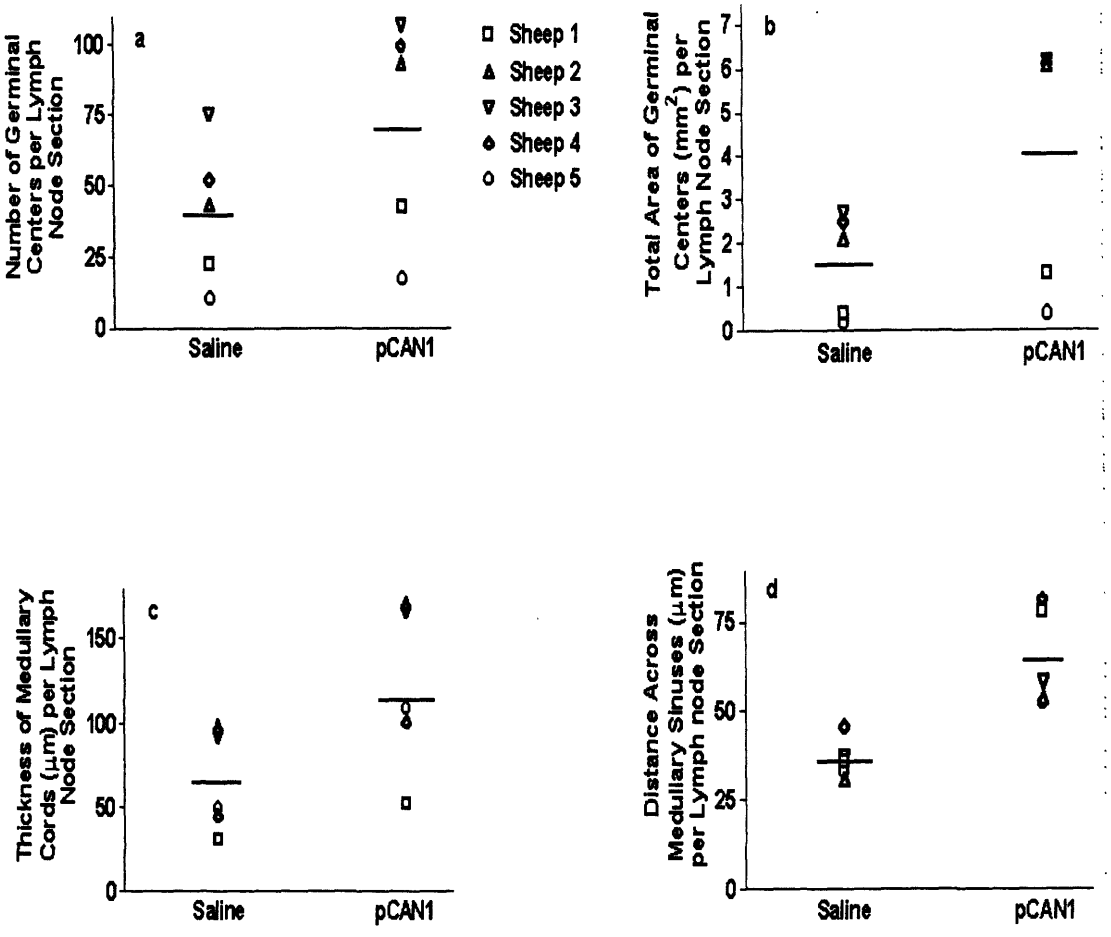

Figure 4.2 Histologic measurements of popliteal lymph nodes 30 days post exposure. **a** Total number of germinal center areas per histological section. **b** Total area of germinal centers per histological section. **c** Thickness of medullary cords in histological sections. **d** Distance across medullary sinuses in histological sections. Plasmid exposed lymph nodes are represented by pCAN1 and control lymph nodes are represented by saline. Each symbol presents data for an individual animal and the bar represents mean values for each group.

Table 4.2. Histological measurements at 30 days post-exposure of popliteal lymph nodes exposed to plasmid DNA.

Lymph Node Measurements	Plasmid DNA[1]	Saline[2]	Percentage Change[3]
Total Lymph Node Area (mm2)	183.0 ± 43.0	141.0 ± 36.1	30
Cortical Area (mm2)	56.4 ± 9.1	50.5 ± 8.6	12
Medullar Area (mm2)	126.7 ± 36.4	90.5 ± 27.2	40
Percentage of Cortical Area to Total Lymph Node Area	34.4 ± 5.1	38.7 ± 8.5	-11
Percentage of Medullary Area to Total Lymph Node Area	65.6 ± 5.1	47.3 ± 6.0	38

Data represents the mean ± SEM for 5 sheep treated with plasmid or saline.
[1]400 μl (1 μg/μl) of plasmid DNA injected intradermally.
[2]Contralateral lymph node was exposed to 400 μl of saline injected intradermally.
[3]Represents a percentage change of the mean values of plasmid DNA treated lymph nodes relative to the mean values of saline treated lymph nodes.

the tissue section were increased significantly (P <0.05) in plasmid-exposed lymph nodes when compared to control lymph node (40.4 ± 11.4 and 1.6 ± 1.2 mm^2, respectively). Figures 4.2c and 4.2d indicate that medullary cords were thickened and medullary sinuses were wider in plasmid-exposed lymph nodes. Medullary cords were significantly thicker (P < 0.01) in plasmid-exposed lymph nodes (114.2 ± 25.2 μm) than in control lymph nodes (62.7 ± 14.9 μm) and medullary sinuses of plasmid treated lymph nodes (64.4 ± 2.5 μm) were significantly more distended (P < 0.01) when compared to control lymph nodes (36.5 ± 1.0 μm). Typical histological changes within lymph nodes are presented with tissue from Sheep 4 (Fig. 4.3). Plasmid treated lymph node from this animal displayed an approximately, two fold increase in germinal center number. The plasmid-exposed lymph node (Fig. 4.3a) had numerous clusters of germinal centers that extended to the deep aspect of the cortex. These germinal centers were packed with round cells with open-faced nuclei and abundant pale finely vacuolated eosinophilic cytoplasm (lymphoblasts). The

number of mitotic figures present varied (0-10 per germinal center) and several macrophages containing phagocytized cell debris (tingible-body macrophages) were apparent. Germinal centers in the control lymph node (Fig. 4.3b) were smaller, seldom extended into the deep aspect of the cortex and had fewer mitotic figures (0-2 per germinal center). Medullary cords in plasmid-exposed lymph node (Fig. 4.3c) as compared to control lymph node (Fig. 4.3d) were markedly thicker, often tortuous, and contained a cell population rich in small lymphocytes and plasma cells with fewer lymphoblasts and macrophages. Within plasmid-exposed lymph nodes, sinuses separating medullary cords were dilated, often free of cells and occasionally had reticular fibers that spanned the sinusoidal space (Fig. 4.3e). In contrast, sinuses in control lymph nodes were narrow, with little separation of reticular fibers (Fig.4.3f). These sinuses were populated primarily with macrophages and fewer lymphocytes were evident.

4.4 Discussion

This investigation is the first report to demonstrate that bacterial DNA can cause long-term changes in draining lymph node weight and morphology (Uwiera *et al.,* 2001). Morphometric and histological measurements of lymph nodes 30 days post-injection, revealed changes in lymph node weight and architecture that were unique to the plasmid treated lymph node. The increased weight of plasmid treated lymph nodes could be caused by either an accumulation of lymph, cells, or a combination of the two. Our results suggested that these lymph node changes correlated with an enlargement of the medulla as total cortical area remained relatively constant (Table 4.2). Histological examination of the lymph nodes revealed that plasmid DNA induced an increase in medullary size that was associated with both increased cellularity and an accumulation of lymph (Fig. 4.3).

Medullary cords are structures that extend into the medulla and are filled with B-lymphocytes, proplasmacytes and plasma cells (Straus, 1981; Wheater, 1987). In plasmid-exposed lymph nodes, medullary cords were thickened and rich in lymphoid cells and medullary sinuses were distended, with reticular fibers being widely separated. Several studies have demonstrated that enlargement of medullary cords can occur after administration of protozoal organisms (Masake, 1980), bacteria (Sato *et al.*, 1990; Spalding & Heath, 1991) and dextran (Spalding & Heath, 1991). The injection of these antigens was associated with large distorted medullary cords filled with plasma cells and lymphoblasts. The sinuses were also dilated, displayed fewer reticular fibers and displayed a prominent orientation towards the hilus. These previous observations suggest that bacterial DNA either induced an active immune response, or activated cellular events that mimic morphologic events induced by foreign antigens.

Histological evaluation of plasmid-exposed lymph nodes also demonstrated a marked enlargement of germinal centers. Germinal centers are composed predominantly of B lymphoblasts and follicular dendritic cells (Morrison, 1986). Enlargement of germinal centers may have been the result of increased B cell proliferation or a reduction in the death of lymphoblasts. In the present experiment, germinal centers of plasmid-exposed lymph nodes were enlarged and rich in lymphoblasts with variable mitotic activity. These observations suggest an enhanced generation of cells within germinal centers. Expansion of germinal centers has been identified in animals challenged with endotoxin (Fujino *et al.*, 1996), protozoa (Masake, 1980), or bacteria (Sato *et al.*, 1990). These researchers have shown that following an insult, germinal centers can respond with increased number, size and lymphoblast proliferation.

Clonal expansion of B-lymphocytes can be caused by the addition of B lymphocyte mitogens such as endotoxin (Hadden, 1990). Endotoxin contamination, therefore, is always a concern in plasmid prepared for DNA immunization. In our experiments, less than 2 pg of endotoxin were detected in each plasmid injection. Most studies have examined the effects of endotoxin on indicators of acute inflammation (Boosman *et al.*, 1989; Conner *et al.*, 1989; Adams *et al.*, 1990). One study in mice, however, demonstrated enlargement of germinal centers and medullary cords after endotoxin administration. The study did not extend beyond 10 days and therefore the long-term effect of endotoxin on germinal centers was not assessed (Fujino *et al.*, 1996). The amount of endotoxin used in these experiments ranged from microgram to milligram quantities and greatly exceeded the quantity of endotoxin delivered in our experiment. It is unlikely, therefore, that the lymph node changes were caused by administering endotoxin. We cannot dismiss, however, the possibility that a low level of endotoxin may interact synergistically with plasmid DNA and accentuate changes seen within the cortex or medulla. Cowdery *et al.* (1996) described that mice pre-treated with bacterial DNA produced large quantities of interferon-γ and interleukin-6 after endotoxin challenge. If bacterial DNA influences cytokine profiles following endotoxin exposure, then it is possible that endotoxin exposure could influence changes in lymph node morphology following the administration of bacterial DNA.

4.5 Summary

Our study demonstrated that purified bacterial plasmid DNA can elicit marked long-term changes in lymph node weight and morphology in sheep. As plasmid DNA is the primary vehicle used in DNA immunization, understanding the effect of plasmid DNA on draining lymph nodes and thereby regional immunity is essential. Further work is needed though, to determine the extent of both low levels of

endotoxin and of immunostimulatory CpG plasmid motifs on draining lymph node morphology. It is not feasible to construct a plasmid devoid of CpG sequences as these sequences are found in areas essential for plasmid replication (Hashimoto-Gotoh & Inselburg, 1979; Naito & Uchida, 1980). In the future, therefore, construction of a plasmid with added numbers of immunostimulatory CpG motifs and the further removal of endotoxin from plasmid preparations would help address these questions.

CHAPTER 5

EFFECTS OF INTRADERMALLY INJECTED PLASMID DNA AND ODN ON CELL TRAFFICKING FROM THE DRAINING LYMPH NODE

5.1 Introduction

The role lymphocytes play in the development and maintenance of an adaptive immune response has been extensively investigated. Many of the cellular and molecular events that regulate the induction of humoral and cell-mediated immunity have been well characterized. It has become evident, however, that the induction and regulation of adaptive immunity is greatly influenced by the response of the innate immune system to infection or inflammation (Fearon & Locksley, 1996; Medzhitov & Janeway, 1997).

The innate immune system utilizes a variety of receptors, known as pattern recognition receptors (PRR), to recognize pathogen-associated molecular patterns (PAMP) that are unique to microorganisms (Fearon & Locksley, 1996). CpG motifs present within bacterial DNA act as PAMP (Krieg & Wagner, 2000) and can activate a variety of cells involved in the induction of an adaptive immune response. Unmethylated CpG motifs present in bacterial DNA or synthetic oligodeoxyribonucleotides (ODN), for example can enhance B cell survival, influence dendritic cell differentiation and induce cytokine secretion by B cells, monocytes, natural killer cells and dendritic cells (Krieg *et al.*, 1995; Ballas *et al.*, 1996; Yi *et al.*, 1998). Further, the presence of immunostimulatory CpG motifs in plasmid DNA vaccines (Roman *et al.*, 1997; Klinman *et al.*, 1997) or immunostimulatory ODN can

substantially enhance antigen-specific humoral and cell-mediated immune responses (Davis *et al.*, 1998). Finally, treatment with ODN containing CpG motifs can protect mice against challenge by a variety of bacterial pathogens (Klinman *et al.*, 1999; Weighardt *et al.*, 2000). Collectively these observations implicate CpG motifs as potent PAMP that effectively activate the innate immune system and modulate the adaptive immune response.

Lymphocytes have the unique ability to migrate continuously from blood to tissue and back to blood. The movement of lymphocytes between different immune compartments is non-random (Abernethy *et al.*, 1991; McKay *et al.*, 1996) and specific mechanisms have evolved to regulate lymphocyte trafficking. The recirculation and homing of lymphocytes allows for continual communication between different immune compartments, facilitates immunosurveillance and the dispersion of 'immunological information' throughout the body (Young, 1999). Although PAMP can induce the migration of inflammatory cells into tissues (Schwartz *et al.*, 1997; Deng *et al.*, 1999), the effect of PAMP on lymphocyte recirculation and, in particular, cell trafficking through draining lymph nodes has not been fully investigated. Only one study has examined the effects of another PAMP, LPS, on lymphocyte movement through a lymph node. The changes in cell trafficking induced by lipopolysaccharide were similar to those reported following antigen treatment (Cahill *et al.*, 1976). Factors that alter lymphocyte trafficking may have a significant effect on the development and dissemination of an adaptive immune response. We therefore, investigated the effects of bacterial DNA on immune surveillance by measuring cell movement through the draining lymph node and determined whether immunostimulatory CpG motifs were responsible for the observed changes.

5.2 Methods and Materials

5.2.1 Animals and Surgery

Suffolk ewes, one to five years of age, were used in all experiments (Department of Animal and Poultry Science, University of Saskatchewan, SK, Canada). The efferent lymphatic vessel of the prescapular lymph node was cannulated as described by Glover & Hall (1976). The method was modified slightly, by forming a loop with the tubing and suturing the loop to the subcutaneous tissue. The tubing then exited the skin at a location ventral to the incision. Clot resistant polyurethane tubing with a 0.95mm outer diameter was used for cannulation (Micro-Renathane implantation tubing, Braintree Scientific Inc., MA, USA).

5.2.2 Plasmid Injection, Lymph Collection and Cell Preparation.

For the construction and preparation of the various plasmids used in cell trafficking experiments, refer to Chapter 3. Lymph was collected in 250 ml polyethylene bottles containing 100 U.S.P. of heparin (Hepalean, Organon, ON, Canada) and 20 mg of sodium ampicillin (Penbritin-2000, Ayerst, QB, Canada) in 1 ml of sterile saline. Depending on the experimental protocol, lymph collection bottles were changed every 30 minutes to twelve hours. Total cell numbers were measured with either a hemacytometer or a particle counter with a 100 μm aperture tube (Coulter Model Z1, Coulter Electronics of Canada, ON, Canada). Animals were rested 48 to 72 hours post-operatively to allow surgery-related changes in lymph flow to subside. Following this rest period, plasmids, linear DNA, calf thymus DNA or ODN 2135 were injected intradermally 10-cm anterior to the *tuber scapulae.* Lymph was collected over a 4-day post-treatment period and the number and phenotype of lymph cells were analyzed. Cells were washed three times with phosphate buffered saline (PBS) and then resuspended in PBS prior to phenotype

analysis. Dendritic cells were isolated using the method described by Knight *et al.* (1983). Briefly, lymph cells were layered on a 14.5% (wt/vol) metrizamide gradient made with medium (Iscove's modified Dulbecco's media, Gibco-BRL, ON, Canada) containing 10% FBS (Gibco-BRL, ON Canada). These lymph cells were centrifuged for 10 min at 600 x *g* and cells at the interface were collected.

5.2.3 Flow Cytometry.

For both single and dual staining immunofluorescence, 2 x 10^6 lymph cells were labeled with primary monoclonal antibody (mAb) in a total volume of 100 µl. Isotype-specific goat anti-mouse Ig (CalTag Laboratories, CA, USA), conjugated with either fluorescein isothiocyanate or phycoerythrin were diluted at 1:100 (v/v) in FAcola (0.1M PBS, 0.2% gelatin, 0.03% NaH_3) was then used to detect mAb in dual and single labeled cell preparations. An isotype matched mouse monoclonal antibody was used as a control for non-specific mAb binding to cells. Labeled cells were fixed in 2% paraformaldehyde in PBS and stored in the dark at 4 °C until analyzed with a FACSscan flow cytometer (Becton Dickinson, CA, USA). The Cell Quest (Becton Dickinson, CA, USA) program was used for data acquisition and analysis.

5.2.4 Monoclonal Antibodies.

The following mAb were used for flow cytometric analysis. The mAbs for ovine sIgM (Plg45A), MHC Class II (TH81A5), CD25 (CACT116A) and monocytes/ granulocytes (DH59B) were purchased from VMRD (Pullman, WA USA). The mAbs for ovine CD1b (VPM5) and mouse control IgG1 were purchased from Serotec (Raleigh, NC, USA), Boehringer Mannheim (Laval, QB, Canada) and Novocastra Laboratories LTD, (Newcastle, UK), respectively. Dr. W. Hein (Agriculture Research, Wallaceville, NZ) generously provided hybridomas from which the mAb specific for

sheep CD5 (ST1a), CD45R (20-96), CD4 (17D-13), CD8 (E95), γδTCR (86D) and LFA1 (F10-150) were produced.

5.2.5 Data Presentation and Statistical Analysis.

In Figs. 5.1, 5.2, and 5.4 to 5.14, the cell number in lymph is expressed as the mean ± standard error of the mean (SEM) for values from 3-6 sheep. Post-treatment values are expressed as a percentage change from the pre-treatment value, which is expressed as 100%. Figure 5.3 presents changes in cell number (cell/hour) for each lymphocyte sub-population identified. Comparison of values between treatment groups was performed with a Kruskal-Wallis analysis and a Dunns comparison (GraphPad Prism, CA, USA). *P* values less than 0.05 were considered significant.

5.3 Results

5.3.1 Plasmid DNA Increases Cell Number in Efferent Lymph.

Purified plasmid DNA was used as a source of bacterial DNA with a known nucleic acid sequence. To determine whether plasmid DNA altered cell trafficking and to monitor possible DNA transfection and expression, the pCAN1 vector, containing the eGFP reporter gene (pCAN1-eGFP), was injected intradermally. Efferent lymph was collected over a 4-day post-treatment period to measure cell traffic (cell/hour) following a single intradermal injection of 4 μg, 40 μg and 400 μg plasmid DNA. An significant increase ($P < 0.05$) in cell trafficking was observed with as little as 4 μg of plasmid and this increase was evident as early as 6 hours post-treatment (Fig. 5.1 a). At 24 hours post-treatment, the increase in cell traffic ranged from a 150 to 650% increase relative to cell number in lymph collected in sheep following the injection of 400 μg calf thymus DNA (CT DNA). There were no significant differences in the cell trafficking response induced by the three

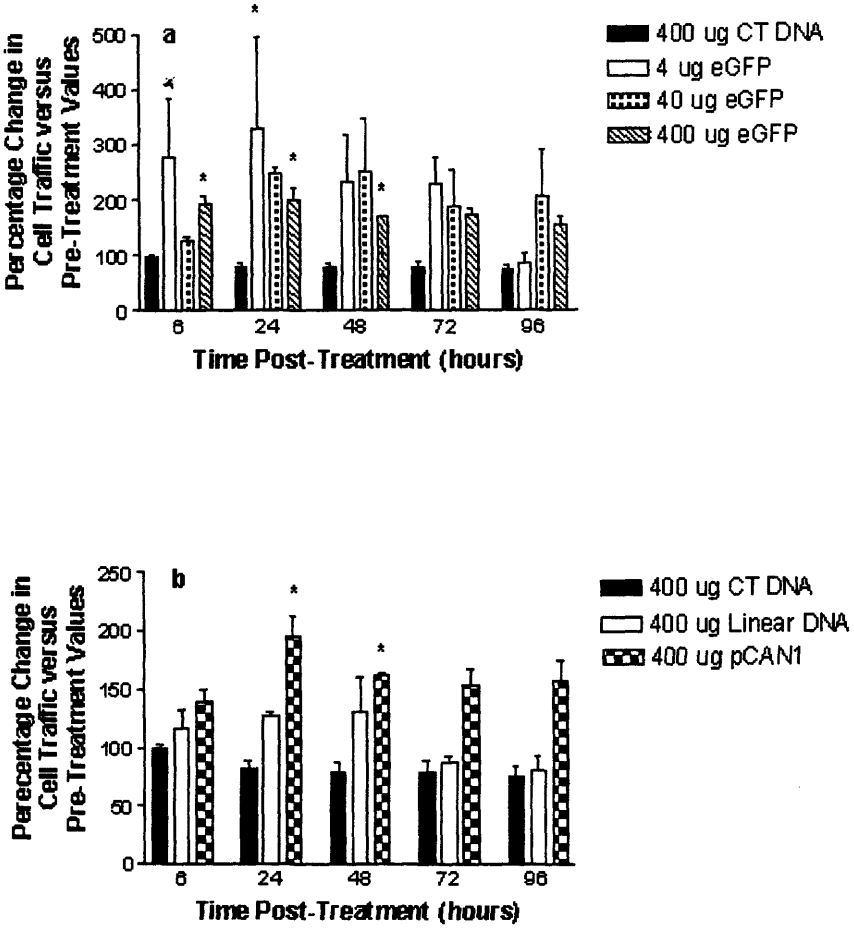

Figure 5.1. Intradermal injection of plasmid DNA increases cell numbers in the efferent lymph of the draining prescapular lymph node. **a** A comparison of cell trafficking following the injection of different doses of pCAN1-eGFP plasmid (eGFP) and calf thymus DNA (CT DNA). **b** A comparison of cell trafficking following the injection of restriction enzyme digested pCAN1-eGFP plasmid (Linear DNA), intact PCAN1 plasmid (pCAN1), and calf thymus DNA (CT DNA). Data are presented as a percentage change in cell number (cells/hour) relative to lymph collected 24 hour prior to injecting DNA. Data are presented as the mean ± SEM of values from each treatment group (n = 6 for CT DNA and n = 3 for all other groups).
* Significant difference between plasmid DNA and CT DNA controls (P <0.05).

concentrations of plasmid. Nonetheless, it did appear that increased cell trafficking persisted longer and was more consistent following treatment with 40 μg and 400 μg plasmid DNA than with 4 μg plasmid DNA (Fig. 5.1 a). Flow cytometric analyses did not detect any fluorescent cells expressing eGFP in samples of total lymph cells. While flow cytometric analysis detected very low numbers of potential $CD1b^+$ cells and $DH59B^+$ cells following centrifugation on a density gradient, there were no significant differences among treatment groups (Table 5.1 and 5.2).

These observations suggested that plasmid DNA might directly increase cell traffic through a lymph node. To determine whether the expression of a foreign protein (GFP) contributed to the observed increase cell trafficking, we injected 400 μg of the pCAN1 vector lacking the eGFP gene insert. The pCAN1 plasmid by itself induced a rapid and prolonged rise in cell traffic that was statistically significant ($P < 0.05$) on days 1 and 2 post-treatment (Fig. 5.1 b). The increased cell flow varied between 150-225% of the pre-treatment values and was not statistically different from cell flow rates observed following the injection of 400 μg of pCAN1-eGFP plasmid. Therefore, these two independent experiments clearly demonstrated that intradermal injection of plasmid DNA, with or without an encoded eukaryotic reporter gene, induced a significant and prolonged increase in cell trafficking through the draining lymph node.

5.3.2 Circular Plasmid is Required to Induce Increased Cell Flow.

As plasmid DNA is a circular form of bacterial DNA, we investigated the possibility that the circular form of plasmid DNA was required to induce a cell traffic response. The pCAN1-eGFP plasmid was digested into two linear segments and 400 μg of digested plasmid DNA was injected intradermally. This linear plasmid DNA did not induce a significant increase in cell trafficking (Fig. 5.1 b). Therefore, we

Table 5.1. The number of $CD1b^{high}P220^{-}$ cells collected from gradient-enriched efferent lymph cells following treatment with calf thymus DNA, plasmid, linear DNA or oligodeoxyribonucleotides.

Number of $CD1b^{+}P220^{-}$ cells collected from Efferent Lymph[a] ($x10^4$ cell/hour)					
	Time Post-Treatment (days)				
Treatment[b]	0	1	2	3	4
CT DNA	0	0.66 ± 0.78	0	0	0
4 μg eGFP	0	0	0	0	2.2 ± 1.2
40 μg eGFP	0	0	1.2 ± 1.0	0.33 ± 0.3	0
400 μg eGFP	0	0	0	3.4 ± 2.0	3.2 ± 1.0
400 μg Linear DNA	0	3.3 ± 2.0	2.3 ± 2.3	2.6 ± 1.7	0
11.7 μg ODN 2135	0	0	0.66 ± 0.58	0	0
585 μg ODN 2135	0	0	2.6 ± 1.5	1.6 ± 1.6	0
400 μg pCAN1	3.0 ± 1.3	0.5 ± 0.5	0.5 ± 0.5	0.7 ± 0.5	0.5 ± 0.5
40 μg pMASIA	0	0	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1
40 μg pBISIA-88	0	0	0	0	1.3 ± 1.3
400 μg pBISIA-88	1.3 ± 1.3	0	0	1.0 ± 1.0	1.3 ± 0.2

Abbreviations: CT DNA calf thymus DNA; eGFP pCAN1-eGFP; Linear DNA digested pCAN1-eGFP; ODN 2135 oligodeoxyribonucleotides.
Data is presented as mean ±SEM of values for each treatment group (CT DNA: n=6, pCAN1: n=4; and for all other treatment groups: n=3).
[a] Cells were collected from gradient enriched lymph and the frequency of $CD1b^{high}P220^{-}$ cells were quantified with flow cytometry. Total $CD1b^{high}P220^{-}$ cell number represents the frequency of $CD1b^{+}P200^{-}$ cells multiplied by the total number of cells collected in lymph per hour.
[b] All treatments were diluted in saline and delivered intradermally in a total 400μl volume.

concluded that circular plasmid DNA was required to induce a significant increase in cell trafficking.

5.3.3 CpG motifs in Plasmid DNA Induce Increased Cell Trafficking.

Since unmethylated CpG motifs in bacterial DNA have been associated with potent immunostimulatory activities, we investigated the possibility that such immunostimulatory CpG motifs were required to induce a cell trafficking response to plasmid DNA. The effect of CpG motifs on cell trafficking was investigated by using both linear immunostimulatory ODN and the same immunostimulatory CpG motifs inserted into plasmid DNA. To facilitate a comparison with previous experiments,

Table 5.2. The number of DH59B^{high}CD1b^{-}cells collected from efferent lymph following treatment with calf thymus DNA, plasmid, linear DNA or oligodeoxyribonucleotides.

Number of DH59B^{+high}CD1b^{-}cells Collected from Efferent Lymph[a] (x10^4 cell/hour)					
	Time Post-Treatment (days)				
Treatment[b]	0	1	2	3	4
CT DNA	4.0 ± 0.8	0	0.3 ± 0.4	0	0
4 μg eGFP	0.26 ± 0.27	0	0	0	0.6 ± 0.7
40 μg eGFP	1.0 ± 1.0	0	3.0 ± 1.0	2.0 ± 1.7	0
400 μg eGFP	7.5 ± 4.0	5.3 ± 5.0	4.0 ± 1.0	0.3 ± 0.3	0
Linear DNA	0	0.3 ± 0.3	2.0 ± 2.0	3.4 ± 1.7	0
11.7 μg ODN	0	0	0.66 ±0.58	0	0
400 μg ODN	0	0	1.6 ± 1.6	0	0
400 μg pCAN1	1.3 ± 1.3	1.3 ± 1.3	0	0.5 ± 0.5	0.5 ± 0.5
40 μg pMASIA	0	0	0	0	0
40 μg pBISIA-88	0	0	0	0	0
400 μg pBISIA-88	2.0 ± 1.7	0	4.7 ± 0.5	1.3 ± 1.3	1.3 ± 1.3

Abbreviations: CT DNA calf thymus DNA; eGFP pCAN1-eGFP; Linear DNA digested pCAN1-eGFP; ODN 2135 oligodeoxyribonucleotides.
Data is presented as mean ±SEM of values for each treatment group (CT DNA: n=6, pCAN1: n=4; and for all other treatment groups: n=3)
[a] Cells were collected from lymph and the frequency of DH59B^{high}CD1b^{-}cells were quantified with flow cytometry. Total DH59B^{high}CD1b^{-} cell number represents the frequency of DH59B^{+high}CD1b^{-} cells multiplied by the total number of cells collected in lymph per hour.
[b] All treatments were diluted in saline and delivered intradermally in a total 400μl volume.

the molarity of CpG dinucleotide delivered was calculated. On this basis, the low dose of ODN 2135 (11.7 μg) had a CpG dinucleotide content equivalent to 400 μg of pCAN1 and the high dose of ODN 2135 (585 μg) had a CpG dinucleotide content equivalent to 20 mg of pCAN1. Cell trafficking was not significantly changed by the intradermal injection of either a low or a high dose of linear ODN 2135 (Fig. 5.2 a). These observations indicated that either CpG motifs were not involved in the cell trafficking response or, as suggested by the previous experiment, that linear bacterial DNA is poor at effectively inducing this biological response.

To address the question whether the form of immunostimulatory CpG motifs influences cell trafficking, it was necessary to first identify a DNA plasmid that, by

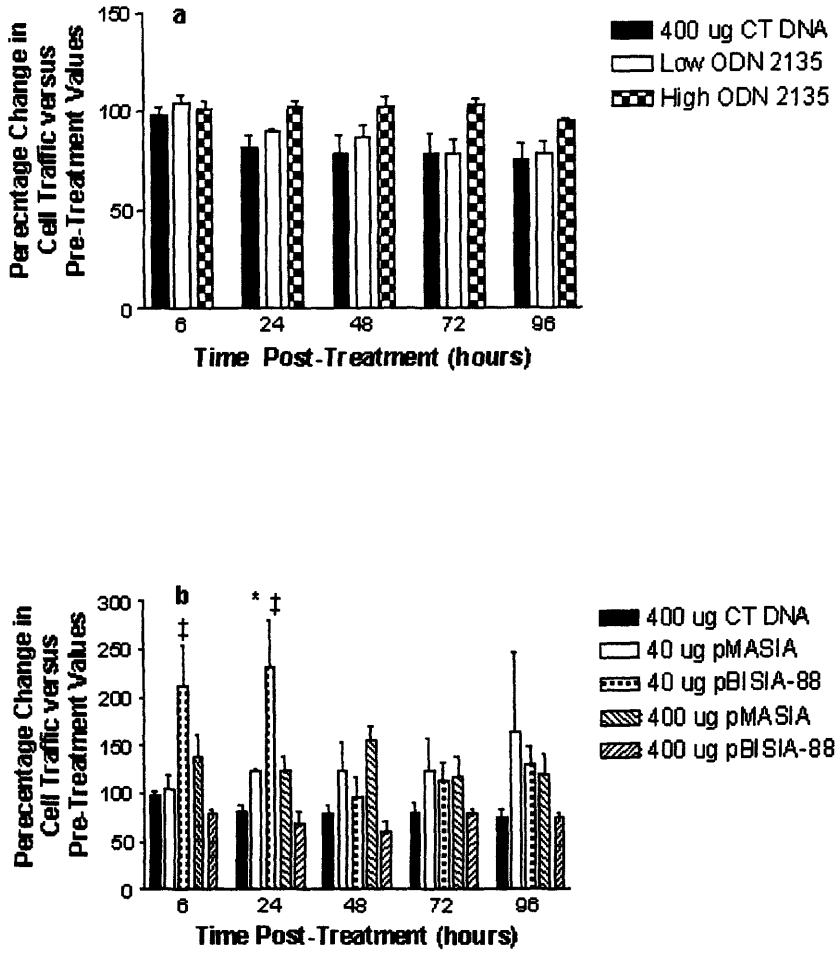

Figure 5.2. CpG motifs, in the context of plasmid DNA, increased cell numbers in the efferent lymph of the draining prescapular lymph node. **a** A comparison of cell trafficking following the injection of 11.7 ug (Low ODN 2135) or 585 ug (High ODN 2135) of immunostimulatory ODN and calf thymus DNA (CT DNA). **b** A comparison of cell trafficking following the injection of 40 ug or 400 ug of either pMASIA plasmid (pMASIA) or pMASIA plasmid containing tandem repeats of the immunostimulatory ODN 2135 sequence (pBISIA-88) and calf thymus DNA (CT DNA). Data are presented as a percentage change in cell number (cells/hour) relative to lymph collected 24 hour prior to injecting DNA. Data are presented as the mean ± SEM of values from each treatment group (CT DNA: n = 6; pCAN1: n = 4; ODN 2135, pMASIA and pBISIA-88: n = 3).
* Significant difference between plasmid DNA and CT DNA controls (P <0.05).
‡ Significant difference between 40 ug pBISIA-88 and 400 ug pBISIA-88 (P<0.05).

itself, did not induce a significant change in cell trafficking. The pMASIA plasmid was selected, as it is similar in size to pCAN1 and both plasmids contain similar numbers of CpG dinucleotides. The pCAN1 plasmid however, does contain a leading 5'-TCGT-CGTT-3' motif located 2 bases upstream from a known immunostimulatory CpG motif. This leading sequence is essential for ODN induced cell activation of blood mononuclear cells of cattle (Pontarollo *et al.*, 2001). The pMASIA plasmid also differs from pCAN1 by incorporating one more known immunoneutralizing CpG motifs. The intradermal injection of either 40 or 400 μg of pMASIA did not induce a significant increase in cell traffic (Fig. 5.2 b). On this basis the pMASIA plasmid, therefore, was deemed suitable and subsequently modified through the insertion of 11 tandem repeats of the immunostimulatory ODN 2135 sequence (pBISIA-88) (Refer to Chapter 3).

Intradermal injection of 40 μg of pBISIA-88 induced a rapid increase in cell trafficking (6 hours) that was significantly different (P <0.05) from CT DNA at 24 hours post-treatment (Fig. 5.2 b). In contrast, injecting 400 μg pBISIA-88 did not induce a significant change in cell trafficking throughout the 4 day post-treatment period (Fig 5.2b). Furthermore, there was a significant difference (P <0.05) between the level of cell trafficking induced by 40 μg and 400 μg of pBISIA-88 at 6 hour and 24 hours post-treatment. These results clearly indicated that CpG motifs, in the context of circular plasmid, could induce an increase in cell traffic through a lymph node. It was apparent also that the induction of increased cell trafficking by immunostimulatory CpG motifs was dose-dependent with a high dose of CpG having no apparent effect on cell trafficking.

5.3.4 Kinetics of Cell Trafficking Response Following Plasmid Treatment.

The kinetics of a biological response can reveal significant information regarding cell signaling mechanisms, the need for gene expression and for *de novo* protein synthesis. Previous experiments indicated that a significant increase in cell trafficking occurred within 6 hours after injecting pCAN1-eFGP plasmid (Fig. 5.1 a). The kinetics of this response was examined further by injecting 400 μg of pCAN1 plasmid intradermally and then collecting lymph 30 min later and at hourly intervals for the next 6 hours. Although not statistically different from pre-treatment values, there was a 50% increase in cell number that was observed within 30 minutes and this gradually increased throughout the 6 hour post-treatment period (Fig. 5.3). This experiment revealed a very rapid onset in the cell traffic response to plasmid DNA but, when viewed collectively with previous observations (Fig. 5.1 and 5.2), it is clearly evident that the maximum cell traffic response observed in this study required at least 24 hours to develop. Thus, while plasmid DNA may have a direct or immediate effect on cell recruitment or cell retention within the lymph node, the gradual increase in cell traffic over a 24 hour period, however, suggests that plasmid DNA induces the production of factors that influence cell recruitment and traffic through a lymph node.

5.3.5 Lymphocyte Populations Involved in the Response to Plasmid DNA.

Cell trafficking responses frequently involve specific cell populations or lymphocyte subsets. The characterization of the specific cell populations present in lymph can provide insight into the nature or specificity of the signals involved in cell recruitment to, and trafficking through, a lymph node. Throughout each of the previous experiments, the phenotype of cells present in lymph was analyzed by flow cytometry. Efferent lymph contained only lymphocytes: while the majority were T

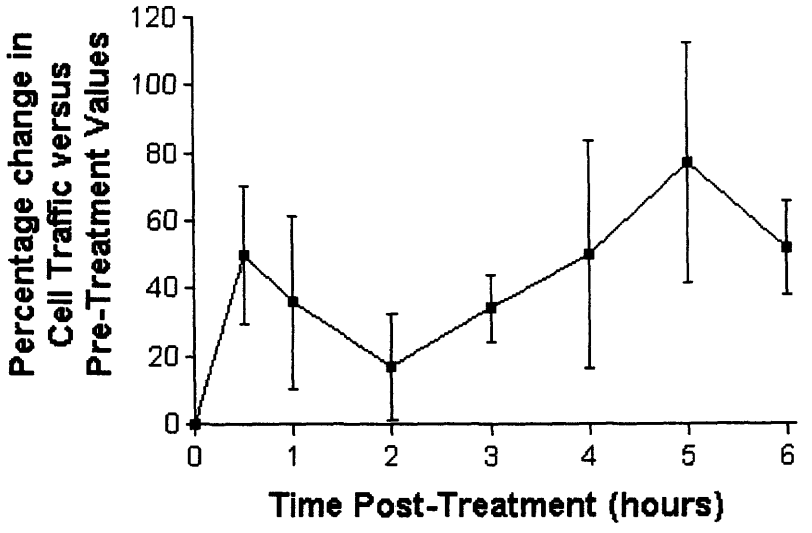

Figure 5.3. The kinetics of lymphocyte trafficking into the efferent lymph of the prescapular lymph node following intradermal injection of 400 μg pCAN1 plasmid. Data are presented as a percentage change in cell number (cells/hour) relative to lymph collected 24 hour prior to injecting DNA. Data are presented as the mean ± SEM of values from four sheep.

cells (Fig. 5.4 a-5.14 a) all T cell and B cell subpopulations analyzed contributed to the increased cell trafficking induced by plasmid DNA (Fig. 5.4 b,c-5.14 b,c).

Furthermore, an analysis of CD25 (IL-2Rα) expression on the total T cell ($CD5^+$) population and of MHC Class II expression on $CD4^+$ T cells did not reveal a significant change in the number of activated T cells leaving the lymph node (Fig. 5.4 d-5.14 d). Thus, the phenotypic analyses indicated that plasmid DNA induced a non-selective trafficking of lymphocytes through the lymph node.

The phenotypic analysis also revealed the presence of extremely small numbers of $CD1b^+$ cells and $DH59B^+$ cells in efferent lymph (Table 5.1 and 5.2). This finding was inconsistent and statistical analyses revealed no significant differences between the various treatment groups. The CD1b cluster determinant is expressed on dendritic cells, B cells, macrophages and monocytes and in this

experiment, almost all $CD1b^+$ corresponded to $CD45R^+$ cells (B cells). Therefore, the presence of $CD1b^+$ cells was not a definitive indicator of dendritic cells.

5.4 Discussion

The innate immune system is a phylogenetically old system that is found in all multicellular organisms and uses receptors encoded in germline DNA (Medzhitov & Janeway, 1997). In vertebrates, cells associated with innate immunity utilize PRR to recognize conserved PAMP and PRR binding then to activate cells involved with acquired immunity (Fearon & Locksley, 1996; Medzhitov & Janeway, 1997). Indeed, it has become apparent that the innate immune system controls both the activation and differentiation of cells involved in the adaptive immune response that arises following exposure to a pathogen (Fearon & Locksley, 1996; Medzhitov & Janeway, 1997). Our hypothesis was that immunostimulatory CpG motifs, a known PAMP, may also influence adaptive immune responses by altering the movement of cells through a lymph node. Increased lymphocyte trafficking through a regional lymph node is one of the hallmarks of an adaptive immune response and provides a mechanism to optimize screening of the available immune repertoire (Smith *et al.*, 1970b; Cahill *et al.*, 1976). The experiments were designed, therefore, to determine whether bacterial DNA could alter lymphocyte trafficking through a regional lymph node. Indeed, the data that immunostimulatory CpG motifs can increase lymphocyte recruitment provides further evidence that recognition of PAMP by the innate immune system can influence adaptive immune responses.

The present investigation identified some factors that might influence the capacity of immunostimulatory CpG motifs to signal an increase in lymphocyte trafficking. A dose-dependent facet to this signal was clearly evident as demonstrated by changes in cell flow following treatment with the CpG enhanced pBISIA-88 plasmid (Fig. 5.2 b). Furthermore, circular plasmid DNA was also

required to induce a significant increase in lymphocyte trafficking (Fig. 5.1 b). It is difficult to interpret the biological significance of these observations without a complete understanding of the mechanism by which CpG motifs bind to and activate cells and without knowing the mechanism by which CpG motifs alter lymphocyte trafficking. Several investigations indicate that cell surface receptors that bind DNA do not activate cells directly and that CpG motifs must be endocytosed for activation to occur (Hacker *et al.*, 1998; Macfarlane & Manzel, 1998). It has also been shown that CpG induced cell activation is dependent on the presence of Toll-like receptor-9 within cells and that this protein could be an intracellular receptor for CpG DNA (Hemmi *et al.*, 2000). It remains to be determined, however, whether DNA binding to cell surface receptors, a process which is not sequence specific (Kimura *et al.*, 1994), facilitates the subsequent uptake of CpG motifs into cells involved in cell trafficking,

The present observations might have relevance for understanding the mechanisms by which DNA vaccines activate the immune system. A dose-dependent effect of CpG motifs on lymphocyte trafficking is consistent with previous reports that the adjuvanticity of CpG motifs is also dose-dependent. As an example, injection of plasmid containing moderate numbers of CpG motifs elicits strong antibody responses, while treatment with plasmids containing high numbers of CpG motifs induced a lower level of antibody production (Krieg *et al.*, 1998). It is apparent that excessive amounts of CpG can suppress biological responses in a variety of systems. It is tempting to speculate that the CpG content of bacterial DNA may regulate physiological processes namely cell trafficking, and antibody production, in a dose-dependent manner. It is possible that by generating vastly different cell trafficking responses to small changes in the amount of CpG motifs

present in bacterial DNA, a sophisticated mechanism has evolved to monitor bacterial insult.

The lack of activity observed with linear DNA may not be explained solely by rapid degradation by nucleases present in skin (Anai *et al.*, 1981) and lymph nodes (Lacks, 1981). It has been shown that both plasmid DNA and digested plasmid DNA induced strong activation of bovine mononuclear cells (Pontarollo *et al.*, 2001). Alternatively, DNA retention and uptake may be greater for plasmid than linear DNA as indicated by transfection studies *in vitro* and *in vivo*. Cultured cells transfected with plasmid DNA for example, had a 10-fold increase in gene expression when compared to cells transfected with linear DNA (Upcroft, 1987). Similarly, in comparison to circular plasmid, there was a markedly lower level of gene expression in muscle following the injection of linear DNA (Danko & Wolff, 1994). Thus, the biological relevance of a cell trafficking response being dependent upon intact plasmid is intriguing. One explanation may be that the circular form of bacterial DNA acts as a signal that alerts the immune system to microbial invasion. It is known that bacteria can release both chromosomal DNA as well as large plasmids into the surrounding area during periods of either bacterial cell growth or lysis (Muto & Goto, 1986). Therefore, the injection of circular plasmid may mimic a physiological condition that occurs during bacterial infection and that is important for regulating cell traffic to the draining lymph node.

The differences observed in cell trafficking responses following treatment with plasmids of similar size and number of CpG motifs suggest that other factors within plasmids might influence these responses. It is interesting that pMASIA plasmid failed to induce an increase in cell trafficking, while pCAN1 evoked marked and prolonged enhancement in cell trafficking (Fig. 5.1b and 5.2 b). One possible explanation for these differences in plasmid activity may be the presence of a

leading 5'-TCGTCGTT-3' motif situated 2 bases upstream from a known immunostimulatory CpG motif. Although only weakly immunostimulatory on its own, this sequence is essential for cell activation by immunostimulatory CpG motifs in bovine mononuclear cells (Pontarollo *et al.*, 2001). As an illustration, in CpG motifs with only moderate immunostimulatory activity, such activity will be markedly improved if this leading sequence is added upstream (Pontarollo *et al.*, 2001). A second possibility is that the presence of an extra immunoneutralizing CpG motif within pMASIA may have dampened the immunostimulatory effects of CpG motifs present within the plasmid. It has been shown that the addition of synthetic immunoneutralizing CpG motifs can substantially impair the induction of immune responses following DNA vaccination (Krieg *et al.*, 1998). These studies and the present observation support the conclusion that cell proliferative responses and cell trafficking responses can be affected by the number of immunostimulatory CpG motifs present within a plasmid, and these activities can be further modulated by other DNA sequences as well.

The kinetics of lymphocyte trafficking following treatment with plasmid DNA revealed a very acute response (30 min) that gradually increased in amplitude over a 24 hour period (Fig. 5.3). This temporal pattern is consistent with more than one cell signaling pathway and/or multiple soluble mediators being involved in the regulation this response. High endothelial venules (HEV) may be one site of a lymph node that is affected by plasmid treatment. The release of cell mediators, namely cytokines and chemokines, that either quickly disperse to HEV or HEV glycocalyx or use the fibroblastic reticular conduit system, could rapidly alter cell migration. Within the last decade, a variety of chemokines that modulate lymphocyte binding to endothelium and movement throughout lymph nodes have been identified (Baggiolini, 1998). Indeed, chemokines can induce the adherence of

lymphocytes to cell adhesion molecules (Gunn *et al.*, 1998) and within minutes cause cells to migrate from blood vessels into the paracortex of the lymph node (Larsen *et al.*, 1989). Immunostimulatory CpG motifs can induce the secretion of numerous chemokines, including MIP-1α, MIP-1β, MIP-2 and RANTES, which are associated with the migration of different lymphocyte populations within lymph nodes (Takeshita *et al.*, 2000). Treatment with immunostimulatory CpG motifs will increase the rapid and prolonged production of these chemokine mRNA in lymph node macrophages (Takeshita *et al.*, 2000). Immunostimulatory CpG motifs will also increase secretion of cytokines such as TNF-α, IFN-α, IFNγ, IL-6 and IL-12 (Ballas *et al.*, 1996; Sato *et al.*, 1996; Yi *et al.*, 1998). Of these only IFN-α has been evaluated *in vivo* in sheep and it has been shown to promote lymphocyte migration into, but not out, of lymph nodes (Kalaaji *et al.*, 1988). Further investigations are required to determine if increases in chemokine or cytokine levels are responsible for the acute and sustained increase in lymphocyte trafficking following plasmid DNA treatment. The likelihood of DNA acting directly on HEV to enhance cell flow is remote since the large net electronegative charge present on both plasmid DNA and the glycoproteins within the endothelial glycocalyx would likely prevent this interaction (Van Damme *et al.*, 1994).

The lymphocyte trafficking response to plasmid DNA is distinct from the response observed following the injection of either antigen or LPS, a known PAMP. The most immediate consequence of antigenic challenge is an abrupt shut down or decrease in emigration of lymphocytes followed first by a significant increase in cell number in efferent lymph and thereby the appearance of blast and activated lymphocytes (Smith *et al.*, 1970b; Hay *et al.*, 1972; Cahill *et al.*, 1976). Depending upon the antigenic stimulus, the cellular composition of lymph may be significantly

altered. In contrast to antigen treatment, the injection of plasmid DNA did not induce a 'shut down' in cell traffic or detectable changes in the cellular composition of efferent lymph or the number of activated lymphocytes (Fig. 5.4-5.14). The results of these phenotypic analyses were consistent with non-selective recruitment of lymphocytes from blood to efferent lymph. One possible explanation for a non-selective increase in cell trafficking is a simple increase in blood flow to the lymph node (Hay & Hobbs, 1977). Hyperemia is a cardinal sign of inflammation and several studies in mice have indicated that CpG motifs can induce an inflammatory response (Schwartz *et al.*, 1997; Deng *et al.*, 1999). It is tempting to speculate that during a bacterial infection, bacterial DNA may serve to enhance cell recruitment from blood while bacterial antigens promote cell retention and cell activation within the lymph node. A coordinated interaction between these two biological responses might ensure the rapid and efficient induction of an adaptive immune response.

As mentioned previously, the effects of LPS and of bacterial DNA on cell trafficking kinetics are markedly different. Exposure to LPS in sheep causes a rapid and prolonged shut down in cell traffic followed by a marked increase in cell flow (Cahill *et al.*, 1976). In contrast, bacterial DNA (plasmid), caused a rapid and prolonged increase in cell trafficking. It is intriguing that two different PAMP (LPS and CpG in bacterial DNA) display such distinct and different effects on lymphocyte trafficking. It is possible that the opposing responses induced by LPS and bacterial DNA may ensure that appropriate numbers of cells enter and leave the lymph node to disseminate information to other immune compartments. These very different responses to PAMP may provide a mechanism to modulate the magnitude and type of host response to individual pathogens.

Previous studies have shown that immunostimulatory CpG motifs can enhance the recruitment of effector cells, such as neutrophils and NK cells, to the

lung (Schwartz *et al.*, 1997). This cell recruitment has obvious implications for immune surveillance, but the recruitment of lymphocytes to a lymph node identifies a novel mechanism by which a known PAMP might serve to integrate innate and adaptive immune responses. It remains to be determined whether changes in cell trafficking have a direct affect the induction and duration of an acquired immune response.

5.5 Summary

Bacterial DNA acts as a PAMP that, primarily through immunostimulatory CpG motifs, induces the secretion of numerous chemokines and cytokines and activates a variety of effector cells (Ballas *et al.*, 1996; Sato *et al.*, 1996; Yi *et al.*, 1998; Takeshita *et al.,* 2000). The possibility that either plasmid DNA or ODN containing CpG motifs might modulate immunosurveillance by altering cell traffic through a regional lymph node was investigated. This is the first study to demonstrate that plasmid DNA and ODN do indeed affect cell trafficking (Uwiera *et al.*, 2002). The intradermal injection of plasmid DNA induced rapid and prolonged increases in the number of lymphocytes present in efferent lymph. This effect on cell trafficking was not dependent on the expression of an encoded reporter gene but varied with plasmid construct and required circular plasmid DNA. The possibility that CpG motifs were responsible for inducing the increase in cell trafficking was further examined. ODN containing immunostimulatory CpG motifs were injected intradermally, either alone or as tandem repeats within a plasmid insert. ODN alone did not alter lymphocyte trafficking but CpG enhanced plasmid induced a dose-dependent increase in cell trafficking. Phenotypic analyses of lymphocytes collected in the efferent lymph revealed that the increase in cell trafficking involved all lymphocyte subpopulations and represented a non-selective movement of cells. These observations reveal that plasmid (bacterial DNA), through immunostimulatory

CpG motifs, can alter immunosurveillance nonspecifically by increasing cell recruitment to a regional lymph node.

CHAPTER 6

SHORT-TERM EFFECTS OF INTRADERMALLY INJECTED PLASMID DNA AND ODN ON DRAINING LYMPH NODE SIZE, MORPHOLOGY AND RESIDENT CELL POPULATIONS

6.1 Introduction

It is well documented that plasmid DNA activates cells involved in the induction and maintenance of an immune response to proliferate and release cytokines (Krieg *et al.*, 1995; Klinman *et al.*, 1996; Sparwasser *et al.*, 1997). Plasmid DNA can also encode antigen and once administered, can generate protective antigen-specific humoral and cell-mediated immunity. It has become apparent that immunostimulatory CpG motifs present within plasmid DNA can influence the induction of these cellular responses *in vivo* and might act as an adjuvant for DNA vaccines.

We have previously shown that injection of plasmid DNA causes long-term changes in the size and architecture of draining lymph nodes (Uwiera *et al.*, 2001). Following plasmid treatment, lymph nodes increased in weight, germinal centers were both larger and more abundant, medullary cords were thicker and medullary sinuses expanded. Injection of plasmid DNA also caused marked changes in cell trafficking through a draining lymph node with a rapid and prolonged (2-4 days) increase in the numbers of cells that migrated into efferent lymph (Uwiera *et al.*, 2002). The presence of tandem repeats of CpG motifs within plasmid DNA influenced cell trafficking. Cell migration through a lymph node increased when a

low amount of plasmid (40 μg pBISIA-88) containing tandem repeats of CpG motif were administered to sheep. In contrast, high amounts (400 μg) of the same plasmid did not enhance cell traffic, suggesting that the presence of CpG motifs affected cell migration in a dose-dependent manner (Uwiera *et al.*, 2002).

To investigate whether changes in cell trafficking were associated with functional or structural changes within a draining lymph node, the short-term effects of plasmid DNA and ODN on the size and morphology of sheep lymph nodes were examined in this study. We analyzed changes in different resident lymph node populations, measured the frequency of antigen-specific antibody cells and assessed lymphocyte responses to cytokines. These measurements will determine whether plasmid DNA or ODN activated lymph node cells and if these changes were indeed associated with cell trafficking responses.

6.2 Methods and Materials

Many of the materials and procedures employed in this study have been already described in Chapters 4 and 5. This section will only deal with methods and materials unique to the present experiment.

6.2.1 Animals and Experimental Design

Female Suffolk sheep ranging from eight months to five years of age (Department of Poultry and Animal Science, University of Saskatchewan, Canada) were used in the experiments. All sheep were injected intradermally with either plasmid DNA, ODN 2135 or calf thymus DNA at a site that was approximately 10 cm anterior to the *tuber scapulae.* Initially, sheep were injected at the same site with 150 μg and 50 μg of hen egg lysozyme (HEL) at 10 days and 4 days prior to treatment, respectively. Sheep were then injected with 400 μg (1μg/μl) of either plasmid DNA, calf thymus DNA or ODN 2135. Four hundred microliters of saline

were injected intradermally on the opposite shoulder and this contralateral lymph node was used as an internal negative control. Sheep were euthanized 4 days post DNA treatment and both prescapular lymph nodes were removed.

6.2.2 Lymph Node Collection, Cell Preparation and Flow Cytometry

For lymph node collection and histological assessment of the lymph nodes, refer to Chapter 4. For cell preparation, flow cytometry and antibody staining, refer to Chapter 5. Following lymph node removal, lymph nodes were cut longitudinally in a medial plane. One half of the lymph node was used for histological measurements while the other half was used to analyze the phenotype and function of different cell populations residing within the lymph node and to quantify the frequency of HEL specific antibody secreting cells. Single cell suspensions were prepared by gently scraping the cut surface of the lymph node while it was immersed in PBS containing 0.1% ethylenediaminetetraacetic acid (EDTA). Lymph node cells were filtered through a 40 μm nylon filter and washed three times with Dulbecco's minimal essential medium (DMEM, Gibo-BRL, ON, Canada) and then resuspended in either PBS for phenotype analysis or AIM-V medium (Gibco-BRL, ON, Canada) with 2% FBS to measure cell proliferative responses and the frequency of antigen-specific antibody secreting cells. Cells numbers were determined with a hemacytometer using trypan blue exclusion for assessing cell viability.

6.2.3 Measurement of Antibody Secreting Cells and Cell Proliferations

For the Enzyme-linked immunospot assay (ELISPOT), nitrocellulose plates (Multiscreen nitrocellulose plates, Millipore, Molshem, France) were coated with HEL (200 ng/well) in coating buffer (O.05M $NaCO_3^-$, 0.05M$NaHCO_3$, 0.003M NaH_3, pH 9.6) at 4 °C for 12 hours. Some wells were treated with coating buffer without HEL to provide controls for the specificity of antibody binding. Plates were washed 3

times with PBST (0.05% Tween 20 in PBS; vol/vol) and then washed 3 times with PBS. Cells were then added to plates at a concentration of 1 $x10^6$ cells/well and incubated at 37 °C for 18 hours. Plates were washed 6 times with distilled water and then washed 4 more times with PBST. Rabbit anti-sheep IgG (light and heavy chain) polyclonal antibodies conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, MD, USA) were diluted in PBST (1/2000 ratio; vol/vol) and 100 µl were added per well. Rabbit anti-sheep IgG was incubated in the wells for 2 hours at room temperature and then the plates were washed 6 times with PBST to remove unbound rabbit anti-sheep polyclonal antibody. The alkaline phosphatase substrate, 5-bromo-4-chloro-3 indolyl phosphate/nitro blue terazolium (Sigma Chemical Co., MO, USA) was added to plates and the reaction was allowed to proceed for 15-30 minutes at room temperature. The reaction was stopped by washing the plates with distilled water. Using a stereoscopic microscope, antigen-specific antibody secreting cells were quantified by counting the number of purple circular spots in each well coated with HEL and subtracting spots identified in control wells. For the ELISPOT, all sample were run in triplicate for each individual sheep.

In the cell proliferation assay, sample were run in triplicate, and 2 x 10^5 viable lymph node cells were added to microtiter wells that contained either medium alone (AIM-V Media) or medium containing 50 ng of cytokines. Cells were then incubated for 72 hours at 37 °C in a humidified atmosphere with 5% CO_2 and 95% O_2 and then pulsed for 8 hours with 0.04 µCi/well of ^{3}[H]-thymidine (50-60 mCi/mmol; Amersham Canada Ltd., ON, Canada). Cells were harvested on to filter mats (Skatron Inc., VA, USA) and each sample was placed in a vial with scintillant (Ready Safe, Beckman, ON, Canada) and counted by a β-scintillation counter (Beckman Instruments, ON, Canada). All cytokines used in the proliferation assays were recombinant human

cytokines purchased from Peprotech (London, UK).

6.2.4 Data Presentation and Analysis

All measurements are expressed as the mean ± standard error of the mean (SEM) for values from 3-6 sheep. Comparison of values between different treatment groups and between treated lymph nodes and contralateral control lymph nodes were performed with a Kruskal-Wallis analysis and a Dunns comparison (GraphPad Prism, CA, USA.). *P* values less than 0.05 were considered significant. The stimulation index was calculated as the proliferative responses (counts per minute) of cytokine stimulated cells divided by the proliferative responses of cells cultured in AIM-V medium.

6.3 Results

6.3.1 Gravimetric and Volumetric Measurements of Treated Lymph Nodes

The biological activity of plasmid DNA and ODN was examined by measuring macroscopic changes in lymph nodes following treatment. Lymph nodes were removed from sheep on the fourth day post-treatment, and gravimetric and volumetric measurements were made. Table 6.1 demonstrates that no significant change in lymph node size, volume or density was observed either between treatment groups, or between the treated lymph node and the contralateral control lymph node.

6.3.2 Histological Measurements of Treated Lymph Nodes

Histological measurements were used to examine lymph node architecture. Morphometric analysis of total lymph node, cortical and medullary area did not reveal any significant differences between the different treatment groups nor between the treated lymph node and the contralateral control lymph node (Table 6.2). Similarly, no changes were seen in the ratio of either cortical or medullary area per total area following the different treatments (Table 6.3). There was, however, an

Table 6.1. Gravimetric and volumetric measurements of prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleotides.

Gravimetric and Volumetric Measurements of Treated Lymph Nodes[ƒ]			
Treatment	Weight (g)	Volume (ml)	Density (g/ml)
CT DNA*	9.2 ± 0.75	10.0 ± 2.0	0.84 ± 0.06
Saline[‡]	10.0 ± 2.0	10.0 ± 1.1	1.04 ± 0.03
pMASIA*	10.2 ± 1.6	11.0 ± 1.5	0.92 ± 0.04
Saline[‡]	11.4 ± 2.5	11.7 ±2.1	0.97 ± 0.04
pBISIA-88*	8.2 ± 1.1	7.2 ± 1.3	0.98 ± 0.08
Saline[‡]	9.6 ± 0.3	9.5 ± 0.3	1.00 ± 0.00
ODN 2135*	7.6 ± 1.5	7.9 ± 1.3	0.98 ± 0.03
Saline[‡]	6.9 ± 1.2	6.3 ± 0.1	1.03 ± 0.08

Data is represented as mean ± SEM of 3 sheep.

[ƒ] Lymph nodes were removed from sheep 4 days post-treatment and gravimetric and volumetric measurements were performed.

* 400 μg (1 μg/μl) of either calf thymus DNA (CT DNA), plasmid (pMASIA, pBISIA-88) or oligodeoxyribonucleotides (ODN 2135) were injected intradermally.

[‡] Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

Table 6.2. Histological measurements of prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleotides.

Histological Measurements of Treated Lymph Nodes[ƒ]					
Treatment	Total Lymph Node Area (mm^2)	Cortical Area (mm^2)	Medullary Area (mm^2)	Percentage of Cortex per Total Lymph Node Area	Percentage of Medulla per Total Lymph Node Area
CT DNA*	609.2 ± 43.5	148.1 ± 2.7	461.0 ± 44.1	24.4 ± 1.7	75.4 ± 1.7
Saline[‡]	630.7 ± 146.5	132.8 ± 5.7	497.7 ± 151.7	27.4 ± 5.2	76.5 ± 5.2
pMASIA*	766.6 ± 184.5	261.7 ± 84.7	616.1 ± 68.8	27.4 ± 3.7	69.7 ± 6.1
Saline[‡]	766.5 ± 125.8	187 ± 43.5	560.0 ± 65.7	23.2 ± 2.0	74.0 ± 3.6
pBISIA-88*	683.7 ± 35.2	165.7 ± 41.5	518.3 ± 11.7	23.7 ± 4.6	76.2 ± 4.6
Saline[‡]	804.6 ± 73.5	188.4 ± 31.7	602.2 ± 80.5	23.4 ± 4.0	74.6 ± 5.8
ODN 2135*	589.3 ± 63.7	148.3 ± 39.4	440.9 ± 35.2	24.2 ± 4.7	75.5 ± 4.7
Saline[‡]	486.5 ± 33.0	127.7 ± 6.4	358.8 ± 28.2	26.2 ± 1.1	73.6 ± 1.2

Data is represented as mean ± SEM of 3 sheep.

[ƒ] Lymph nodes were removed from sheep 4 days post-treatment and lymph nodes were fixed and sectioned and histologic measurements were performed

* 400 μg (1 μg/μl) of either calf thymus DNA (CT DNA), plasmid (pMASIA, pBISIA-88) or oligodeoxyribonucleotides (ODN 2135) were injected intradermally.

[‡] Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

Table 6.3. Measurement of germinal center, medullary cord and medullary sinus in prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleotides.

	Histological Measurements of Treated Lymph Nodes[ƒ]			
Treatment	Total Germinal Center Area (mm^2)	Total Germinal Center Number	Thickness of Medullary Cords (μm)	Distance Across Medullary Sinuses (μm)
CT DNA*	1.9 ± 1.0	58.0 ± 23.4	53.0 ± 17.0	59.0 ± 15.0
Saline‡	3.4 ± 2.7	66.0 ± 20.0	74.0 ± 17.0	65.0 ± 14.0
pMASIA*	17.6 ± 9.5	240.3 ± 61.7 †	93.0 ± 32.0	44.0 ± 3.0
Saline‡	12.6 ± 6.7	153.0 ± 41.7	89.0± 23.0	46.0 ± 3.0
pBISIA-88*	3.1 ± 1.1	79.7 ± 18.5	44.0 ± 5.0	52.0 ± 4.0
Saline‡	5.2 ± 0.9	111.3 ± 7.6	60.0 ± 22.0	44.0 ± 5.0
ODN 2135*	7.7 ± 6.7	106.7 ± 54.1	80.0 ± 13.0	61.0 ± 9.0
Saline‡	8.6 ± 4.5	99.0 ± 37.6	57.0 ± 11.0	49.0 ± 4.0

Data is represented as mean ± SEM of 3 sheep.
[ƒ] Lymph nodes were removed from sheep 4 days post-treatment and lymph nodes were fixed and sectioned and histologic measurements were performed
* 400 μg (1 μg/μl) of either calf thymus DNA (CT DNA), plasmid (pMASIA, pBISIA-88) or oligodeoxyribonucleotides (ODN 2135) were injected intradermally.
‡ Contralateral lymph node was exposed to 400 μl of saline injected intradermally.
† Significant difference between pMASIA and CT DNA (P <0.05).

80% increase in cortex area in pMASIA treatment group as compared to CT DNA treatment. While not statistically different, however, this difference may have biological importance. Similarly, while not statistically different, there was an approximate 9-fold increase in the total area of germinal centers in pMASIA treated sheep. Treatment with pMASIA also caused a significant 5-fold increase in the numbers of germinal centers when compared to treatment with CT DNA. (Table 6.3; $P < 0.05$).

6.3.3 Phenotypic Analysis of Isolated Lymph Node Cells

Examining cell populations in lymph nodes can provide insight into the cell populations that might be recruited or retained within a lymph node following different treatments. The percentages of different cell populations present in

prescapular lymph nodes were analyzed and data are presented in Table 6.4. None of the various treatments caused significant changes in B or T cells, macrophages ($DH59B^{high}$) or dendritic cells ($CD1b^{+}P220^{-}$). Although treatment with ODN 2135 caused an increased frequency in B cells, this increase was not significantly different from CT DNA treatment (Table 6.4). A few differences were observed in the frequency of various lymphocyte sub-populations isolated from lymph (Tables 6.5, 6.6).

In general, treatment with pMASIA and ODN 2135 did not cause significant changes in B cell sub-populations when compared to CT DNA treated lymph nodes. There was, nonetheless, a 52% and 119% increase in $CD72^{+}CD21^{+}$ B cells following pMASIA and ODN 2135 treatment respectively, and this may have biological importance (Table 6.6). Notably, there was a significant 4-fold increase in the ratio of $CD21^{+}$ versus $CD21^{-}$ B following injection with pMASIA (P <0.05; Table 6.6). Furthermore, a 35% and 49% increase in B cells expressing surface IgM (sIgM) was detected in lymph nodes isolated from sheep treated with ODN 2135 and pMASIA, respectively. These findings suggest that treatment with bacterial DNA markedly altered B cell populations but not T cell populations. It is noteworthy that although activation of B cells by CpG motifs is well known (Krieg *et al.*, 1995; Klinman *et al.*, 1996), the effects of CpG motifs on specific B cell sub-population has not been reported.

Analyzing the expression of surface markers provides information on the state of cellular activation or differentiation. Data presented in Table 6.7 shows no significant difference in T cell expression of either MHC II or CD25 between either treatment groups or treated lymph nodes and contralateral controls. The one notable exception was a 48% increase in $CD5^{+}CD25^{+}$ T cells detected in lymph nodes treated with ODN 2135 when compared to lymph nodes treated with CT DNA.

Table 6.4. The percentage of cell populations isolated from prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleotides.

Percentage of Cell Populations Isolated from Treated Lymph Nodes[f]				
Treatment	B-cells	T-cells	$DH59B^{high+}$ cells	$CD1b^{+}P220^{-}$ cells
CT DNA*	33.8 ± 4.5	63.1 ± 3.1	2.3 ± 0.8	2.5 ± 1.4
Saline‡	30.6 ± 4.4	62.5 ± 3.6	2.9 ± 0.7	2.4 ± 1.0
pMASIA*	30.7 ± 8.7	60.0 ± 3.7	4.6 ± 1.4	3.2 ± 2.1
Saline‡	33.3 ± 2.1	58.7 ± 1.5	4.5 ± 1.8	2.6 ± 0.8
pBISIA-88*	27.2 ± 4.7	59.1 ± 4.4	2.3 ± 0.6	2.7 ± 0.4
Saline‡	35.6 ± 4.4	58.1 ± 1.1	3.2 ± 1.5	2.6 ± 0.5
ODN 2135*	43.1 ± 8.3	56.0 ± 2.1	1.3 ± 0.3	5.3 ± 1.9
Saline‡	38.8 ± 4.6	58.1 ± 1.1	1.3 ± 0.3	7.2 ± 1.5

Data is represented as mean ± SEM of 3 sheep treated with oligodeoxyribonucleotide (ODN 2135) and plasmid (pMASIA, pBISIA-88) and 6 sheep treated with calf thymus DNA (CT DNA).

[f] Cells were isolated from excised sheep lymph nodes 4 days post-treatment. The percentage of B-cells, T-cells, $DH59B^{high+}$ cells and $CD1b^{+}P220^{-}$ cells isolated from lymph nodes was determined with flow cytometry.

* 400 μg (1 μg/μl) of either calf thymus DNA (CT DNA), plasmid (pMASIA, pBISIA-88) or oligodeoxyribonucleotides (ODN 2135) were injected intradermally.

‡ Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

Table 6.5. The percentage of T cell sub-populations isolated from prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleo-tides.

Percentage of T-cell Sub-populations Isolated from Treated Lymph Nodes[f]			
Treatment	$CD4^{+}$ T-cells	$CD8^{+}$ T-cell	γ/δ TCR^{+} T-cell
CT DNA*	46.4 ± 3.0	12.1 ± 0.7	4.6 ± 0.7
Saline‡	46.2 ± 4.2	11.7 ± 1.0	4.6 ± 0.8
pMASIA*	45.7 ± 1.7	10.8 ± 2.2	3.7 ± 1.9
Saline‡	47.8 ± 1.9	11.9 ± 0.9	2.9 ± 1.2
pBISIA-88*	45.6 ± 3.0	10.3 ± 1.3	3.2 ± 0.7
Saline‡	41.0 ± 2.6	9.0 ± 1.7	2.8 ± 0.4
ODN 2135*	40.4 ± 1.8	11.8 ± 0.6	3.9 ± 0.2
Saline‡	41.0 ± 1.5	13.8 ± 0.6	3.3 ± 0.3

Data is represented as mean ± SEM for 3 sheep treated with oligodeoxyribonucleotide (ODN 2135) and plasmid (pMASIA, pBISIA-88) and 6 sheep treated with calf thymus DNA (CT DNA).

[f] Cells were isolated from excised sheep lymph nodes 4 days post-treatment. The percentage of $CD4^{+}$, $CD8^{+}$ and γ/δ TCR^{+} T-cells isolated from lymph nodes was determined with flow cytometry

* 400 μg (1 μg/μl) of either CT DNA, plasmid (pMASIA, pBISIA-88) or ODN 2135 was injected intradermally.

‡ Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

Table 6.6. The percentage of B cell sub-populations and percentage of sIgM expressing B cells isolated from prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleotides.

Percentage of B-cell Sub-populations Isolated from Treated Lymph Nodes[ƒ]				
Treatment	sIgM	$CD21^-CD72^+$ B-cells	$CD21^+CD72^+$ B-cells	Ratio of $\frac{CD21^+CD72^+}{CD21^-CD72^+}$ B-cells
CT DNA*	13.5 ± 2.1	9.7 ± 1.1	13.7 ± 5.2	1.2 ± 0.3
Saline‡	10.3 ± 2.5	9.1 ± 1.3	11.9 ± 4.6	1.0 ± 0.3
pMASIA*	20.1 ± 1.9	4.9 ± 1.4	20.9 ± 2.4	4.9 ± 1.5†
Saline‡	23.6 ± 3.2	7.6 ± 1.0	22.5 ± 1.2	3.0 ± 0.4†
pBISIA-88*	17.4 ± 2.2	5.5 ± 1.3	17.4 ± 4.3	3.7 ± 1.3
Saline‡	19.3 ± 1.6	8.5 ± 1.2	22.0 ± 3.7	2.7 ± 0.8
ODN 2135*	18.3 ± 1.7	14.1 ± 1.4	30.1 ± 5.6	2.2 ± 0.5
Saline‡	17.0 ± 1.2	10.6 ± 1.3	14.5 ± 4.9	1.6 ± 0.5

Data is represented as mean ± SEM for 3 sheep treated with oligodeoxyribonucleotide (ODN 2135) and plasmid (pMASIA, pBISIA-88) and 6 sheep treated with calf thymus DNA (CT DNA).

ƒ Cells were isolated from excised sheep lymph nodes 4 days post-treatment. The percentage of $CD21^-CD72^+$ and $CD21^+CD72^+$ B-cells as well as $sIgM^+$ B-cells isolated from lymph nodes was determined with flow cytometry. The change in frequency of $CD21^+$ B-cells following treatment is demonstrated by $CD21^+CD72^+$ B-cells divided by $CD21^-CD72^+$ B cells.

* 400 μg (1 μg/μl) of either calf thymus DNA (CT DNA), plasmid (pMASIA, pBISIA-88) or oligodeoxyribonucleotides (ODN 2135) were injected intradermally.

‡ Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

†Significant difference between both CT DNA and pMASIA and CT DNA the contralateral lymph node (saline) of pMASIA treated sheep ($P<0.05$).

6.3.4 Quantifying the Frequency of Antigen-specific Antibody Producing Cells Isolated from Treated Lymph Nodes

It has been shown that CpG motifs either alone or present within plasmid can enhance antibody production by B cells (Krieg *et al.*, 1995: Klinman *et al.*, 1997). We therefore investigated the possibility that treatment with either plasmid or ODN 2135 altered the frequency of B cells producing antibody to a specific antigen. Sheep were immunized with HEL and then injected with pMASIA, pBISIA-88 or ODN 2135. Quantitative measurements of cells secreting antibody to HEL did not detect

Table 6.7. The percentage of T cells expressing activation markers following isolation from prescapular lymph nodes treated with calf thymus DNA, plasmid or oligodeoxy-ribonucleotides.

Percentage of T-cell sub-populations Expressing Activation Markers Isolated from Treated Lymph Nodes[f]				
Treatment	$CD4^+MHCII^+$ T-cells	$CD5^+CD25^+$ T-cells	$CD5^+LFA1^+$ T-cells	$CD8^+CD25^+$ T-cells
CT DNA	23.0 ± 4.5	20.2 ± 1.6	29.9 ± 3.5	3.2 ± 0.5
Saline	23.6 ± 2.9	21.7 ± 4.6	32.6 ± 2.7	3.4 ± 0.4
pMASIA	18.4 ± 2.5	18.4 ± 1.3	29.7 ± 5.8	2.4 ± 0.8
Saline	21.2 ± 3.1	14.9 ± 2.0	39.6 ± 3.9	2.1 ± 0.2
pBISIA-88	14.1 ± 2.9	17.1 ± 2.3	41.6 ± 8.8	2.2 ± 0.3
Saline	18.1 ± 1.9	13.7 ± 2.1	28.9 ± 2.3	1.6 ± 0.5
ODN 2135	13.1 ± 2.6	30.1 ± 6.4	30.1 ± 6.4	2.1 ± 0.5
Saline	18.4 ± 0.2	26.7 ± 2.6	26.7 ± 2.6	2.3 ± 0.3

Data is represented as mean ± SEM for 3 sheep treated with oligodeoxyribonucleotide (ODN 2135) and plasmid (pMASIA, pBISIA-88) and 6 sheep treated with calf thymus DNA (CT DNA).

[f] Cells were isolated from excised sheep lymph nodes 4 days post-treatment. The percentage of $CD4^+MHCII^{+-}$, $CD5^+CD25^+$, $CD5^+LFA1^+$ and $CD8^+CD25^+$ T-cells isolated from lymph nodes was determined with flow cytometry.

* 400 μg (1 μg/μl) of either calf thymus DNA (CT DNA), plasmid (pMASIA, pBISIA-88) or oligodeoxyribonucleotides (ODN 2135) were injected intradermally.

‡ Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

any significant differences among the various treatment groups or the treated lymph node and the contralateral control lymph node (Table 6.8). The ODN 2135 treated lymph nodes had an approximate 4.4 fold increase in the frequency of B cells producing HEL specific antibody when compared to CT DNA treated lymph node. Treatment with ODN 2135 may be the only treatment group that has any biological significant results, as this group had the smallest variability between individual sheep.

6.3.5 Proliferation of Cells Following Cytokine Treatment

Lymphocyte activation is frequently associated with the expression of cytokine receptors and exposure to the appropriate cytokine can then induce a proliferative response. Thus, measuring cytokine-induced proliferative responses can provide information on cell activation and the expression of functional cytokine

Table 6.8. The number of hen egg lysozyme (HEL) antibody-secreting cells isolated from prescapular lymph nodes following treatment with calf thymus DNA, plasmid or oligodeoxyribonucleotides.

Number of HEL Specific Antibody Secreting Cells Isolated from Treated Lymph Nodes[f]	
Treatment	HEL Antibodies Secreting Cells (positive cells per 10^6 cells)
CT DNA*	1.6 ± 0.8
Saline‡	2.2 ± 0.8
pMASIA*	12.5 ± 11.0
Saline‡	4.1 ± 1.7
pBISIA-88*	18.5 ± 9.0
Saline‡	12.5 ± 10.7
ODN 2135*	7.1 ± 1.2
Saline‡	6.3 ± 5.2

Data is represented as mean ± SEM for 3 sheep treated with oligodeoxyribonucleotide (ODN 2135) and plasmid (pMASIA, pBISIA-88) and 6 sheep treated with calf thymus DNA (CT DNA).

[f] Cells were isolated from excised sheep lymph nodes 4 days post-treatment. The numbers of HEL specific antibody secreting cells were quantified by subtracting the number of positive cells (purple spots) from negative controls.

* 400 μg (1 μg/μl) of either CT DNA, plasmid (pMASIA, pBISIA-88) or ODN 2135 was injected intradermally.

‡ Contralateral lymph node was exposed to 400 μl of saline injected intradermally.

receptors (Goldsmith & Greene, 1994). Isolated lymph node cells were exposed to IL-2, IL-4, IL-7 and IL-15, a group of cytokines that bind to γ chain common subunit receptors and are important for lymphocyte development. No significant differences in the cytokine-induced stimulation indexes between either treatment groups or treated lymph nodes versus contralateral control lymph nodes were observed (Fig. 6.1). Though not statistically significant, the proliferative responses of cells isolated from lymph nodes treated with bacterial DNA were consistently greater than the responses of cells isolated from CT DNA treated lymph nodes. Cells isolated from pBISIA-88 treated lymph nodes demonstrated a 73%, 104%, 32% and 88% increase in the stimulation index in response to IL-2, IL-4, IL-7 and IL-15, respectively. Lymph node cells isolated from sheep injected with ODN 2135 showed a 77%, 116% and

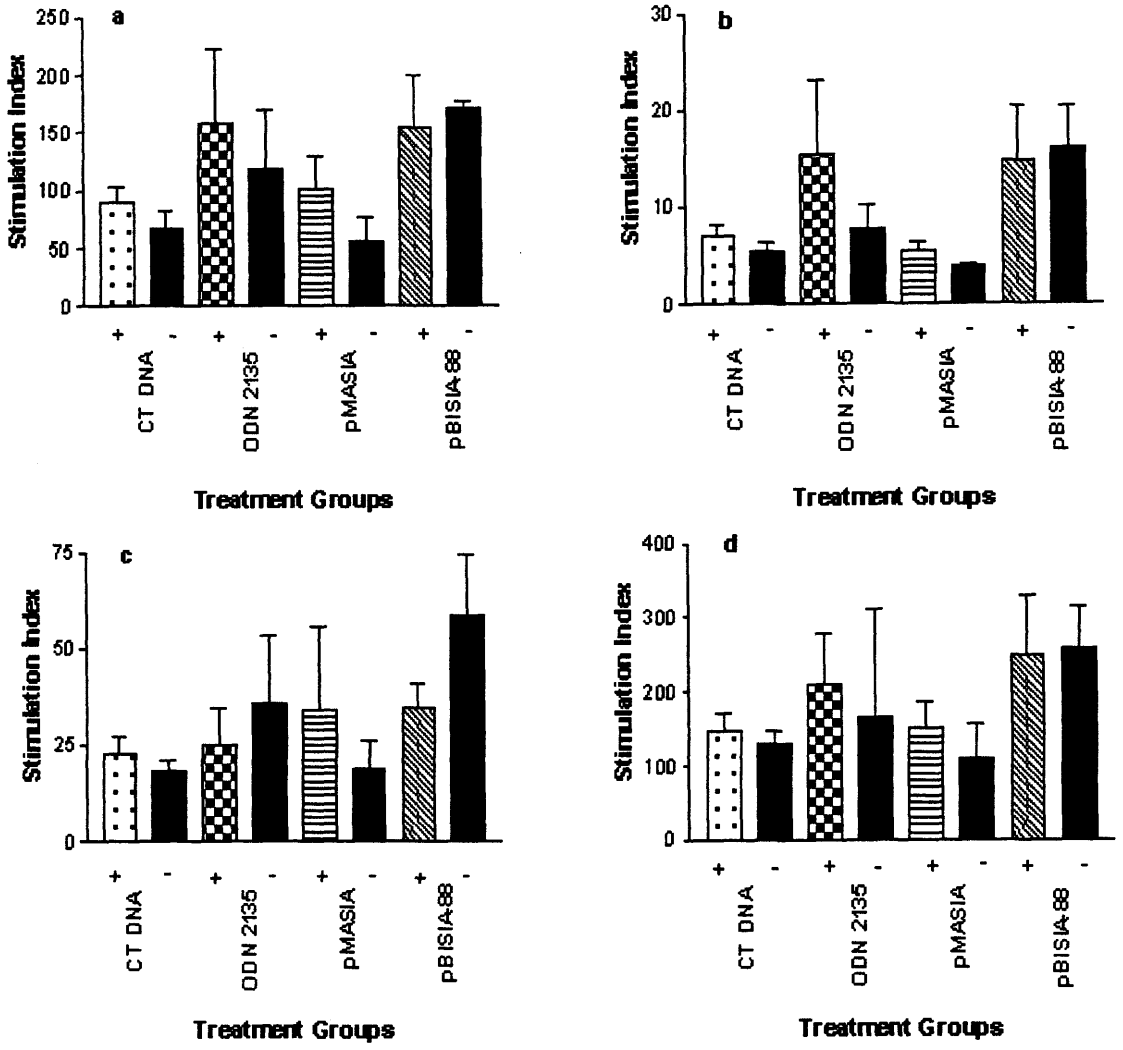

Figure 6.1 The stimulation indexes for cyokine-induced proliferative responses of cells isolated from sheep following a 4-day treatment with plasmid (pMASIA, pBISIA-88), oligodeoxyribonucleotides (ODN 2135) and calf thymus DNA (CT DNA). **a** Cells exposed to 50 ng of IL-2. **b** Cells exposed to 50 ng of IL-4. **c** Cells exposed to 50 ng of IL-7. **d** Cells exposed to 50 ng of IL-15. Treated lymph nodes are shown as (+) and contralateral saline treated lymph nodes are represented by (-) and are placed to the right of data for each treatment group. The bar represents the mean ± SEM and all samples were run in quadruplicate. Six sheep were treated with CT DNA and all other treatments had 3 sheep per group.

41% increase in the stimulation index with IL-2, IL-4 and IL-15, respectively. Cells from pMASIA treated lymph nodes also had a 54% increase in the IL-7 induced stimulation index.

The stimulation index is a ratio between cytokine treated and medium treated cells, therefore any large variations in background levels of medium treated cells could affect stimulation index values. Background proliferation values in all groups were similar (Figure 6.2) suggesting that the stimulation index (Figure 6.1) of cytokine treated cells did not reflect differences in spontaneous background proliferative responses between treatment groups.

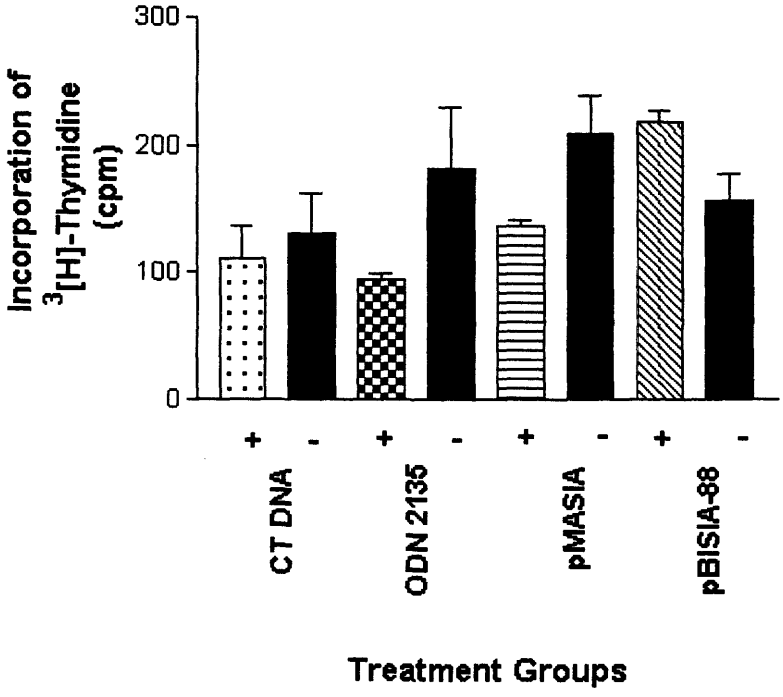

Figure 6.2. Background proliferation of cells isolated from the lymph nodes of sheep following a 4-day treatment with plasmid (pMASIA, pBISIA-88), oligodeoxyribonucleotides (ODN 2135) and calf thymus DNA (CT DNA). Treated lymph nodes are shown as (+) and contralateral saline treated lymph nodes are represented by (-) and are placed to the right of data for each treatment group. The bar represents the mean ± SEM and all samples were run in quadruplicate. Six sheep were treated with CT DNA and all other treatments had 3 sheep per group.

6.4 Discussion

This study was conducted to determine whether the previously observed changes in cell trafficking could be related to structural or functional changes occurring within the lymph node. Sheep were treated with immunostimulatory ODN or plasmid with added immunostimulatory CpG motifs and then the architecture and cellular composition of collected lymph nodes were examined. These measurements included assessing the changes in lymph node size and morphology as well as changes in the resident cell populations. Measurements of cell proliferative responses and the numbers of antigen-specific antibody producing cells present in lymph nodes were also determined.

One of the most consistent findings in this study was that regardless of treatment, changes that occurred within the treated lymph nodes were very similar to changes observed in the contralateral control lymph nodes. This observation supports the conclusion that plasmid and ODN treatment effects were not localized to the draining lymph node but also affected lymphoid tissue at distant sites. This conclusion, however, is not supported by observations made by Lipford *et al.* (2000), when examining the effects of ODN on mouse lymph nodes. Their experiment differed from our study as they delivered both a smaller amount (30 μg) of ODN and used a subcutaneous route of delivery. Nevertheless, they showed that treatment with ODN increased the total cell number present in the draining lymph nodes while saline-treated contralateral lymph nodes remained relatively quiescent. They demonstrated that at Day 2 post-treatment, there was an approximate 5-fold increase in total cell number, which was followed by a gradual increase in all cell populations that generally peaked at Day 10 post-treatment and then rapidly declined by Day 14. The discrepancy between our findings and the work by Lipford *et al.* (2000) suggests that quantitative histological assessment of lymph nodes may

not be sufficiently sensitive to identify early changes in lymphocyte cell number following treatment with ODN. Isolating and counting all cells from a single lymph node may be a better method for evaluating lymph node cellularity. This method, however, may be impractical in sheep, as sheep lymph nodes are considerably larger than mouse lymph nodes. Similarly, isolating cells from a large fragment of lymph node may also not be ideal method either. Although this method could provide a large number of cells, it may not provide an accurate assessment of resident cell populations, as the distribution of cell populations varies within the lymph node. Furthermore, a disadvantage of isolating cells to evaluate lymph node change is the inability to assess lymph node morphology.

Lipford *et al.* (2000) also demonstrated marked short-term increases in B cell number following ODN treatment. They did not determine, however, whether these cells were found in medullary cords or in germinal centers, an assessment that could give insight into the type of B cell present and its stage of maturation. Both immature and mature B cells are present in germinal centers for example, but only mature B cells and plasma cells are found in medullary cords (Junqueira, 1986; Wheater, 1987). Our experimental design did reveal that pMASIA and ODN 2135 treatment caused an enlargement of germinal centers, and there was a significant increase in $CD72^{+}CD21^{+}$ B cells following treatment with pMASIA. This increase in naïve B cells was reflected in an increased ratio of $CD72^{+}CD21^{+}$ B cells to $CD72^{+}CD21^{-}$ B cells (Table 6.6). The CD72 cell marker is expressed on all developing B cells, some populations of macrophages, follicular dendritic cells and T cells, but is not expressed on plasma cells (Gordon, 1994; Parnes & Pan, 2000). The CD72 surface protein assists in B-T cell interactions by binding to CD5 on T cells and is also believed to play a role in B cell maturation (Gordon, 1994). The CD21 molecule is a complement receptor that has been identified on B cells, immature T cells and

follicular dendritic cells (Tsoukas & Lambris, 1993). This surface protein may be involved in B-cell regulation as CD21 is down regulated following B cell activation and differentiation (Tsoukas & Lambris, 1993). The expression of CD21 on B cells has been used to identify a stable population of recirculating B cells that often expresses L-selectin (Young *et al.*, 1997). Cell trafficking studies have revealed that $CD21^+$B cells represent either a population of recirculating non-memory B cells, or of B cells that were recently activated by antigen (Young *et al.*, 1997). The increase in $CD72^+CD21^+$ B cells following treatment with ODN 2135 or pMASIA indicates that non-memory and possibly immature B cells could be the B cell population that responds to the immunostimulatory effect at CpG motifs. It is possible hat $CD72^+CD21^+$ B cells may have been either recruited from blood into the lymph node by L-selectin binding to high endothelial venules or have been generated in germinal centers. Currently, it is unknown whether CpG motifs or plasmid DNA preferentially stimulate specific B cell sub-populations.

The sIgM expressed by mature B cells can signal differentiation and activation of B cells (Haseman & Capra, 1989) and an increased level of sIgM expression may indicate B cell activation. Enhanced secretion of sIgM has been reported in mice treated with ODN (Klinman *et al.*, 1996) and the secretion of other isotypes is also increased following injection with ODN (Krieg *et al.*, 1995) and plasmid (Klinman *et al.*, 1997). These observations indicate that ODN and plasmid DNA can activate B cells that produce different antibody isotypes. In our experiment, an increased frequency of $sIgM^+$ B cells was observed for all treatment groups, with the highest frequency of $sIgM^+$ B cells present in lymph nodes collected from pMASIA treated sheep. The correlation between CD21, CD72 and sIgM expression was not analyzed. Thus, we can not determine whether the increase in $CD72^+CD21^+$ B cells was a direct consequence of an increased frequency of $sIgM^+$

B cells.

Injection of ODN 2135 into sheep caused an approximately 50% rise in the frequency of T cells ($CD5^+$) expressing a detectable level of IL-2 receptor (IL-2Rα) relative to CT DNA lymph node controls. The specific sub-population or sub-populations of T cells expressing IL-2Rα was not determined, but it was clear that these $CD25^+$T cells did not express $CD8^+$ (Table 6.7). Oligodeoxyribonucleotides are not thought to activate T cells directly, but instead to induce dendritic cells to release IL-12 and IFN-γ and these cytokines are believed to then activate Th1 cells (Chattergoon *et al.*, 1997; Krieg & Wagner, 2000). To determine whether dendritic cells did indeed induce T cell activation, it would be necessary to measure the cytokines produced by dendritic cells and assay the capacity of isolated dendritic cells to activate T cells. This analysis was not carried out in our experiment and further work is therefore needed to determine the mechanism by which bacterial DNA or CpG increased IL-2Rα expression on T cells.

Changes in cell phenotype can assist in determining whether a specific treatment has had a biological effect. Phenotypic analysis of cells, however, does not always provide information regarding cell function. As an example, the IL-2Rα by itself is a non-functional receptor and is unable to transmit a signal from the cell surface (He & Malek, 1998). Therefore, increased expression of IL-2Rα on lymphocytes does not necessarily ensure that a lymphocyte will respond to IL-2. Measuring cell proliferation following exposure to exogenous cytokines, however, provides information on both cell activation and cell function. To determine whether treatments with plasmid or ODN 2135 altered cell function, isolated lymph node cells were stimulated with cytokines and cell proliferative responses were measured. Although not statistically significant, treatment with ODN 2135 and pBISIA-88

resulted in increased cell proliferative responses following stimulation with IL-2, IL-4, IL-7 and IL-15. Thus, the increased proliferative responses of cell exposed to IL-2 confirmed that increased expression of CD25 (IL-2Rα) on T cells reflected an increased capacity to respond to cytokines.

It is interesting that cells isolated from lymph nodes exposed to ODN 2135 and pBISIA-88 displayed a responsiveness to γ chain common cytokines relative to lymph nodes exposed to CT DNA. This general increase in cytokine-induced cell proliferative responses may be related to the structural similarities of the above mentioned cytokines. The four cytokines used are members of a cytokine family that bind to receptors with a γ chain common subunit and all these cytokines play a role in lymphopoiesis (Giri *et al.*, 1994; Kondo *et al.*, 1994; Kimura *et al.*, 1995;). These receptors are present on virtually all hematopoietic cells and when bound by a cytokine will induce cell activation using the JAK/STATS signaling pathway (O'Shea, 1997). The intracellular signals generated by γ chain common subunit receptors demonstrate a redundancy in receptor function, as members of this cytokine family produce many similar cellular responses (Giri *et al.*, 1994; Kondo *et al.*, 1994; Kimura *et al.*, 1995; He & Malek, 1998). The relatively uniform increase in cell proliferative responses following pBISIA-88 and ODN 2135 treatment suggested that either these treatments induced expression of all four γ chain common subunit receptors on the cell surface or that the recombinant cytokines cross-reacted with two or more cytokine receptors. Further work is needed to determine whether IL-4, IL-7 and IL-15 cytokine receptors were increased on cell surfaces and to identify the specific cell populations that had increased expression levels of these receptors following bacterial DNA treatment.

As mentioned, this experiment was carried out in part to determine whether changes in cell trafficking could be related to structural and functional changes occurring within the lymph node following plasmid treatment. In Chapter 5, it was demonstrated that increased cell trafficking involved a non-selective movement of lymphocytes through the lymph node (Uwiera *et al.*, 2002). The observations in this study indicated that non-memory, immature $CD72^{+}CD21^{+}$ B cells were increased in frequency following plasmid DNA or ODN treatment. A minor increase in $CD72^{+}CD21^{+}$ B cells, however, could not account for the increased numbers of cells present in efferent lymph and there was not a selective increase in B cell trafficking. Therefore, increased cell trafficking was likely not related to either *de novo* generation of cells or decreased retention of cells within the lymph node. Thus, the cells collected from efferent lymph most likely migrated directly from blood into efferent lymph. This interpretation is supported by a study in sheep that showed that less than 4% of cells that migrate into efferent lymph originated from within the lymph node (Hall & Morris, 1965).

The Increased frequency of $CD72^{+}CD21^{+}$ B cells and $CD25^{+}$ T cells within the lymph node and the enhanced proliferative responses of lymph node cells indicated that bacterial DNA and ODN had a biological effect within the draining lymph node. These responses did not correlate with an increase in cell trafficking suggesting that plasmid DNA and CpG motifs can affect cells within the lymph node and yet not alter cell trafficking. CpG motifs may directly activate mechanisms involved in inflammation that mediate an increase in blood flow and thereby increase cell trafficking. Alternatively, CpG motifs may activate lymphocytes, macrophage or dendritic cells to release mediators that influence cell traffic. Thus, it is difficult to relate the increased B cell populations or the activation of T cells within lymph nodes to increased cell trafficking following plasmid treatment. It is documented, however,

that CpG motifs can induce resident lymph node macrophages to increase production of mediators involved in lymphocyte migration (Takeshita *et al.*, 2000). It is possible, therefore, bacterial DNA could indirectly influence lymphocyte trafficking by inducing the release of mediators from cells closely involved in regulating cell flow.

6.5 Summary

In Chapter 5, we demonstrated that intradermal injection of plasmid DNA induced significant changes in cell trafficking. This cell trafficking response was related to both the form of DNA and the quantity of immunostimulatory CpG motifs present within plasmids. To determine whether changes in cell trafficking were related to short-term changes in the draining lymph node, sheep were injected with plasmid (pMASIA and pBISIA-88) and ODN 2135 and lymph nodes were then removed and analyzed. Treated prescapular lymph nodes were collected and gravimetric, volumetric and histologic measurements of lymph nodes were made. Cells were isolated from lymph nodes and the frequency of various cell populations and the levels of cell activation and cytokine responsiveness were assessed. Treatment with pMASIA was associated with an increased number of germinal centers, suggesting that B cells and possibly recirculating, non-memory B cells were affected by treatment. The increased expression of IL-2Rα, as well as increased cytokine-induced proliferative responses, suggested that ODN 2135 and possibly pBISIA-88 treatment were associated with an increased expression of γ chain common subunit receptors on cells, which possibly reflected enhanced cell activation

Several experiments generated results that seemed to be potentially biologically significant though not statistically significant. Increasing the numbers of

animals in treatment groups, using animals of similar age as well as reducing exposure to bacteria present within the environment may reduce the variations in results. The increased number of animals needed to address these variations, however, may not be feasible in out-bred animals such as sheep. For example, the data from CT DNA treated animals (6 sheep) were in many instances as variable as data from the other treatments groups (3 sheep). Therefore, to reduce inter-group variation, sheep in treatments groups should be age-matched and have minimal exposure to environmental bacteria or products that induce immune stimulation.

CHAPTER 7

GENERAL DISCUSSION

In 1798, Jenner published the first clinical study showing that an inoculation (vaccination) with cowpox could cross protect individuals from lethal infections with smallpox. Since then, vaccines have nearly eradicated many infectious diseases, such as smallpox and polio, and consequently have saved countless lives through induction of immunity against infectious agents. Vaccines have also improved animal health bolstering livestock production and increasing revenue for livestock producers. Indeed, it is argued that the development of vaccines has been one of most beneficial health related technologies created to improve the well being of both people and animals. One of the latest innovations in vaccine development is the use of plasmid DNA to deliver vaccine antigens to induce protective humoral and cell-mediated immunity. The relative ease and low expense of large-scale plasmid production and the simplicity of inserting genes encoding antigen or immune modulating proteins makes plasmid DNA a good candidate for use in vaccination programs. Although initially bacterial DNA was considered inert, it has become apparent that CpG motifs present within plasmids are responsible for the activation of cells involved in adaptive immune responses and CpG motifs can act as adjuvants to enhance immune responses to DNA vaccines. Small linear segments of immunostimulatory CpG motifs (ODN) can also activate cells involved in innate immunity and promote protection against disease. Indeed, researchers have shown

that these ODN can protect animals against challenge by a variety of infectious microorganisms (Klinman *et al.*, 1999; Weighardt *et al.*, 2000).

To date, research examining the effects of DNA vaccines and immunostimulatory ODN on the induction of immune responses and disease protection has taken place predominantly in mice. Both the low cost of these animals and an extensive knowledge of mouse genetics and immune function have facilitated their use as the primary animal model for testing DNA vaccines and ODN. New product testing should also be done within the target species to ensure that the products are immunologically active within the proper species. Great potential exists for implementing DNA vaccines and immunostimulatory ODN in the livestock industry to improve animal health. It is necessary, therefore, to study the effects of plasmid DNA and immunostimulatory ODN in food producing animals.

The primary objective of this work was to investigate the effects of plasmid DNA and ODN on ruminant immune function. This investigation was focused on three main research areas. 1) The first objective was to examine the long-term effects of plasmid DNA on lymph node architecture, using the draining lymph node as a model system. 2) The second objective was to determine whether bacterial DNA might alter immune surveillance by changing the migration of cells involved in the induction and maintenance of an immune response through a draining lymph node. This was carried out by examining whether the structure of DNA, the presence of known immunostimulatory CpG motifs and expression of foreign protein influenced cell trafficking following treatment with plasmid DNA. A lymphatic cannulation model was used to investigate these short-term changes in cell trafficking. Furthermore, it was hoped that understanding the interaction between bacterial DNA and the mammalian immune system might provide further insight into the host response to bacterial infection. Using the sheep cannulation model, short-

term changes in cell trafficking were measured following treatment with plasmid DNA and ODN. 3) Finally, we wanted to determine whether changes in cell trafficking following treatment with bacterial DNA were associated with functional and structural changes within the draining lymph node. Such associations might provide further insight into the mechanisms by which bacterial DNA altered cell migration into or through the lymph node and into efferent lymph.

Sheep were chosen as the animal model for these investigations for several reasons. Firstly, cell trafficking during the induction and maintenance of an immune response has been well characterized in sheep. Secondly, mouse monoclonal antibodies specific for a variety of sheep leukocyte antigens were available and this facilitated a phenotypic analysis of cells present in lymph and lymph nodes. Thirdly, synthetic CpG motifs with known immunostimulatory activity in sheep had been identified and were commercially available. Finally, surgical techniques used in cell trafficking studies are well established in sheep (Glover & Hall, 1976).

Plasmids used in the experiments were designed to have both enhanced adjuvanticity and support optimal gene expression. Chapter 3 describes the creation of these plasmids and the evaluation of protein expression in plasmids that encoded GFP and eGFP reporter genes. These reporter genes were inserted to facilitate the detection of cells transfected with plasmid in cell trafficking studies. To assess gene expression and protein synthesis, plasmids were transfected into COS7 fibroblasts and production of the green fluorescence of protein was measured. Cells transfected with pCAN1-GFP and pCAN1-eGFP expressed sufficient protein to generate a fluorescent signal that was readily detected by flow cytometry. These *in vitro* experiments suggested that cell transfection *in vivo* could produce sufficient amounts of GFP to be detectable with flow cytometry. Since dendritic cells can also express GFP following transfection with plasmid encoding GFP (Paglia *et al.*, 1998;

Strobel *et al.*, 2000), we assumed that our *in vitro* transfection of fibroblasts (COS7 cells) provided an indication that *in vivo* transfection of dendritic cells should also be detectable with flow cytometry.

To investigate whether bacterial DNA altered immune surveillance in the draining lymph node, the long-term effects of plasmid (bacterial) DNA on popliteal lymph node size and morphology were first examined. The popliteal lymph node was examined as its size, shape and subcutaneous location made it readily accessible to measure with ultrasound equipment. In Chapter 4, ultrasound was used to follow changes in lymph node size over a 28-day period. At 30 days post-treatment, all lymph nodes were then removed and assessed both macroscopically and histologically. Ultrasound was selected for this experiment to facilitate the monitoring of changes in lymph node size over time and allowed for repeated lymph node measurements within an individual animal. This approach also reduced the numbers of sheep used by 4-fold, as otherwise it would have been necessary to perform euthanasia on sheep at weekly intervals to measure progressive changes in lymph node size.

There was a relatively equal and gradual increase in the size of both the lymph node exposed to plasmid and the contralateral lymph node exposed to saline. Ultrasound measurements demonstrated an approximately 66 and 75 percent increase in lymph node size in plasmid and saline-treated lymph nodes, respectively. The lack of difference between the lymph nodes suggested that either plasmid treatment did not have a significant effect on lymph nodes or that the effect was not localized to the lymph node draining the site of DNA injection. Macroscopic and histological measurements, however, revealed that plasmid DNA treatment did indeed significantly alter the structure of the lymph node draining the DNA injection site. Relative to saline-treated lymph nodes, plasmid treated lymph nodes weighed

more, had an increased medullary area and demonstrated an approximately 2-fold increase in germinal center number and area. Furthermore, medullary cord thickness and medullary sinus width was significantly increased following exposure to plasmid DNA. Medullary expansion as well as increased medullary cord thickness and increased width of medullary sinuses suggested that the increased lymph node weight was caused by both an accumulation of lymph and an increase in cell number. These changes in lymph node morphology were similar to changes that occur following exposure to antigen and mitogens, known activators of humoral immunity. These observations suggested, therefore, that plasmid DNA was also a potent and long-term activator of cells involved in humoral immune responses.

Long-term (30 days) changes in lymph node structure following plasmid treatment have implications for the use of DNA vaccines within the livestock industry. As mentioned, plasmid treated lymph nodes were heavier and had distinct architectural changes compared to saline-treated lymph nodes. Alterations in lymph node size and structure following DNA vaccination might be interpreted by food inspection personnel as indicators of a response to infectious disease or injury. Thus, the induction of lymph node enlargement by a DNA vaccine might lead to unnecessary carcass trimming or whole animal condemnation.

Changes in lymph node function following plasmid treatment could have an impact on concurrent immune responses. The enlargement of germinal centers associated with plasmid injection may increase the population of B cells that produces antibodies to the antigens or infectious organisms. This may have a beneficial effect by improving the amplitude of immune responses as suggested by the modest increase in numbers of antigen-specific antibody secreting cells (Table 6.8). Conversely, expansion of B cells without stringent antigen selection may result in the production of autoantibodies. Therefore, lymph node changes following

plasmid treatment may lead to changes in immune function and potentially adversely affect animal health.

The acute effects of plasmid DNA and immunostimulatory ODN were then examined by monitoring cell trafficking through a lymph node (Chapter 5). In this study, the efferent lymphatic vessel of the prescapular lymph nodes was cannulated and cells were collected following the intradermal injections of plasmid DNA or ODN. Although the popliteal lymph node was used in the long-term lymph node study, the prescapular lymph node was used for cell trafficking studies, since it was much easier to consistently cannulate the efferent lymphatic vessels of this lymph node.

Intradermal injections of as little as 4 μg of plasmid DNA caused a marked increase in cell trafficking through the prescapular lymph node. A dose-dependent facet existed for this response, although significant differences were not observed with a 100-fold increase in dose of plasmid (400 μg), the increased cell trafficking response persisted longer with 40 μg and 400 μg of plasmid than with 4 μg. As mentioned, the eGFP gene was inserted into pCAN1 to facilitate the possible detection of DNA transfected cells. Flow cytometric analysis failed to detect any cells expressing eGFP in efferent lymph even when using techniques to enrich for dendritic cells. This suggests that either cells expressing eGFP did not enter lymph or the frequency of cells expressing eGFP was below the level of detection (0.01%) with flow cytometry. To ensure that increased cell trafficking was not caused by the expression of a foreign protein (eGFP), pCAN1 without the eGFP gene insert was injected into sheep and the cell trafficking response was again measured. The increased cell trafficking in efferent lymph was similar following treatment with either pCAN1-eGFP or pCAN1. This observation indicated that the increase in cell traffic

was not dependent upon expression of a foreign protein but was probably a direct response to plasmid DNA.

Increased cell traffic induced by bacterial DNA was then further characterized by determining whether the form of bacterial DNA was critical for this response. Treatment with plasmid (circular) DNA induced the greatest increase in cell traffic. In contrast, when plasmid DNA was digested into linear fragments of DNA, cell trafficking was not significantly increased. The apparent lack of activity observed in response to linear DNA, could not be explained simply by rapid nuclease digestion since nuclease-resistant ODN failed to alter cell trafficking. An alternative explanation might be greater DNA retention and uptake for plasmid than linear DNA. The latter possibility is supported by *in vitro* and *in vivo* transfection studies. Another intriguing possibility is that the immune system may have developed receptors that recognize circular DNA as an indicator of bacterial infection. It has been shown that bacteria can release both chromosomal DNA as well as large plasmids into the surrounding area during either periods of growth or bacterial cell lysis (Muto & Goto, 1986). Therefore, the injection of circular plasmid may mimic a bacterial infection and the increase in cell trafficking induced by intact plasmid may reveal a relevant limitation to the host response to bacterial DNA.

Numerous studies have shown that immunostimulatory CpG motifs present in plasmid or synthesized as ODN can activate cells *in vitro* and can improve protective immune responses *in vivo*. Chapter 5 addressed the question of whether immunostimulatory CpG motifs were required to alter cell migration through a lymph node. The results of these experiments clearly showed that the addition of immunostimulatory CpG motifs to a plasmid could indeed increase cell trafficking in a dose-dependent manner. The injection of 40 μg of pBISIA-88 plasmid induced a marked increase in cell trafficking while treatment with 400 μg of pBISIA-88 failed to

significantly increase the level of cell traffic in efferent lymph. These changes in cell trafficking are consistent with previous reports that excessive levels of many CpG motifs in plasmid DNA can reduce immunological responses. A plasmid containing 50 CpG sequences for example, induced a lower immune response than a plasmid construct containing only 16 CpG motifs (Krieg *et al.*, 1998). Thus, an excessive amount of CpGs can suppress both the amplitude of cell trafficking response and the induction of a humoral immune response. It is tempting to speculate that the CpG content in bacterial DNA might also regulate other physiological processes, such as cell trafficking, in a dose-dependent manner. It could be hypothesized that by modulating cell trafficking responses to the amount of CpG motifs present in bacterial DNA, vertebrates may have evolved a mechanism to monitor the level of bacterial infection. Another interesting observation was that although plasmid with tandem repeats of ODN 2135 induced increased cell trafficking, linear ODN 2135 failed to produce any significant alterations in cell traffic. Similar to observations following treatment with linear fragments of plasmid DNA, the lack of activity observed with linear ODN 2135 might be related to less retention and uptake of linear ODN by cells relative to ODN present in circular plasmid DNA. From the present study, it is apparent that CpG motifs present within a plasmid, but not in excessive numbers, can induce increased cell trafficking through a draining lymph node.

Another interesting observation was that, regardless of plasmid treatment, there were no specific changes in the cellular composition of efferent lymph, indicating that plasmid treatment induced a nonselective increase in lymphocyte traffic. Plasmid treatment also caused a rapid (30 minutes) increase in cell traffic that reached maximal levels by 24 hours. This pattern of cell trafficking is unique to treatment with plasmid (bacterial) DNA. Exposure to antigen or mitogen (LPS) for

example, induced an initial shutdown in cell traffic within the first 24 hours, followed by a selective release of lymphoblasts and activated lymphocytes into efferent lymph. The differences in responses following plasmid and antigen treatment suggest that different mechanisms are influencing cell trafficking responses to immunostimulatory CpG motifs and antigen. Plasmid DNA specifically enhances cell recruitment without altering cell retention in the lymph node. This indicates that bacterial DNA likely does not activate specific mediators involved in cell retention. It is possible therefore, that a coordinated interaction between antigen and bacterial DNA with CpG motifs might ensure the rapid and effective induction of an adaptive immune response.

In Chapter 6 we investigated the effects of bacterial DNA on lymph node architecture and cellular composition. The objective was to characterize events occurring within the lymph node and determine whether these changes could elucidate the mechanisms involved in altered cell flow. Previous studies in mice have shown that five days after ODN injection, lymph nodes were increased in size and cellularity. In contrast, our study demonstrated no such changes four days after intradermal injection. Although there were differences in the amount, composition and method of ODN delivery in the mouse study, the discrepancy between our study and the mouse study, suggests that total cell counts may be a more sensitive method for measuring lymph node cellularity *in vivo.* Analysis of cells isolated from lymph nodes, however, does not provide information regarding lymph node morphology. While assessment of total cell counts is currently impractical in sheep, future studies including both total cell counts and assessment of lymph node morphology would provide the most complete and useful information.

The short-term lymph node study identified several significant changes in lymph node architecture and cellular composition. It also identified however, some

other changes that may have biological significance. Treatment with ODN 2135 and pMASIA was associated with an increased number of germinal centers and a possible expansion in naive, or recently activated B cells ($CD72^{+}CD21^{+}$ B-cells). Although not statistically significant, treatment with ODN 2135 and pBISIA-88 may have also induced an increased expression of γ chain common receptors on T cells and enhanced cytokine induced proliferative responses. These receptors are found on many lymphocytes and are essential for the development, maturation and activation of lymphoid cells (He & Malek, 1998). It appears, therefore, that ODN and pBISIA-88 induced activation of lymph node cells following treatment.

The short-term lymph node experiment demonstrated that treatment with plasmid DNA or ODN induced multiple cellular effects within a lymph node. These effects, however, did not appear to correlate with increased cell trafficking, suggesting that bacterial DNA may have separate effects on cells involved in cell trafficking and on resident lymph node cells.

In summary, the effects of plasmid (bacterial) DNA and immunostimulatory CpG motifs on immunosurveillance were investigated using the lymph node as a model system. Immunosurveillance was monitored by analyzing the dynamics of cell migration through a lymph node and then determining whether changes in cell trafficking were associated with changes within the lymph node. This research was divided into three groups of experiments. 1) A long-term lymph node study that provided the first evidence that exposure to plasmid DNA induced prolonged changes in lymph node size and architecture. 2) A cell trafficking study that examined the kinetics of cells migrating through a lymph node and provided clear evidence that exposure to CpG motifs within plasmid DNA could enhance cell traffic through a lymph node. These are the first observations to determine that bacterial DNA can alter immune surveillance. 3) A short-term study of lymph node

architecture and cellular composition demonstrated that although CpG motifs caused several cellular changes within a lymph node, these changes could not be directly correlated with the increase in cell trafficking. Taken together these observations support the conclusion that bacterial DNA can significantly enhance cell recruitment and movement through a lymph node and the increased cell number in efferent lymph is likely not directly related to changes in resident lymph node cell populations. In the future, DNA vaccines and immunostimulatory ODN may be used to both protect against and treat diseases that affect both man and domestic animals. It is therefore essential to develop a full understanding of the mechanisms whereby these products affect the function of the immune system. This work has provided substantial information on both the short and long-term effects of plasmid DNA and immunostimulatory CpG motifs on immune surveillance and a basis for future studies.

CHAPTER 8

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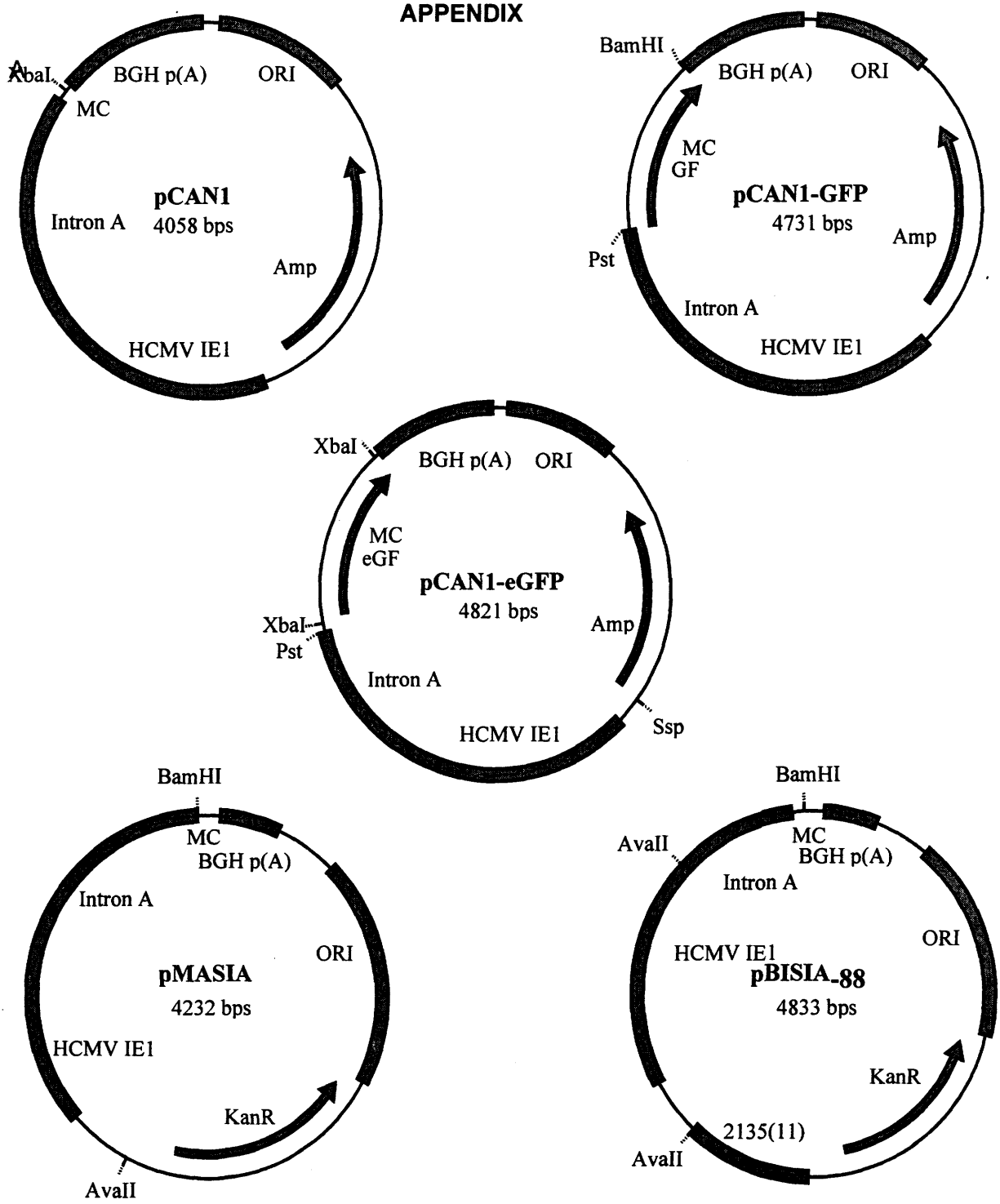
APPENDIX



Appendix 1. Plasmid constructs of pCAN1; pCAN1-GFP; pCAN1-eGFP; pMASIA; pBISIA-88. All plasmids contain five regions: multiple cloning site (MCS), intron A, bovine growth hormone poly A [BGH p(A)], origin of replication (ORI), and human cytomegalovirus promoter/enhancer (HCMV IE1). For plasmid selection in culture, the plasmids pCAN1, pCAN1-GFP and pCAN1-eGFP encoded β-lactamase (Amp) while the plasmids pMASIA and pBISIA-88 encoded kanamyacin resistance factors. Plasmids pCAN1-GFP and pCAN1-eGFP encode the GFP and eGFP reporter genes, respectively. pBISIA-88 contains an 11 tandem repeat insert of ODN 2135 [2135(11)]. Enzyme restriction sites used to digest plasmids (Fig. 3.1) are displayed on the plasmids.