MODULATION OF IMMUNE RESPONSES INDUCED BY VACCINATION AGAINST BOVINE RESPIRATORY SYNCYTIAL VIRUS

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By

JOHN WILLIAM MAPLETOFT

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ABSTRACT

As respiratory syncytial virus (RSV) is a respiratory pathogen that causes significant morbidity and mortality in infants, there has always been great interest in the development of a vaccine. In the 1960s, children were immunized with formalin-inactivated (FI)-RSV vaccines. Not only did these vaccines fail to prevent infection, but in most cases they resulted in enhanced disease upon subsequent exposure to the virus. In the intervening years, studies in mice have led to the hypothesis that the enhanced disease is due to an aberrant Th2-biased immune response. Thus, we hypothesized that formulating FI-RSV vaccines with a Th1 promoting adjuvant, such as CpG oligodeoxynucleotides (ODN), would result in the induction of protective immunity against RSV without any risk of deleterious effects. We observed in calves that parenterally delivered FI-bovine RSV (BRSV) formulated with CpG ODN resulted in a shift towards a Th1-biased or more balanced immune response that was protective against BRSV.

As RSV infects the lung mucosa, vaccines that induce mucosal immunity are desirable. Parenterally delivered vaccines typically induce systemic immunity with low mucosal immune response levels, whereas mucosally delivered vaccines induce systemic and mucosal immunity. However, upon mucosal delivery there is an increased chance of vaccine components being degraded or washed away prior to the induction of immunity. Thus, we added polyphosphazenes (PP) to our mucosal vaccine formulations. PP are synthetic polymers that form non-covalent complexes with other vaccine components, increasing their stability. Intranasally delivered FI-BRSV co-formulated with CpG ODN and PP performed better than FI-BRSV alone, or FI-BRSV

formulated with either adjuvant individually, in terms of inducing protective immunity against BRSV in mice. Furthermore, mice that received intranasally-delivered FI-BRSV or BRSV F protein co-formulated with CpG ODN and PP developed higher levels of immunity and protection than mice that received parenterally delivered vaccines. Because of the similarities between BRSV and HRSV, co-formulation of intranasally delivered HRSV vaccines with CpG ODN and PP could prove important in the development of a safe vaccine against HRSV in humans.

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DEDICATION

To my parents, for being there from the beginning

To Aileen, for standing with me now and forever

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Ab	antibody
Ag	antigen
AP	alkaline phosphatase
ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BHV	bovine herpesvirus
BRSV	bovine respiratory syncytial virus
BT	bovine turbinate
CMIS	common mucosal immune system
CpG ODN	CpG oligodeoxynucleotides
CTL	cytotoxic T-lymphocytes
Da	dalton
ddH_2O	double-distilled water
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
ds	double-stranded
ECP	eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ENA	epithelial cell-derived neutrophil attractant
ERK	extracellular receptor kinase
F protein	fusion protein
FBS	fetal bovine serum
FI	formalin inactivated
FI-BRSV	formalin inactivated bovine respiratory syncytial virus
FI-HRSV	formalin inactivated human respiratory syncytial virus
FI-RSV	formalin inactivated respiratory syncytial virus
G protein	attachment protein
GBK	Georgia bovine kidney
GRO	growth-regulated gene
HBV	hepatitis B virus
HBVsAg	hepatitis B virus surface antigen
His	histidine
HIV	human immunodeficiency virus
HRSV	human respiratory syncytial virus
HSV	herpes simplex virus
Ig	immunoglobulin
IFN	interferon
IFNAR	interferon α/β receptor

IKK	IκB kinase
IL	interleukin
IN	intranasal
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISCOM	immune-stimulating complex
ISRE	interferon-stimulated regulatory element
I-TAC	interferon inducible T cell α chemoattractant
JAK	Janus kinase
kDa	kilodalton
L protein	large protein
LARC	liver and activation-regulated chemokine
LNC	lymph node cell
LPS	lipopolysaccharide
M protein	matrix protein
mAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
MAP	mitogen activated protein
MCP	monocyte chemotactic protein
MDC	macrophage-derived chemokine
MEM	minimal essential medium
MIP	macrophage inflammatory protein
N protein	nucleocapsid protein
NAP	neutrophil-activating protein
NF-κB	nuclear factor-κB
NS	non-structural
OAS	2'-5'-oligoadenylate sythetase
ODN	oligodeoxynucleotides
P protein	polymerase phosphoprotein
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PFU	plaque forming unit
pIgR	polymeric immunoglobulin receptor
PKR	dsRNA-activated protein kinase
PP	polyphosphazenes
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RANTES	regulated upon activation, normal T cell expressed and secreted
RNA	ribonucleic acid
RNase	ribonuclease
RSV	respiratory syncytial virus
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
rVV	recombinant vaccinia virus
sAg	surface antigen

SAE	small airway epithelial
SC	subcutaneous
SH protein	small hydrophobic protein
STAT	signal transducer and activator of transcription
STCP	stimulate T cell chemotactic protein
TARC	thymus and activation-regulated chemokine
TGF	transforming growth factor
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF-α receptor activated factor
TYK	tyrosine kinase
VV	vaccinia virus

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 RESPIRATORY SYNCYTIAL VIRUS

Respiratory Syncytial Virus (RSV) was first isolated in 1957 (Hacking & Hull, 2002). It has a helical nucleocapsid and pleomorphic envelope with a diameter of approximately 150 nm, results in yearly epidemics of respiratory disease, and is transmitted by large particle aerosol and fomites (Graham *et al.*, 2002). It is a seasonal virus – outbreaks occur during winter in temperate climates, and during the rainy season in tropical climates (McNamara & Smyth, 2002). The RSV genome is a 15.2 kilobase negative sense strand of RNA, which is transcribed into 10 mRNAs encoding 11 proteins (both the M2-1 and M2-2 proteins are translated from overlapping open reading frames in the same M2 mRNA) (Collins & Murphy, 2002). The proteins synthesized from the RSV genome are: the nucleocapsid (N) protein; the polymerase phosphoprotein (P); the large (L) protein; the M, M2-1 and M2-2 matrix proteins; the NS1 and NS2 nonstructural proteins (recently identified to be antagonists of the interferon-α/β mediated anti-viral state (Schlender *et al.*, 2000)); and the attachment (G), fusion (F) and small hydrophobic (SH) transmembrane glycoproteins (Collins & Murphy, 2002). The G protein is involved in the attachment of the virion to the host cell, and F, the fusion protein, is involved in syncytia formation. Syncytia, a hallmark cytopathic effect of RSV infection, are large, multi-nucleated cells which result from the fusion of RSV-infected host cells with neighboring cells. The exact function of SH remains unknown. G and F are the only viral antigens to which neutralizing antibodies are raised (Collins & Murphy, 2002).

RSV infects epithelial cells of the respiratory tract and induces pulmonary inflammation; it seldom infects other tissues (Varga & Braciale, 2002). It is the leading cause of respiratory disease in infants and young children worldwide (Heilman, 1990) and is responsible for significant economic loss. In 2000 alone, there were 86,000 RSV infection-related hospitalizations in the United States, costing a total of 394 million dollars (Paramore *et al.*, 2004). Nearly 98% of these hospitalizations occurred in children less than 5 years old. Between 1997 and 2000, RSV bronchiolitis was the leading cause of infant hospitalization, and in 1999, an estimated 360 RSV-associated postneonatal (28-365 days old) deaths occurred in the United States (Leader & Kohlhase, 2003). Other studies have resulted in similar observations. In the United States RSV accounts for virtually one-quarter of all pediatric respiratory disease hospitalizations, 85,000 to 144,000 infant hospitalizations per year, and 500 infant deaths per year (Shay *et al.*, 1999). Furthermore, treatment and hospitalization for RSV disease are estimated to cost United States taxpayers 365 to 585 million dollars per year (Stang *et al.*, 2001). Eighty-seven percent of children have been exposed to RSV by the age of eighteen months, and by the age of three virtually all children have seroconverted (Simoes, 1999). The immunity developed following infection with RSV is incomplete; reinfections occur throughout life (Hacking & Hull, 2002). RSV is also an important cause of respiratory disease in the immunocompromised and elderly, where its impact is approaching that of nonpandemic influenza (Collins & Murphy, 2002). Direct viral cytopathology plays only a part in the disease observed during infection with RSV; the host's own immune/inflammatory response mediates damage as well (Varga & Braciale, 2002). There is no safe and effective vaccine in humans. Should infection and disease occur, supporting therapy, including oxygen, humidification and ventilation, is prescribed when necessary. Because of the significant health and economic

burdens associated with RSV-mediated respiratory disease, the development of a safe and effective vaccine is a priority worldwide.

Like human RSV (HRSV), bovine RSV (BRSV) is an enveloped, non-segmented, single-stranded RNA *Pneumovirus* of the family *Paramyxoviridae* and order *Mononegavirales*. BRSV is responsible for significant economic loss to the cattle industry (Stott & Taylor, 1985), and is one of the four known viral components of bovine shipping fever. HRSV and BRSV have similar clinical outcomes in their respective host species, ranging from asymptomatic infection, to bronchiolitis and pneumonia, and sometimes death (Philippou *et al.*, 2000). As with HRSV, distribution of BRSV is worldwide.

While several commercial BRSV vaccines are available for cattle, better vaccines that are more efficacious in the face of maternal antibodies and induce longer lasting protection would be desirable. More importantly, there is currently no vaccine against HRSV. Several studies on parenterally-delivered formalin-inactivated (FI)-HRSV vaccines were carried out in children in the 1960s (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kim *et al.*, 1969; Weibel *et al.*, 1966). Not only did FI-HRSV vaccines fail to protect upon natural infection, but in most cases, disease was enhanced. In the study with the youngest age group, 80% of the vaccinated children required hospitalization and some died (Kim *et al.*, 1969). In the years immediately following this tragedy, many other human vaccine studies, against several other disease-causing organisms were delayed or arrested completely for fear of repeating the same mistakes. At the same time, these events motivated the development of the current guidelines for vaccine development, which require that potential vaccines are extensively evaluated in animal models prior to testing in humans (Graham *et al.*, 2002). While these guidelines have allowed us to avoid further

tragedies, they may be one of the reasons a vaccine against HRSV has yet to be developed, as vaccine candidates that look promising in animals are prevented from being tested in humans.

1.2 RSV PROTEINS

1.2.1 G protein

The RSV G protein is a type II transmembrane protein with an N-terminal transmembrane domain. It is comprised of 289 to 299 amino acids (Meanwell & Krystal, 2000) and does not display any structure or sequence homology with the attachment proteins of the other members of the family *Paramyxoviridae*, though all are type II transmembrane proteins (Langedijk *et al.*, 1997; Langedijk *et al.*, 1996; Wertz *et al.*, 1985). Variability in the G protein is what distinguishes RSV strain A from RSV strain B (Graham *et al.*, 2000). The RSV G protein appears as a trimer on the virion surface and is responsible for the attachment of the virions to the host cell (Levine *et al.*, 1987), probably via the interaction of its heparin-binding domains with the glycosaminoglycans on the surface of the host cell membrane (Teng *et al.*, 2001). The attachment function can also be provided by the RSV F protein alone (Karron *et al.*, 1997). The extracellular domain of the RSV G protein is very heavily glycosylated, adding approximately 55 kDa to the mass of the polypeptide, (Melero *et al.*, 1997).

The RSV G protein is also produced in a second, smaller, secreted form that lacks the transmembrane domain, and is generated by the initiation of translation at an internal ribosome entry site (i.e. AUG initiation codon) that is in the same reading frame as the full-length RSV G protein (Roberts *et al.*, 1994). This secreted form of the RSV G protein accounts for approximately 80% of the total G protein produced 24 hours into an RSV infection (Hendricks *et al.*, 1988). Not only does RSV have the unusual capacity of being able to re-infect previously

infected individuals throughout the course of their lives, but the virus can also infect neonates despite the presence of virus-neutralizing maternal antibodies (Yang *et al.*, 2007). It has been previously suggested that the secreted form of the RSV G protein may act as a decoy by binding neutralizing anti-G antibodies before they have a chance to interact directly with virion membrane-anchored G, allowing the virus to escape neutralization (Easton *et al.*, 2004; Valarcher & Taylor, 2007). Recently, secreted G protein was indeed shown to act as a virus neutralizing antibody decoy (Yang *et al.*, 2007). In this study, the authors used a recombinant virus with a mutation that eliminated the expression of the secreted form of G. They found that this recombinant virus was substantially more sensitive to neutralization by polyvalent RSV and G-specific neutralizing antibodies, both *in vitro* and in mice. In another recent study, glycosylation of the C-terminal domain of the RSV G protein reduced its reactivity with human sera containing anti-RSV antibodies (Rawling & Melero, 2007). This allowed the authors to conclude that variable glycosylation of the RSV G protein may function to reduce the antigenicity of the protein by shielding it with host-specified sugars, potentially accounting for RSV's ability to evade RSV G-specific neutralizing antibodies.

1.2.2 F protein

The RSV F protein is responsible for both the fusion of the viral membrane with a host cell, as well as the fusion of an infected cell with its neighbors to mediate direct cell-to-cell viral transmission (Domachowske & Rosenberg, 1999). The latter effect results in the formation of syncytia, giant multinucleated cells that are the hallmark cytopathic effect in RSV infection. In fact, the RSV F protein was originally identified by immunoprecipitation using monoclonal antibodies that inhibited syncytium formation *in vitro* (Walsh & Hruska, 1983). The F protein is

first synthesized in an inactivated form, a 574 amino acid trimeric coiled-coil called F0. Activation occurs following cleavage at two furin consensus sites, resulting in the formation of the F1 and F2 subunits, which remain linked by a disulfide bond (Gonzalez-Reyes *et al.*, 2001; Zimmer *et al.*, 2001; Zimmer *et al.*, 2002).

The RSV F protein's contribution to the virus' evasion of the host immune response is conceptually simple but extremely effective. By facilitating the fusion of an infected host lung epithelial cell with neighboring uninfected host cells, the virus bypasses the host's extracellular immune defense mechanisms, e.g. RSV-specific antibodies, while continuing to spread from cell to cell.

As a vaccine candidate, the RSV F protein has already been used successfully in a number of studies in children. In a study in RSV-seropositive children aged 18-36 months, significant increases in geometric mean antibody titer were demonstrated one month after intramuscular immunization, and none of the vaccine recipients had any RSV infections documented between the time of vaccination and six months later (Tristram *et al.*, 1993). In another study in children aged 24-48 months, increases in ELISA and neutralizing antibody levels were shown, with no serious RSV illness in vaccine recipients through at least one, and in some cases two, RSV seasons (Paradiso *et al.*, 1994). The RSV F protein has also shown success in children with cystic fibrosis. In one study (mean age = ~5 years) significant neutralizing antibody titers were induced in two-thirds of vaccine recipients and significant ELISA antibody titers were induced in nearly all vaccine recipients (Piedra *et al.*, 1996). In another study (age range = 2.6 to 8.9 years), in which the vaccine was given once, or twice in successive years, the children that received the vaccine twice in successive years experienced significantly lower incidence of lower repiratory illness, fewer antibiotic courses, and fewer days of illness,

compared to the children immunized only the previous year (Piedra *et al.*, 1998). In all of the above studies, there was no evidence of vaccine-associated disease enhancement, and vaccination site reactions were mild, transient and not significantly different from those in the placebo-treated children.

1.2.3 SH protein

The transmembrane SH envelope protein consists of 64 amino acids and is not required for replication or syncytia formation, but is necessary for fusion (Domachowske & Rosenberg, 1999). The exact role of SH is uncertain.

1.2.4 Non-structural proteins

Among the paramyxoviruses, only the members of the genus *Pneumovirus* have the genes for the non-structural NS1 and NS2 proteins. These genes are located at the most 3' position of the genome and, therefore, are the most abundantly transcribed genes. The corresponding proteins are relatively small; the RSV NS1 protein is only 139 amino acids in size and the RSV NS2 protein is only 124 amino acids in size (Collins & Wertz, 1985a; b). These proteins are present in only trace amounts in mature RSV virions, but are abundant in RSV-infected cells (Olmsted & Collins, 1989). Until recently, the exact functions of NS1 and NS2 remained unknown, but they appear be antagonists of the type I IFN mediated anti-viral state (Schlender *et al.*, 2000). Type I IFNs (also known as interferon α/βs) are an important first line of defense against viruses. They are produced by eukaryotic cells in response to viral infections. Once the type I IFNs have been synthesized and secreted, they transcriptionally induce expression of a series of antiviral proteins: expression of the dsRNA-activated protein kinase

(PKR), for example, results in the phosphorylation, and therefore inactivation, of the translation initiation factor eIF-2a, thereby inhibiting protein synthesis and blocking viral replication (Williams, 1999). The 2'-5'-oligoadenylate synthetases (OAS), another class of proteins whose expression is induced by type I IFNs, catalyze the synthesis of 2',5'-oligoadenylates, which bind, and thereby activate, RNaseL, which in turn cleaves RNA and inhibits viral replication (Rebouillat & Hovanessian, 1999). The Mx proteins, which belong to the superfamily of dynamins with GTPase activity (Horisberger, 1992), bind to viral nucleocapsids from a variety of different virus families, blocking their transport and/or other biological functions, thereby inhibiting viral replication (Weber *et al.*, 2000).

Type I IFNs accomplish the above by binding to the interferon α/β receptor (IFNAR) on the surface of the infected cells, as well as neighboring cells, resulting in the activation of the intracellular Janus (JAK) kinases JAK1 and tyrosine kinase 2 (TYK2) (Colamonici *et al.*, 1994; Novick *et al.*, 1994). JAK1 and TYK2 then phosphorylate, and therefore activate, the latent transcription factors STAT1 and STAT2, which then dimerize, associate with interferon regulatory factor-9 (IRF-9), translocate to the nucleus, and bind to the interferon-stimulated regulatory element (ISRE) promoter, activating transcription of the downstream genes (Stark *et al.*, 1998).

The RSV NS proteins, therefore, appear to suppress the type I IFN response by inhibiting STAT2 production. At least two studies have demonstrated that wild-type RSV infection or expression of recombinant NS proteins in respiratory epithelial cells decreases STAT2 levels (Lo *et al.*, 2005; Ramaswamy *et al.*, 2006). One of these studies also featured a system in which they used a vaccinia virus vector to express the RSV NS2 gene in respiratory epithelial cells (Ramaswamy *et al.*, 2006). They found that decreasing the levels of vaccinia virus-expressed

NS2 using RNA interference resulted in the inhibition of the NS2-mediated suppression of STAT2 expression. The authors of the other study also found that, compared to wild-type RSV, mutant viruses lacking the RSV NS1 or NS2 genes no longer decreased STAT2 levels or type I IFN responsiveness (Lo *et al.*, 2005).

The inhibition of type I IFN responsiveness through a downstream transcription factor makes sense, as treatment of cells infected with bovine RSV NS1 and/or NS2 deletion mutants with recombinant IFN-α or IFN-β results in the inhibition of the growth of the mutant viruses, but failed to inhibit the growth of wild-type bovine RSV (Schlender *et al.*, 2000). The ability of the NS proteins expressed by wild-type bovine RSV to suppress type I IFN responsiveness even following treatment of the cells with recombinant IFN-α or IFN-β indicates that the RSV NS proteins do not affect the expression of the type I IFNs themselves, but instead must act on a downsteam element (e.g. STAT2).

By suppressing the type I IFN antiviral response, the RSV NS proteins are able to contribute to host immune evasion. Recombinant RSV lacking the NS1 and NS2 genes was demonstrated to be a strong inducer of the type I IFN response in human pulmonary epithelial (A549) and human peripheral blood monocyte-derived macrophages (Spann *et al.*, 2004). This agreed with a previous study, in which recombinant bovine RSV lacking the NS1 and NS2 genes was also demonstrated to be a strong inducer of the type I IFN response in bovine nasal fibroblasts and bovine bronchoalveolar macrophages (Valarcher *et al.*, 2003). Furthermore, while recombinant bovine RSV lacking the NS1 and NS2 genes replicated as well as wild-type bovine RSV in cells that lack a functional type I IFN system, its replication was severely inhibited in cells with a functioning type I IFN system, as well as in young calves (Valarcher *et al.*, 2003).

Type I IFNs have also been implicated in adaptive immunity. The suppression of the type I IFN response (a response traditionally considered to be primarily innate) could affect the adaptive immune response against RSV in such a manner that it would allow re-infections to occur in previously infected individuals because type I IFNs have been demonstrated to enhance humoral immunity (Le Bon *et al.*, 2001) and promote the proliferation of memory T cells (Tough *et al.*, 1999). Furthermore, any time that innate immune mechanisms are inhibited, it puts more responsibility on the adaptive immune system in terms of resolving the infection. If the adaptive immune response is being hampered as well (as it is by the G- and F proteins during an RSV infection), this acts as a double-blow, which therefore contributes to the ability of RSV to cause re-infections throughout the course of an individual's life.

1.2.5 Other proteins

The N protein encapsulates the negative sense strand RNA genome. Along with the N protein, the P and L proteins are required for replication of the RNA genome. Transcription from the genome requires the M2 protein. The M protein mediates the interaction between the helical nucleocapsid and the envelope (Domachowske & Rosenberg, 1999).

1.3 FI-RSV IN CHILDREN

In the 1960s, four studies were carried out in which children received formalin-inactivated (FI)-RSV vaccines (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kapikian *et al.*, 1969; Kim *et al.*, 1969). Not only did these vaccines fail to protect the children upon subsequent natural infection, but in most cases, disease was enhanced. In one of the studies, 80% of vaccinees required hospitalization (compared to 5% of those that received a control

parainfluenza vaccine) and 2 children died (Kim *et al.*, 1969). Mean durations of hospitalization in this study were 10.5 days for FI-RSV vaccinees and 6.7 days for age-matched controls naturally infected with RSV (Kim *et al.*, 1969). During natural infection, FI-RSV vaccinees experienced pneumonia and/or bronchiolitis, while control vaccinees experienced rhinitis, pharyngitis, and/or bronchitis (Kim *et al.*, 1969). Autopsy reports from the children that died described a lung infiltrate consisting mostly of neutrophils, with eosinophils 'in some areas' (Prince *et al.*, 2001a), indicating the importance of the host inflammatory response in the enhancement of disease. The 1969 published paper in the *American Journal of Epidemiology* put the emphasis on a 'peribronchiolar monocytic infiltration with some excess in eosinophils' (Kim *et al.*, 1969). Prince *et al.* offered two explanations as to why the presence of eosinophils, by far the smallest component of the granulocytic infiltrate observed in the lungs of the children that died, was emphasized over the severe neutrophilia (Prince *et al.*, 2001a). First, at that time, neutrophilia was not considered a feature of RSV pathogenesis (Aherne *et al.*, 1970). Second, as *Klebsiella* was cultured from the lungs of one of the autopsies, and *E. coli* was cultured from the lungs of the other autopsy, the neutrophilia may have been attributed to bacterial infection. Prince *et al.* stained recut sections from the autopsy materials and could detect no bacteria, allowing them to conclude that the positive cultures were due to numbers of bacteria too small to be responsible for the influx of neutrophils (Prince *et al.*, 2001a). The studies carried out in the 1960s were done without prior animal testing, indicating the importance of such experiments (Openshaw & Tregoning, 2005). Finally, analysis of sera from FI-RSV vaccinees revealed production of non-neutralizing antibodies against the F and G proteins, suggesting that a lack of neutralizing antibodies (perhaps due to alteration of F and G protein epitopes during formalin

inactivation) may contribute to the enhancement of disease by delaying the clearance of virus from the lungs (Murphy *et al.*, 1986b).

1.4 ANIMAL MODELS AND VACCINE-ENHANCED DISEASE

1.4.1 Mice

BALB/c mice serve as an animal model for RSV infection, as they are somewhat more permissive to RSV replication than other strains of mice (Graham *et al.*, 2002). That being said, RSV is not a natural pathogen in mice, and an inoculum of greater than 0.5-2 million plaque forming units (pfu) is required to establish infection, with a peak lung viral titer less than the inoculum. By comparison, the inoculum required to infect a human is presumed to be very low, perhaps no more than a few pfu, with a peak viral titer of 10^6 pfu/ml of nasal secretion (Domachowske *et al.*, 2001). To initiate infection, mice are anesthetized and inoculated intranasally. The inoculum is aspirated into the lungs. Because of the unnaturally high doses required for infection, the mouse model has not been useful for studying direct viral cytopathology in a primary RSV infection (Graham *et al.*, 2002). This concession has little bearing on the choice to use the mouse model, as much of the disease associated with human RSV infections is mediated by the host immune system, not the virus itself. Furthermore, BALB/c mice primed with FI-RSV, then challenged with live RSV, exhibit similar lung pathology to the children that died during the FI-RSV vaccine trials, with a lung infiltrate composed of monocytes, lymphocytes and eosinophils (Graham *et al.*, 2002). This makes BALB/c mice an ideal model for understanding the mechanism behind the immune-mediated lung pathology during post-immunization RSV infections.

FI-RSV-mediated enhancement of pulmonary histopathology has been reproduced in mice (Connors *et al.*, 1992). This study demonstrated the importance of $CD4^+$ T cells in disease enhancement, as this pathology was completely abrogated in FI-RSV-immunized mice if the $CD4^+$ T cell subset was depleted immediately prior to challenge. A subsequent study by the same authors demonstrated that depletion of IL-4 and IL-10 (Th2-type cytokines) from FI-RSV-immunized mice immediately before RSV infection also results in abrogation of enhanced pathology, allowing formulation of the hypothesis that, in mice, FI-RSV disease enhancement is caused by an imbalanced Th2/Th1 immune response (Connors *et al.*, 1994). In a later study, FI-RSV-immunized, RSV-infected mice had increased numbers of granulocytes, eosinophils and $CD4^+$ cells, as well as a decreased number of $CD8^+$ cells in bronchoalveolar lavages (BAL) obtained four and eight days after infection (Waris *et al.*, 1996). These BAL cell population changes were accompanied by increased mRNA expression of IL-5, IL-13 and IL-10 (Th2-type cytokines), as well as decreased mRNA expression of IL-12 (a Th1-type cytokine), lending further support to the Th2 hypothesis. Indeed, in a subsequent paper, the same authors demonstrated that a Th1-skewed immune response (characterized by reductions in $CD4^+/CD8^+$ ratios and Th2 cytokine mRNA expression in BAL cells) induced by priming with live RSV (prior to immunization with FI-RSV) could prevent enhancement of pulmonary inflammation upon subsequent RSV infection (Waris *et al.*, 1997). Increased pulmonary immunopathology has also been prevented by immunizing mice with FI-BRSV formulated with CpG oligodeoxynucleotides (ODN), an adjuvant known to stimulate Th1-type immune responses (Oumouna *et al.*, 2005). This provides further evidence that a balanced or Th1-biased immune response (in this case characterized by CpG ODN-induced increases in serum IgG2a and IFN-γ production by *in vitro*-restimulated splenocytes, as well as decreases in serum IgE) is protective

in mice. Vaccines other than FI-RSV (e.g. recombinant vaccinia virus expressing RSV proteins) have been shown to induce enhanced RSV disease in mice, but these will be discussed in a subsequent section of this review.

The advantages that the RSV mouse model possesses when compared to those of other species (especially large animals) include the relative low cost of the animals, the ease of handling and housing, as well as the comprehensive availability of immunological reagents. These advantages are tempered by the fact that RSV is not a natural pathogen of mice. Pneumovirus of mice (PVM), a natural pathogen of mice that reproduces the features of RSV infection, belongs to the same family (*Paramyxoviridae*), subfamily (*Pneumovirinae*), and genus (*Pneumovirus*) as RSV (Rosenberg *et al.*, 2005). PVM and RSV share the same genome size and structure, and the various genes of PVM share 29-62% nucleotide sequence identity and 10-60% amino acid sequence identity with RSV and BRSV (Krempl *et al.*, 2005). PVM may be a more appropriate model for RSV, but this topic is outside of the scope of this review.

1.4.2 Cotton Rats

The cotton rat (*Sigmodon*) is a member of the order Rodentia and family Cricetidae (New World rats and mice and true hamsters). Cotton rats are therefore more closely related to hamsters, gerbils, voles and lemmings than to laboratory rats and mice (members of the family Muridae; Old World rats and mice) (Coe & Prince, 1996). Cotton rats are notable among laboratory rodents for being susceptible to infection with many common human pulmonary viruses, including RSV, influenza A and B viruses, parainfluenza viruses 1, 2 and 3, and adenovirus types 2, 4, 5 and 7. The pulmonary pathology induced by these viruses in cotton rats is similar to that observed in humans (Ginsberg & Prince, 1994; Prince *et al.*, 1978).

The first evidence of enhanced pulmonary pathology in FI-RSV-immunized, RSV-challenged cotton rats was published in 1986 (Prince *et al.*, 1986). This was the first time FI-RSV-enhanced disease had been demonstrated in a small laboratory animal. As parenteral immunization of cotton rats with FI-RSV induced high levels of non-neutralizing antibodies directed against the F and G proteins, the authors suggested that the neutralizing epitopes on these proteins may have been altered by formalin treatment (Prince *et al.*, 1986). Subsequent studies demonstrated immunopathology despite a 90-99% reduction in virus replication in FI-RSV-immunized, RSV-challenged cotton rats, indicating a dissociation between viral load and disease enhancement (Prince *et al.*, 2001a; Prince *et al.*, 1999). Other studies have revealed that immunization of cotton rats with FI-RSV formulated with monophosphoryl lipid A (an adjuvant derived from lipopolysaccharide from the gram-negative bacterium *Salmonella Minnesota* that activates cells via toll-like receptor 4) reduces both polymorphonuclear leukocyte infiltration of lung alveoli (Prince *et al.*, 2001b), and mRNA expression of a number of Th1- and Th2-type cytokines and chemokines normally associated with FI-RSV-primed RSV challenge (Boukhvalova *et al.*, 2006). A lack of immunological reagents, however, has limited the usefulness of this model in dissecting the specific mechanisms of disease enhancement.

1.4.3 Cattle

The BRSV model is attractive because it features an outbred, large animal species in which the virus is a natural pathogen. Issues that one must be aware of include the cost of the animals and their care, as well the availability of appropriate large animal facilities. BRSV was first isolated from cattle in the 1970s, and it is the closest phylogenetic relative to HRSV (van den Hoogen *et al.*, 2001). Like HRSV infections in humans, which are typically more severe in

infants and small children, BRSV infections in calves are usually more severe than infections in adults (Van der Poel *et al.*, 1994). Unlike HRSV, however, natural BRSV infections are often accompanied by bacterial infections (*Mannheimia haemolytica, Pasteurella multocida,* or *Haemophilus somnus*) (Easton *et al.*, 2004). It has been estimated that BRSV is responsible for more than 60% of epizootic respiratory diseases observed in dairy herds (Baker *et al.*, 1986; Elvander, 1996; Uttenthal *et al.*, 1996), and up to 70% of beef herds (Caldow *et al.*, 1988; Stott *et al.*, 1980).

The incubation time for BRSV in cattle is estimated to be between two and five days. BRSV infection can be asymptomatic, limited to the upper respiratory tract, or involve both the upper and lower respiratory tracts. Infection is characterized by a cough with nasal and ocular discharges. More severe infections can result in pyrexia, anorexia, depression, increased respiratory rate, shortness of breath, weezing, and open mouth breathing (Verhoeff *et al.*, 1984). Interstitial pneumonia can be observed at necropsy (Viuff *et al.*, 1996). The cranio-ventral areas of the lungs are often consolidated and a mucopurulent discharge can be visible from the bronchus and small bronchi. The caudo-dorsal areas of the lungs are often distended and bronchotrachial and medistinal lymph nodes are often enlarged (Valarcher & Taylor, 2007). Microscopic lesions are characterized by exudative and proliferative bronchiolitis with alveolar collapse and peribronchiolar infiltration by mononuclear cells (Valarcher & Taylor, 2007). Syncytia may be visible in the epithelium or lumen of the bronchi, or in the alveolar walls and lumina (Viuff *et al.*, 2002). The lumen of bronchi, bronchioles and alveoli can be blocked by cellular debris, consisting of desquamated epithelial cells, neutrophils, macrophages, and sometimes eosinophils (Viuff *et al.*, 2002).

Evidence of FI-BRSV-enhanced disease in calves has been documented in only a few studies. A study featuring two immunizations with low doses of FI-BRSV (0.2 mg of protein per immunization) induced enhanced disease, accompanied by production of non-neutralizing IgG antibodies, upon infection with BRSV (Gershwin *et al.*, 1998). A subsequent study by the same authors demonstrated that two immunizations with a ten-fold higher dose of FI-BRSV (2.0 mg of protein per immunization) did not induce disease enhancement upon infection with BRSV (Kalina *et al.*, 2004). Analysis of sera from the two studies revealed that animals that received the low dose vaccine produced more IgE than animals that received the high dose vaccine (Kalina *et al.*, 2004). Another study demonstrated that immunization with FI-BRSV prior to BRSV infection results in earlier onset of clinical disease (day 2 versus day 4-5, characterized by pyrexia and dyspnea), but reduced pulmonary pathology compared to control animals (West *et al.*, 1999). The vaccine protein dose in this study was 1.3 mg per immunization, indicating, again, a possible role for vaccine dose in disease enhancement. A later study documented production of IgE, reduction in virus replication, and enhancement of disease in FI-BRSV-immunized, BRSV-infected calves, but this was accompanied by significant lung eosinophilia, a feature that has not been described in any other study in cattle (Antonis *et al.*, 2003).

1.4.4 Non-human primates

Despite being the most relevant animal model for human disease, there are a number of obvious logistical concerns that must be addressed in order to carry out experiments in non-human primates (e.g. availability of animals, cost, approval, appropriate housing, handling, etc.), thereby limiting the number of studies that have been completed thus far. The first evidence of FI-RSV-enhanced disease in non-human primates was published in 1993. FI-RSV-immunized

African green monkeys experienced enhanced pulmonary pathology, accompanied by a reduction in virus replication, when challenged with RSV (Kakuk *et al.*, 1993). A subsequent study carried out in cynomolgus macaques demonstrated the presence of RSV-specific T cells producing the Th2-type cytokines IL-13 and IL-5 in FI-RSV-immunized, RSV-challenged animals (De Swart *et al.*, 2002). Intratracheal challenge three months following the third immunization induced a hypersensitivity response associated with lung eosinophilia, and two out of seven animals that received the FI-RSV vaccine died twelve days after challenge (De Swart *et al.*, 2002). A model using bonnet monkeys (*Macaca radiata*) also demonstrates disease enhancement in FI-RSV-immunized, RSV-challenged animals, but with increased virus replication in immunized animals (Ponnuraj *et al.*, 2001). A further study revealed evidence of antibody-dependent disease enhancement, as serum samples from FI-RSV-immunized bonnet monkeys increased *in vitro* RSV infection of U937 cells (Ponnuraj *et al.*, 2003).

1.5 RECOMBINANT VACCINIA VIRUS EXPRESSING RSV PROTEINS

A system that allows the examination of the roles of different RSV antigens in disease enhancement involves sensitization of mice by dermal scarification with recombinant vaccinia virus (rVV) expressing individual RSV proteins. Following challenge with RSV, BALs of mice scarified with rVV-G featured 14-25% eosinophils, compared to less than 3% in mice scarified with rVV-F or rVV-N (Openshaw *et al.*, 1992). Mice scarified with rVV-G or rVV-F developed lung hemorrhage, and mice scarified with rVV-G, rVV-F, or rVV-N displayed pulmonary polymorphonucleocyte efflux (Openshaw *et al.*, 1992). A later study demonstrated that the effects of scarification with rVV-G or rVV-F in mice can be abrogated by intraperitoneal injection with 0.5 mg of anti-TNF antibodies beginning one day before, and on the day of RSV

infection (Hussell *et al.*, 2001). Further studies, using frameshift mutations altering the COOH terminus of the G protein gene in rVV-G cDNA, were able to pinpoint the region responsible for the induction of eosinophilia and disease enhancement upon subsequent challenge with RSV (Sparer *et al.*, 1998). This study also demonstrated that the altered G protein could induce protection from RSV without inducing immunopathology. Incidentally, route of administration seems to be critical in determining whether enhanced disease is induced by immunization of mice with rVV carrying RSV proteins. While scarification often primes for enhanced disease, it appears as though protection against live RSV can be induced by immunizing mice intranasally or intraperitoneally with rVV-G (Stott *et al.*, 1986), or rVV-F (Wertz *et al.*, 1987). Intranasal or intraperitoneal immunization with rVV-N also induces protection against RSV challenge, though not to the same extent as rVV-G or rVV-F (King *et al.*, 1987). Interestingly, scarification with rVV-G does not prime for enhanced disease in all strains of mice (Hussell *et al.*, 1998). Upon RSV challenge of rVV-G-primed mice, eosinophilia occurs in H-2^d mouse strains (BALB/c, DBA/2n, and B10.D2), but not in H-2^k mouse strains (CBA/Ca, CBA/J, C3H, BALB.K, or B10.BR). Among H-2^b mouse strains, 129 and BALB.B developed eosinophilia, whereas C57BL/6 and C57BL/10 did not. Depletion of $CD8^+$ T cells or IFN-γ prior to challenge with RSV results in induction of eosinophilia in normally eosinophilia-resistant mice, specifically in rVV-F-primed BALB/c mice, or in rVV-G-primed C57BL6 mice (Hussell *et al.*, 1997; Hussell *et al.*, 1998). Eosinophilia is also induced in rVV-F-scarified, RSV challenged, $CD8^+$ T cell-deficient β_2 microglobulin knock-out mice (Srikiatkhachorn & Braciale, 1997). These results fit with earlier experiments demonstrating that F-specific T cells are a mixture of $CD8^+$ and $CD4^+$ cells with a Th1-type cytokine secretion pattern (high IL-2, some IL-3, and low IL-4 and IL-5), and G-specific T cells are almost entirely $CD4^+$, with a Th2-type cytokine secretion pattern (high

IL-3, IL-4 and IL-5, and low IL-2) (Alwan *et al.*, 1994; Alwan *et al.*, 1993). In fact, approximately half of $CD4^+$ cells that infiltrate the lungs of rVV-G-primed, RSV challenged mice use the same Vβ gene (Vβ14), and depletion of these $CD4^+$ $V\beta14^+$ T cells one day prior, as well as four and eight days after scarification, abolishes pulmonary injury (Varga *et al.*, 2001). These studies highlight the importance of the interplay between the RSV G protein and the host $CD4^+$ T cell population with regards to disease enhancement in mice.

1.6 HOST CELLS INVOLVED IN RSV PATHOGENESIS

1.6.1 Respiratory epithelial cells

The epithelial cells of the respiratory tract are the target cells for RSV infection. In response to infection, these cells secrete many chemokines. Chemokines act as attractants to guide the different types of blood leukocytes to sites of infection and inflammation. In circumstances in which the presence of particular leukocytes at the site of infection exacerbates disease, the prior release of the chemokines that attracted those leukocytes has actually been detrimental to the individual's health. Generally, a target cell secretes a chemokine, and a blood leukocyte that expresses that chemokine's corresponding receptor will encounter the chemokine and move along its concentration gradient towards the original target cell (which is presumably at a site of infection or inflammation). All chemokines have four conserved cysteine residues that form two essential disulfide bonds. The nomenclature for chemokines is based on the proximity of the first two cysteine residues. The first two cysteine residues can be adjacent (as is the case in CC chemokines), separated by a single amino acid (CXC chemokines), or as in the case of the chemokine-like molecule fractalkine, separated by three amino acids (CX_3C chemokines) (Baggiolini, 1998). Chemokine receptors are simply named for the class of chemokine to which

they bind. In vitro, using human cDNA microarrays, Zhang et al. (Zhang *et al.*, 2001) were able to detect inducible expression of many chemokines in infected cells. In RSV-infected human type II alveolar A549 cells they detected inducible expression of CXCL1 (GRO1/GROα/MGSA-α/NAP-3/GRO/MIP-2/KC), CXCL2 (GRO2/GROβ/MGSA-β/MIP-2α/GRO/MIP-2/KC), CXCL3 (GRO3/GROγ/MGSA-γ/MIP-2β/GRO/MIP-2/KC), CXCL5 (ENA-78/GCP-2/LIX), CXCL8 (IL-8), CXCL11 (I-TAC), CX3CL1 (frackalkine/neurotactin/ABCD-3), CCL1 (I-309/TCA-3/P500), CCL2 (MCP-1/MCAF/TDCF/JE), CCL3 (MIP-1α/LD78α), CCL4 (MIP-1β), CCL5 (RANTES), CCL17 (TARC/ABCD-2), CCL20 (MIP-3α/LARC/exodus-1), and CCL 22 (MDC/STCP-1/ABCD-1). In RSV-infected human Small Airway Epithelial (SAE) cells, they detected inducible expression of CXCL1, CXCL2, CXCL3, CXCL8, CXCL11, CX3CL1, CCL3, CCL4, CCL5, and CCL20. In these cells inducible expression of CCL2, CCL17, and CCL22 was not detected.

1.6.2 Macrophages

Macrophages, along with the target respiratory epithelial cells, are the first cells to interact with RSV in infected lungs (Kimpen, 2001). When circulating monocytes migrate into specific tissues, they differentiate into macrophages. Monocytes are recruited by CX3CL1, CCL2, CCL3, CCL4, and CCL 5. Macrophages play roles in both the innate and adaptive immune defense systems. They act as phagocytic cells that are able to engulf and digest invading pathogens, but they can also act as antigen presenting cells (APCs) that are able to take part in the activation of both T helper and cytotoxic T-lymphocytes. Furthermore, upon interaction with invading pathogens, macrophages are able to secrete cytokines such as TNF-α and IL-6 (Franke-Ullmann *et al.*, 1995).

1.6.3 Neutrophils

In infants, neutrophils represent 93% of leukocytes found in the upper airway and 76% in the lower airway during RSV infection (Everard *et al.*, 1994). During a viral infection, neutrophils aid in the destruction of virus-infected cells via degranulation, which results in the release of lytic enzymes and other cytotoxic substances into the proximity of infected cells. Unfortunately, this often results in damage to uninfected neighboring cells, such as in the case of an RSV infection. In the bloodstream, neutrophils develop a loose adherence to the endothelial wall that allows them to "roll" along the vascular endothelium. When a neutrophil encounters certain chemokines, which may be secreted from infected cells, the neutrophil will then adhere firmly to the endothelium and begin to squeeze between endothelial cells and move along the chemokine concentration gradient towards the site of infection. In humans, neutrophil chemotaxis is controlled by CXCL8, a chemokine secreted by respiratory epithelial (the cells infected by RSV) and macrophages (Wang & Forsyth, 2000). The murine homolog of CXCL8 has yet to be identified, but evidence suggests that it may be either KC or MIP-2 (CXCL1, CXCL2, or CXCL3) (Rollins, 1997). The importance of CXCL8 in the lung pathology associated with RSV infection, and by extension the importance of neutrophil chemotaxis in this process, is possibly confirmed by a study that showed that a polymorphism near the human CXCL8 gene (which presumably affects CXCL8 expression) is a determinant in disease severity (Hull *et al.*, 2000). Higher levels of CXCL8 have been correlated with RSV disease severity (Smyth *et al.*, 2002), and infants with RSV bronchiolitis display elevated levels of CXCL8 (Bont *et al.*, 1999). Neutrophils are also recruited by CXCL1, CXCL2, CXCL3, and CXCL5.

1.6.4 Eosinophils

Similar to neutrophils, eosinophils are motile cells that mediate their cytotoxic effects via degranulation at a site of infection. Again, this often results in damage to healthy uninfected bystander cells. Evidence for the involvement of eosinophils in the immunopathogenesis of RSV infection comes in the form of studies that show the upregulation of eosinophil chemoattractants CCL3 and CCL5 during infection of epithelial cells (Harrison *et al.*, 1999). Additionally, eosinophil cationic protein (ECP), a cytotoxic protein contained in the granules of eosinophils, is present in the nasopharyngeal secretions and serum of children with RSV bronchiolitis (Garofalo *et al.*, 1992; Sigurs *et al.*, 1994). Another correlate with eosinophilia is the production of the eosinophil chemoattractant CCL11 (eotaxin) (Humbles *et al.*, 1997).

1.6.5 Cytotoxic T-lymphocytes

While cytoxic T-lymphocytes (CTLs) are vital to the immune response against the majority of intracellular pathogens, the presence of RSV-specific CD8+ CTLs is important for both viral clearance and the immunopathogenesis of RSV disease. The effector functions of CTLs during RSV infection include cytokine secretion and lysis of infected cells by perforin/granzyme or Fas/Fas-mediated apoptosis (van Drunen Littel-van den Hurk *et al.*, 2007). In mice, when CTLs are depleted, RSV replication is significantly prolonged, yet no evidence of disease is outwardly visible. In the presence of RSV-specific CTLs, viral clearance is accelerated, but lung pathology is enhanced (Cannon *et al.*, 1988; Domachowske & Rosenberg, 1999).

The CTL response is directed against several RSV proteins, including F, SH, N and M2 (Bangham & McMichael, 1986; Cherrie *et al.*, 1992; Gaddum *et al.*, 2003; Simoes & Carbonell-Estrany, 2003; Taylor *et al.*, 1997), with M2-specific CTLs inducing disease enhancement in mice upon subsequent challenge with RSV (Cannon *et al.*, 1988). Despite recent contradictory results (Bukreyev *et al.*, 2006), the RSV G protein is traditionally considered not to be able to induce a CTL response in BALB/c mice (Bangham & McMichael, 1986; Graham *et al.*, 2002; Nicholas *et al.*, 1990) or humans (Simoes & Carbonell-Estrany, 2003). In calves, however, a G-specific CTL response can be detected during infection with BRSV (Antonis *et al.*, 2006).

IFN-γ, which is produced by CTLs, has also been implicated in RSV clearance and immunopathogenesis. IFN-γ has been demonstrated to be important for protection against RSV following immunization of mice with a G-protein fragment (Plotnicky-Gilquin *et al.*, 2002), and severe RSV infections are associated with low IFN-γ production in infants (Aberle *et al.*, 1999; Joshi *et al.*, 2003), indicating a role for IFN-γ in protection against RSV. RSV-infected IFN-γ knockout mice, however, display a reduction in airway obstruction compared to wild-type mice (van Schaik *et al.*, 2000), and an overproduction of IFN-γ appears to be associated with RSV-induced wheezing in infants and children (van Schaik *et al.*, 1999). Again, these observations provide evidence that the lung pathology that accompanies RSV infection is immune-mediated.

While CTLs may play a role in RSV immunopathogenesis, they are clearly necessary for viral clearance. A deficiency in the RSV-specific CTL response could explain why RSV infections are usually more severe in infants. While RSV-specific CTL activity is displayed in 65% of infected children over six months of age, this activity is observed in only 35 to 38% of infected children under five months of age (Chiba *et al.*, 1989). Based on the results of these studies, it seems as though there is a delicate balance between protection and disease

augmentation with regards to the specific effects of CTLs in RSV infection. Ultimately, however, CTLs appear to be necessary for viral clearance and a successful vaccine against RSV, therefore, would probably have to induce a robust anti-RSV CTL response.

1.6.6 T helper cells

CD4+ T helper (Th) cells have also been implicated in both protection from RSV and disease augmentation. Mice given RSV-specific CD4+ T cell enriched splenocytes prior to primary infection with RSV display accelerated viral clearance, but also experience enhanced disease (Alwan *et al.*, 1992). Furthermore, the lung immunopathology induced by challenge of FI-RSV-vaccinated mice is abrogated by the depletion of CD4+ T cells (Connors *et al.*, 1992). CD4+ Th cells have been classified into the Th1 and Th2 subsets based on the cytokines they secrete. Th1 cells secrete IL-2, IFN-γ and TNF, and Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (van Drunen Littel-van den Hurk *et al.*, 2007). Activated Th1 cells are recruited by CXCL11 and CCL4, and activated Th2 cells are recruited by CCL1, CCL17 and CCL22. In mice, the response to a primary RSV infection, or immunization with the F protein, tends to be more Th1-biased (Bangham & Askonas, 1986; Bangham *et al.*, 1986; Jackson & Scott, 1996). On the other hand, immunization with FI-RSV, or the G protein, tends to result in a more Th2-biased immune response (Hancock *et al.*, 1996; Jackson & Scott, 1996; Johnson *et al.*, 1998). It is widely believed that a Th2-skewed immune response is responsible for the enhanced disease associated with FI-RSV vaccines. This is supported by a study in which the increased lung pathology induced by challenge of FI-RSV-immunized mice was reduced by the administration of anti-IL-4 and anti-IL-10 antibodies (Connors *et al.*, 1994). Antibodies against IFN-γ and IL-2, however, had no effect. Furthermore, while IFN-γ receptor knockout mice display enhanced lung

pathology and increased IL-4, IL-5 and IL-13 expression upon infection with RSV, IL-4 knockout mice display no enhanced pathology or changes in Th1 cytokine expression (Boelen *et al.*, 2002). As well, IFN-nonresponsive STAT1 knockout mice show enhanced disease and preferential IL-4, IL-5 and IL-13 induction following challenge with RSV (Durbin *et al.*, 2002). While CD4+ T cells from the wild-type mice in this study produce primarily IFN-γ upon exposure to RSV antigen, CD4+ T cells from the STAT1 knockout mice produce IL-13.

In mice, it seems clear that RSV disease enhancement is associated with a Th2-type immune response. Human studies, however, feature contradictory findings, some of which may be due differences in experimental design. In a study in infants younger than three months, infection with RSV was associated with increased IL-4 levels in nasopharyngeal secretions (Kristjansson *et al.*, 2005). In contrast, higher levels of IFN-γ in nasopharyngeal secretions are associated with RSV-induced wheezing (van Schaik *et al.*, 1999). While further research is clearly necessary, it is expected that a successful vaccine against RSV would induce a balanced or more Th1-biased immune response. The F protein and/or Th1-promoting adjuvants are components worth considering for inclusion in such a vaccine.

1.7 HUMORAL IMMUNITY AGAINST RSV

Serum and mucosal IgM, IgG and IgA are induced during primary RSV infection in humans (Gimenez *et al.*, 1987; Ogra, 2004; Vainionpaa *et al.*, 1985; Ward *et al.*, 1983). IgM production is induced in the first five to ten days after primary infection and can be detected for up to three months. The IgG response, which consists mostly of the IgG1 and IgG3 subclasses, reaches it peak titer twenty to thirty days after symptoms have begun. IgG production begins earlier and is of a greater magnitude during secondary RSV infection, reaching its peak titer

between five and seven days post-infection. The IgA response begins between two and five days after the initiation of a primary infection with RSV and reaches its peak titer between eight and thirteen days post-infection. IgG and IgA responses are increased in children older than six months in age, suggesting that age is an important factor in production of antibodies against RSV (Meurman *et al.*, 1984; Welliver *et al.*, 1980). While antibodies are produced against most RSV proteins during infection, it is thought that antibodies against the F and G proteins are most important for protection (Murphy *et al.*, 1994).

IgE production can also be induced during infection with RSV, but it is typically associated with wheezing. In a study on the nasopharyngeal secretions of RSV infected children, IgE could be detected in only one out of nineteen patients without wheezing, but in the majority of sixty patients with wheezing (Welliver *et al.*, 1981). Furthermore, histamine, whose release is associated with the presence of IgE, was detected more often and in greater amounts in the patients with wheezing. IgE production and histamine release appear to be associated with severe RSV disease and it is likely that a safe RSV vaccine would avoid their induction.

It is believed by some that the enhanced disease observed upon subsequent infection of FI-RSV-immunized children was antibody-mediated. This view gained support following several studies in which it was suggested that antibody may increase viral replication (Gimenez *et al.*, 1996; Gimenez *et al.*, 1989; Ponnuraj *et al.*, 2001; Ponnuraj *et al.*, 2003). One hypothesis is that the presence of antibody against RSV could enhance disease by forming immune complexes and activating complement (Polack *et al.*, 2002). However, the role of antibodies in protection against RSV has been shown in several animal models, including cotton rats (Prince *et al.*, 1985) and primates (Hemming *et al.*, 1985), and was confirmed in humans by the observation that administration of RSV immune globulin decreases the incidence and severity of bronchiolitis in

high-risked children (Groothuis *et al.*, 1993). Furthermore, mice depleted of B-cells are more permissive to secondary infection with RSV (Graham *et al.*, 1991). It is expected, therefore, that a successful vaccine against RSV would induce the production of antibodies, especially in the mucosa.

1.8 MUCOSAL IMMUNIZATION AGAINST RSV

Two thousand years ago, the earliest attempts to protect against infections were made by administering foreign material to a mucosal surface (Mestecky & McGhee, 1989). Mucosal surfaces, which have an estimated surface area of 400 m^2 in humans (McGhee & Kiyono, 1992), are the entry and departure point for the vast majority of infectious agents. It is therefore easy to see the potential importance of mucosal immune responses in terms of preventing infection. In fact, almost 80% of all immunocytes in a healthy adult human reside in, or are in transit between the various mucosa-associated lymphoid tissues (MALT) (Holmgren & Czerkinsky, 2005). Delivery of vaccines to the mucosal surface typically results in the induction of both mucosal and systemic immune responses, whereas parenteral delivery typically results in systemic immunity with weak or no mucosal immune responses (Lamm, 1997; Levine, 2000). Parenteral immunization, therefore, typically induces immunity that resolves established infections, rather than immunity that prevents the initial interactions at the mucosal surface. And once a pathogen has maneuvered through the mucosal surface, the host-pathogen interaction has often moved in favor of the pathogen. Furthermore, mucosal epithelial cells express low levels of MHC class II molecules (Ryan *et al.*, 2001). These levels are increased during inflammation, allowing epithelial cells to present antigen to both $CD4^+$ and $CD8^+$ T cells. Mucosal immunization also offers several logistical advantages, such as ease of administration, the potential for self-

administration, removal of the possibility of reusing infected needles, and minimization of adverse effects due to systemic dispersion of potentially toxic vaccine components. Despite all of this, most clinical vaccine research has traditionally focused on parenterally delivered vaccines, partly because of the ease of analyzing humoural and cell-mediated immune responses in serum and blood leukocytes, respectively. Mucosal immune responses, on the other hand, are much more difficult to assess. Antibodies in mucosal secretions are comparatively difficult to quantitate, and isolation and analysis of mucosal T cells is technically difficult and labour intensive. Most importantly, the efficiency of mucosal immunization is low unless the vaccines are formulated with the proper adjuvants. The ultimate consequence is that only a few mucosal vaccines have been approved for human use, including a nasal vaccine against influenza virus (Belshe *et al.*, 1998) and oral vaccines against poliovirus (Modlin, 2004), rotavirus (Kapikian *et al.*, 1996), *Salmonella typhi* (Levine, 2000), and *Vibrio cholerae* (Levine, 2000). As many infectious diseases have been successfully controlled over the past 50 years via parenterally delivered vaccines, the development of mucosal vaccines may not necessarily be the only way to combat the pathogens that pose the greatest threats today. That being said, the average child receives 20-25 parenterally delivered vaccines by the time they are 18 months old (Ogra *et al.*, 2001). The development of mucosally delivered vaccines may provide a relatively painless way to replace some of these injections.

A critical hallmark of the adaptive mucosal immune response is the production and secretion of dimeric/multimeric IgA antibodies. Compared to other isotypes, antibodies of the IgA isotype are more resistant to degradation in protease-rich mucosal environments. IgA's protease resistance is due to its dimerization and heavy glycosylation (Neutra & Kozlowski, 2006), as well as its association with the secretory component, a protein fragment derived from

the epithelial polymeric immunoglobulin receptor (pIgR), which is responsible for the transportation of IgA through epithelial cells to the lumen (Kaetzel *et al.*, 1991). IgA essentially has three major functions: promoting the entrapment of potential pathogens in the mucus, preventing attachment to host epithelial cells via the blocking of microbial surface molecules (Hutchings *et al.*, 2004), and transporting microorganisms that have penetrated the epithelial wall back into the lumen via pIgR transport (Robinson *et al.*, 2001). In addition, as it binds only weakly to complement, IgA protects the mucosa from excessive immune-mediated damage (Meeusen *et al.*, 2004). Furthermore, the importance of IgA is illustrated by the observation that, in humans, more IgA is produced than all other antibody isotypes combined (van Egmond *et al.*, 2001). In fact, around 75% of all antibody producing cells in the body produce IgA (Dietrich *et al.*, 2003), in amounts that exceed 50 mg per kg of body weight per 24 h (Holmgren & Czerkinsky, 2005). The induction of mucosal IgA secretion is dependent on T helper cells (Hornquist *et al.*, 1995; Lycke *et al.*, 1987). In fact, T cells isolated from MALT have been demonstrated to be superior to T cells isolated from the blood in terms of their ability to help IgA production (Elson *et al.*, 1979; Kawanishi *et al.*, 1982). TGF-β in concert with IL-10 and IL-4 have been demonstrated to induce B-cell isotype switching to the IgA subclass in the presence of T cell help (Asano *et al.*, 2004; Goodrich & McGee, 1998), and IL-5, IL-6 and IL-10 are able to increase IgA production and proliferation of IgA committed B cells in the absence of T cells (Beagley *et al.*, 1988; Defrance *et al.*, 1992; Kunimoto *et al.*, 1989; Sonoda *et al.*, 1992). This is supported by experiments that demonstrated that IL-6 knockout mice mount poor IgA responses, but that this could be overcome by IL-6 gene therapy (Husband *et al.*, 1999; Ramsay *et al.*, 1994).

IgG antibodies can also be detected in mucosal secretions following mucosal immunization and contribute to mucosal immune defense (Eriksson *et al.*, 1998; Kozlowski *et al.*, 2002). It was previously assumed that serum IgG diffused through the epithelial wall by paracellular leakage (Neutra & Kozlowski, 2006), but it has recently been shown that epithelial cells express an IgG-specific Fc receptor that can mediate transport of IgG through the epithelial wall to the luminal surface (Yoshida *et al.*, 2004). CTLs in mucosal tissues can also play an important part in mucosal immunity, especially against viruses. This is demonstrated by the observation that mucosally vaccinated, but not parenterally vaccinated, mice are protected following mucosal infection with rVV expressing HIV gp160, and this protection can be abrogated by delivery of anti-CD8 antibodies (Belyakov *et al.*, 1998).

Previously, it was assumed, as some evidence suggested, that stimulation of the common mucosal immune system (CMIS) would induce roughly equal immune responses at all mucosal sites, regardless to which mucosal site the vaccine was administered (Bienenstock *et al.*, 1978; Czerkinsky *et al.*, 1987; McDermott & Bienenstock, 1979). More recent evidence, however, suggests that stimulation of the various mucosal sites results in the induction of unequal immunity at those sites (Wu & Russell, 1997). For example, while oral vaccination induces responses in the small intestine, ascending colon, and salivary and mammary glands, it induces poor responses in the large intestine, female genital tract, or tonsils (Eriksson *et al.*, 1998; Kozlowski *et al.*, 1997; Quiding *et al.*, 1991). Rectal vaccination, however, induces strong responses in the rectum, but poor responses in the proximal colon and small intestine (Eriksson *et al.*, 1998; Jertborn *et al.*, 2001; Kozlowski *et al.*, 1997). Furthermore, nasal vaccination induces responses in the upper airway and cervicovaginal mucosa, without inducing responses in the gut (Johansson *et al.*, 2004; Johansson *et al.*, 2001; Kozlowski *et al.*, 1997; Nardelli-

Haefliger *et al.*, 2003). Choice of immunization route, therefore, is of critical importance, and typically, the route through which a particular pathogen infects the body is the optimal route of immunization.

In the case of RSV, parenteral delivery of FI-RSV to infants resulted in enhanced disease upon subsequent natural infection (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kim *et al.*, 1969; Weibel *et al.*, 1966). The development of a mucosal RSV vaccine is one way in which enhanced disease might be avoided, and several studies have already been completed using intranasally delivered vaccines in rodents. A study with recombinant RSV F protein adjuvanted with CpG ODN resulted in an increase in virus neutralizing antibodies and a decrease in virus production in cotton rats (Prince *et al.*, 2003). In two studies in mice using recombinant F protein adjuvanted with cholera toxin, increases in mucosal IgA and protection from live challenge were observed (Tebbey *et al.*, 2000; Walsh, 1993). In another study using the F protein, this time adjuvanted with caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate, increases in serum IgG, IgG1, IgG2b, and IgA, as well as mucosal IgA were observed (Tebbey *et al.*, 1999). These antibodies were found to be RSV-neutralizing, and the mice were protected from a live viral challenge. Live viral (hYu *et al.*, 2008; Kahn *et al.*, 2001; Matsuoka *et al.*, 2002; Stott *et al.*, 1987)} and bacterial (Cano *et al.*, 2000; Falcone *et al.*, 2006) vectors expressing whole RSV proteins or peptides have also been explored.

In two more recent studies, a novel intranasal vaccine strategy based on chimeras of portions of the RSV F protein fused with the ctxA_2B gene of cholera toxin was evaluated (Singh *et al.*, 2007a; Singh *et al.*, 2007b). In these studies, they observed increases in systemic and mucosal antibodies against RSV, as well as enhanced protection from viral challenge. In two additional recent studies, one in BALB/c mice (Cyr *et al.*, 2007a) and another in C57Bl/6 mice

(Cyr *et al.*, 2007b), a subunit RSV antigen enriched for the F and G proteins and formulated with the proteosome-based adjuvant Protollin (meningococcal outer membrane proteins mixed with LPS from *Shigella flexner*) was examined. Here, they observed increases in systemic and mucosal RSV-specific antibodies and full protection from live viral challenge, with no induction of lung eosinophilic pulmonary pathology. Clearly, mucosally delivered vaccines against RSV have potential.

One of the major challenges of mucosal immunization is that potential vaccines face all of the same defenses as pathogens do, such as dilution/capture in mucus, mechanical clearance, or degradation by proteases. Large vaccine doses are therefore required, and it is impossible to quantitate the amount of a vaccine component that crosses the mucosa. As was the case in many of the above examples, adjuvants and/or delivery vehicles are required in order to achieve optimal immune responses for mucosally delivered vaccines.

1.9 POTENTIAL ADJUVANTS FOR RSV VACCINES

1.9.1 Toll-like receptors and CpG oligodeoxynucleotides

Vertebrates have two types of defense mechanisms to cope with infections: innate defenses and adaptive defenses. The adaptive defense system, which is mediated by B and T cells, is specific and provides a memory response to prevent or reduce repeated infections by the same pathogen. Adaptive defenses are generally slow to develop. The innate defense system, on the other hand, acts as a first line of defense, immediately detecting and categorizing pathogens as virus, bacteria, fungus or parasite upon the first signs of infection (Krieg, 2006). Cells of the innate defense system can accomplish this function because of the presence of pattern-recognition receptors, the best known being the TLRs.

TLRs recognize a variety of pathogen-associated molecular patterns (Akira & Takeda, 2004). TLR1 recognizes bacterial lipopeptides (Takeuchi *et al.*, 2002). TLR2 recognizes lipoprotein/lipopeptides (Aliprantis *et al.*, 1999), peptidoglycan (Takeuchi *et al.*, 1999), glycolipids (Opitz *et al.*, 2001), and atypical lipopolysaccharide (LPS) (Hirschfeld *et al.*, 2001; Werts *et al.*, 2001). TLR3 recognizes double-stranded RNA (Alexopoulou *et al.*, 2001). TLR4 recognizes LPS (Poltorak *et al.*, 1998). TLR5 recognizes bacterial flagellin (Hayashi *et al.*, 2001). TLR6 recognizes lipopeptides from mycoplasma (Takeuchi *et al.*, 2001). TLR7 and TLR8 recognize single-stranded RNA (Diebold *et al.*, 2004), and TLR9 recognizes CpG oligodeoxynucleotides (Hemmi *et al.*, 2000).

After they have been internalized into an endosomal compartment, CpG ODN bind TLR9, leading to the recruitment of MyD88, IL-1 receptor-associated kinase 1 (IRAK1), interferon regulatory factor-7 (IRF7), and TNF-α receptor activated factor-6 (TRAF6) (Ahmad-Nejad *et al.*, 2002; Hacker *et al.*, 2000; Muzio *et al.*, 1998; Muzio *et al.*, 1997; Rutz *et al.*, 2004; Schnare *et al.*, 2000). This leads to the activation of several mitogen-activated protein (MAP) kinases, including extracellular receptor kinase (ERK), p38, Jun-N terminal kinase, and IκB kinase (IKK), whose pathways ultimately result in increased activation of transcription factors such as NF-κB that alter the gene expression of various cyto/chemokines (Choudhury *et al.*, 2002; Hartmann & Krieg, 2000; Takeshita & Klinman, 2000; Tsujimura *et al.*, 2004; Yi & Krieg, 1998; Yi *et al.*, 2003; Yi *et al.*, 2002).

It has been reasonably well established that the failure of FI-RSV vaccines and other vaccines including recombinant vaccinia virus (rVV) encoding the RSV G protein (Openshaw *et al.*, 1992) in mice is due to the induction of a Th2-biased immune response (Connors *et al.*, 1994; Connors *et al.*, 1992; Waris *et al.*, 1997; Waris *et al.*, 1996). An adjuvant that induces Th1-

type or balanced immune responses would therefore be a candidate component of a successful vaccine formulation. CpG oligodexynucleotides (ODN) are short pieces of DNA that contain unmethylated CG dinucleotides flanked by two 5' purines and two 3' pyrimidines. CpG ODN binding of TLR9 induces production of IL-1, IL-6, IL-12, and tumor necrosis factor-α by dendritic cells and macrophages, as well as production of IFN-γ, IL-6, and IL-10 by natural killer cells (Ballas *et al.*, 1996; Hartmann & Krieg, 2000; Hartmann *et al.*, 2000). CpG ODN are often phosphorothioate-modified to increase their resistance to nucleases, thereby enhancing their *in vivo* activity. Vaccines adjuvanted with CpG ODN generally induce an overall Th1-type immune response characterized by production of IFN-γ and antigen-specific IgG2a in mice (Chu *et al.*, 1997; Davis *et al.*, 1998; Ioannou *et al.*, 2002a; Jakob *et al.*, 1998). Increased production of IgG2 and secretion of IFN-γ have also been reported in calves vaccinated with CpG ODN-formulated bovine herpesvirus-1 (BHV-1) glycoprotein D (Ioannou *et al.*, 2002b). CpG ODNs do not cause adverse vaccine site reactions (Ioannou *et al.*, 2003) and are therefore safe as vaccine adjuvants. CpG ODN also have potential in therapies against cancer (Krieg, 2004) and asthma (Racila & Kline, 2005), both being disease situations in which Th1-type/cell-mediated immune responses are usually desirable.

CpG ODN have also been used as a mucosal adjuvant in several vaccines delivered intranasally to mice. When hepatitis B surface antigen was formulated with CpG ODN it resulted in increases in humoral and cell-mediated systemic immune responses, as well as increases in IgA in the lungs and feces (McCluskie & Davis, 1998). Intranasal delivery of herpes simplex virus (HSV)-1 recombinant glycoprotein B formulated with CpG ODN resulted in increases in IgA in the genital tract, as well as protection from intravaginal challenge with HSV-2 (Gallichan *et al.*, 2001). Furthermore, an intranasally delivered vaccine consisting of glycoprotein 120-

depleted human immunodeficiency virus (HIV)-1 immunogen coformulated with CpG ODN induced increases in IgG and IgA in serum and vaginal washes, as well as protection from intravaginal challenge with rVV expressing the HIV-1 *gag* protein (Dumais *et al.*, 2002). CpG ODN clearly shows potential as a mucosal adjuvant in mice.

1.9.2 Polyphosphazenes

An alternate or complementary approach to avoid enhanced disease while maintaining or increasing protection against RSV infection is to change the route of immunization. Several intranasal immunization strategies have been employed to protect rodents from RSV challenge, including recombinant F protein adjuvanted with CpG ODN (Prince *et al.*, 2003), cholera toxin (Tebbey *et al.*, 2000; Walsh, 1993), or caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate (Tebbey *et al.*, 1999), as well as live viral (Kahn *et al.*, 2001; Matsuoka *et al.*, 2002; Stott *et al.*, 1987) or bacterial (Cano *et al.*, 2000; Falcone *et al.*, 2006) vectors expressing whole RSV proteins or peptides. One of the challenges of intranasal immunization is delivering the vaccine components in such a manner that they are not degraded or removed prior to the initiation of the immune response. Polyphosphazenes (PP) are synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms and organic side groups attached at each phosphorus atom (Payne & Andrianov, 1998). PP form non-covalent complexes when mixed with compounds of interest, i.e. antigens and other adjuvants, increasing their stability and allowing for multimeric presentation. Delivery of antigens formulated with PP has been successful in enhancing murine antibody responses to influenza (Mutwiri *et al.*, 2007; Payne *et al.*, 1995; Payne *et al.*, 1998), rotavirus (McNeal *et al.*, 1999) and cholera (Wu *et al.*,

2001). Protection was also enhanced in the latter two models. Recently, PP has also been co-formulated with CpG ODN as part of a vaccine against hepatitis B in mice (Mutwiri *et al.*, 2008).

1.10 CHOOSING A TARGET POPULATION FOR HRSV VACCINATION

RSV is a leading cause of respiratory disease in infants and young children worldwide (Heilman, 1990) and is responsible for significant economic loss; in 2000 alone, there were 86,000 RSV infection-related hospitalizations in the United States, costing a total of $394,000,000 USD (Paramore *et al.*, 2004). Nearly 98% of these hospitalizations occurred in children less than 5 years old. Between 1997 and 2000, RSV bronchiolitis was the leading cause of infant hospitalization, and in 1999, an estimated 360 RSV-associated postneonatal (28-365 days old) deaths occurred in the United States (Leader & Kohlhase, 2003). Despite these statistics, the vast majority of RSV infections pass in less than a week, with no need for hospitalization or other interventions (Openshaw & Tregoning, 2005). Only 1 to 2% of infants require hospitalization for RSV bronchiolitis, and of these, only 2 to 5% require mechanical ventilation (Leader & Kohlhase, 2002). In fact, mortality from RSV infection has been estimated as only 0.005 to 0.02% in affluent countries (Openshaw & Tregoning, 2005). Thus, the vast majority of children may simply not be predisposed to develop of severe RSV infections.

As it turns out, there are several risk factors associated with the development of severe RSV lower respiratory tract infections. They include male sex, age of less than 6 months, birth during the first half of the RSV season, multiple siblings living in the same house or sleeping in the same bedroom, day care exposure, and possibly tobacco smoke exposure (Simoes, 2003). Children with several of the above factors may be suitable candidates for vaccination, but another interesting risk factor is genetic predisposition. Single nucleotide polymorphisms in the

genes encoding TLR4 (Awomoyi *et al.*, 2007); surfactant protein B (in the German population) (Puthothu *et al.*, 2007); IL13 (Puthothu *et al.*, 2006); VDR, JUN, IFNA5, and NOS2 (Janssen *et al.*, 2007); and CD14 (in the Japanese population) (Inoue *et al.*, 2007); as well as polymorphisms in other genes have been associated with severe/symptomatic RSV infections.

More research is necessary, especially studies that simultaneously correlate traditional risk factors and multiple genetic risk factors with severe disease, but eventually, children that have multiple risk factors or certain risk factor patterns could be selected for vaccination, minimizing the risk of vaccine-associated complications in children that do not need the vaccine, while minimizing the risk of severe RSV infections in those that do.

1.11 HYPOTHESES AND OBJECTIVES

Our overall hypothesis was that formulation of an FI-BRSV vaccine with CpG ODN will shift the immune response from a Th2-biased response to a Th1-biased or more balanced response, thereby avoiding induction of host immunopathology. To evaluate this hypothesis we carried out three sets of studies.

The hypothesis for the first set was that addition of CpG ODN to a parenterally delivered FI-BRSV vaccine will result in an increase in cellular immunity and protection against BRSV in newborn calves. Thus, our objective here was to determine the effects of adding CpG ODN to an FI-BRSV vaccine in calves.

Our next hypothesis was that a combination of CpG ODN and PP would be the best adjuvant formulation for intranasal delivery of FI-BRSV, in terms of enhancing humoral, cell-mediated, and mucosal immunity, as well as protection, against BRSV in BALB/c mice. Thus, our objective here was to determine the best adjuvant formulation for intranasal delivery of FI-

BRSV, in terms of enhancing humoral, cell-mediated, and mucosal immunity, as well as protection, against BRSV in BALB/c mice.

Our final hypothesis was that intranasal immunization would be the best route of delivery for BRSV vaccines co-formulated with CpG ODN and PP, in terms of enhancing humoral, cell-mediated, and mucosal immunity, as well as protection, against BRSV in BALB/c mice. Thus, our objective here was to determine the optimal route of delivery for FI-BRSV and purified recombinant BRSV F protein vaccines co-formulated with CpG ODN and PP, in terms of enhancing humoral, cell-mediated, and mucosal immunity, as well as protection, against BRSV in BALB/c mice.

2.0 Formulation with CpG oligodeoxynucleotides increases cellular immunity and protection induced by vaccination of calves with formalin-inactivated bovine respiratory syncytial virus

Published in *Virology* (Mapletoft *et al.*, 2006).

2.1 ABSTRACT

Vaccination of calves with formalin-inactivated bovine respiratory syncytial virus (FI-BRSV) induces low levels of cellular immunity that may not be protective. Since inactivated and subunit vaccines formulated with CpG oligodeoxynucleotides (ODNs) have been shown to induce cellular immune responses, we studied the ability of a FI-BRSV vaccine formulated with CpG ODN to elicit cellular immunity against BRSV. Neonatal calves were immunized parenterally with FI-BRSV, FI-BRSV formulated with CpG ODN or medium and challenged with BRSV after two immunizations. Calves vaccinated with FI-BRSV formulated with CpG ODN developed increased numbers of IFN-γ secreting cells in the peripheral blood and broncho-tracheal lymph nodes and enhanced BRSV-specific serum IgG2 in comparison to FI-BRSV immunized animals. Calves that received the FI-BRSV vaccine formulated with CpG ODN also experienced a reduction in the amount of BRSV in the lung tissue. Based on these observations, CpG ODN appears to be a suitable candidate adjuvant for inactivated BRSV vaccines.

2.2 INTRODUCTION

Human respiratory syncytial virus (HRSV) is the leading cause of respiratory disease in infants and small children in the world (Heilman, 1990) and HRSV infection has been associated with the development of asthma later in life (Sigurs *et al.*, 2000). HRSV also is an important cause of respiratory disease in the immunocompromised and in the elderly, where its impact is approaching that of nonpandemic influenza (Collins & Murphy, 2002). Like HRSV, bovine respiratory syncytial virus (BRSV) is a single-stranded, negative-sense RNA *Pneumovirus* of the family *Paramyxoviridae* and order *Mononegavirales*. BRSV primarily infects neonatal calves and is one of the four known viral constituents of bovine "Shipping Fever", which causes significant economic loss to the cattle industry (Stott & Taylor, 1985). Clinical outcomes for these two viruses are similar in their respective host species and range from asymptomatic infection to bronchiolitis and pneumonia, and sometimes death (Philippou *et al.*, 2000).

In the 1960s, studies on formalin-inactivated (FI)-HRSV vaccines were carried out in children (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kim *et al.*, 1969; Weibel *et al.*, 1966). Not only did the vaccines fail to protect the children from natural infection, but in most cases, disease upon subsequent exposure was exacerbated (Graham *et al.*, 2002). Histological examination of the lungs of children that died led to the conclusion that increased numbers of lung eosinophils were responsible for the enhanced immunopathology (Chin *et al.*, 1969). A recent review of these autopsy materials, however, revealed that neutrophils, not eosinophils, might have been the predominant granulocyte in the lungs (Prince *et al.*, 2001a). Exacerbation of disease, characterized by increases in clinical signs of infection and BRSV-specific serum IgE (Kalina *et al.*, 2004), and decreases in IFN-γ production (Woolums *et al.*, 1999), have been demonstrated in

calves infected with BRSV following vaccination with FI-BRSV. It is thus hypothesized that BRSV-specific cellular immune responses, characterized by interferon-γ (IFN-γ) production and BRSV-specific IgG2, could be protective against subsequent infections.

While there is evidence that DNA vaccines can induce protective immune responses (Hassett *et al.*, 1997; Van Drunen Littel-van den Hurk *et al.*, 1999; Zhang *et al.*, 2002), neonates generally develop weak systemic immune responses that can be at least partially attributed to a deficiency in cellular immunity (Marodi, 2006). This is exemplified by the impaired ability of their T cells to secrete IL-2 and IFN-γ (Melvin *et al.*, 1995), and the hypo-responsiveness of their macrophages to activation by IFN-γ (Marodi *et al.*, 1994; Marodi *et al.*, 2000). Thus, to achieve protection in neonates, it is necessary to develop vaccine formulations that promote strong, cellular immune responses. CpG oligodeoxynucletides (ODNs) are short DNA sequences consisting of unmethylated CG dinucleotides flanked by two 5' purines and two 3' pyrimidines. CpG ODNs have been shown to possess immunomodulatory activity (Krieg *et al.*, 1995), but they require phosphorothioate modification to increase nuclease resistance and activity (Klinman *et al.*, 1996; Samani *et al.*, 2001). CpG ODNs bind Toll-like receptors, thereby stimulating the secretion of cytokines by various cells of the immune system: IL-1, IL-6, IL-12 and tumor necrosis factor-α by macrophages and dendritic cells, as well as IL-6, IL-10 and IFN-γ by lymphocytes and natural killer cells (Ballas *et al.*, 1996; Hartmann & Krieg, 2000; Hartmann *et al.*, 2000). Overall, CpG ODNs induce strong Th1-type immune responses, characterized by high IFN-γ secretion and an increase in antigen-specific IgG2a production in mice (Chu *et al.*, 1997; Davis *et al.*, 1998; Ioannou *et al.*, 2002a; Jakob *et al.*, 1998). Increased production of IgG2 and secretion of IFN-γ have also been reported in nine-month-old calves vaccinated with CpG ODN-

formulated bovine herpesvirus-1 (BHV-1) glycoprotein D (Ioannou *et al.*, 2002b). CpG ODNs do not cause adverse reactions (Ioannou *et al.*, 2003) and are therefore safe as vaccine adjuvants.

Formulation of inactivated BRSV vaccines with CpG ODN induces BRSV-specific cell-mediated immune responses in mice that are protective against BRSV infection (Oumouna *et al.*, 2005). In the present study, we investigated the ability of a FI-BRSV vaccine formulated with CpG ODN to increase cell-mediated immunity against BRSV in neonatal calves.

2.3 MATERIALS AND METHODS

2.3.1 Cells and virus - The Iowa strain of BRSV was propagated in Georgia Bovine Kidney (GBK) cells, maintained in modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 1% heat-inactivated fetal bovine serum (FBS; SeraCare Life Sciences, Inc., Oceanside, CA), 0.1 mM non-essential amino acids (Gibco-BRL), 10 mM HEPES buffer (Gibco-BRL) and 50 μg/ml gentamicin (Gibco-BRL). Infected cells were incubated at 37 ^{o}C in modified atmosphere with 5% CO_2. Four to seven days after infection, infected cells were collected by scraping and either frozen at -70 ^{o}C until vaccine preparation or challenge, or used at a dilution of 1:10 to infect more cells for up to five infection cycles. Virus titers were determined by a micro-isolation plaque assay on GBK cells. Briefly, ten-fold serial dilutions in modified Eagle medium with 1% FBS were added to 70-80% confluent GBK monolayers in 96-well tissue culture plates (Corning Incorporated). Cells were incubated for 7 days at 37 ^{o}C in modified atmosphere with 5% CO_2, and BRSV plaques were visualized by immunostaining. Cells were fixed with 80% acetone in 0.1M PBS (38 mM sodium phosphate monobasic, 61 mM sodium phosphate dibasic heptahydrate, 0.15 M sodium chloride, pH 7.2). Polyclonal goat anti-

BRSV IgG (VMRD, Inc.) at a dilution of 1:5,000, followed by biotinylated rabbit anti-goat IgG (Vector Laboratories, Inc.) at a dilution of 1:750 were used to detect BRSV plaques. Vectastain Avidin-Biotin Complex horseradish peroxidase (Vector Laboratories, Inc.), followed by diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories, Inc.) were used to visualize BRSV plaques. Viral titers were expressed as PFU/ml.

2.3.2 Vaccination and challenge - FI-BRSV was prepared as previously described (Kim *et al.*, 1969). Three to four week-old Holstein calves were randomly allocated to three groups of six animals each and vaccinated twice intramuscularly at a 3-week interval with a total volume of 2 ml of MEM containing either FI-BRSV formulated with EMULSIGEN® (MVP Laboratories Inc., Ralston, NB) (FI-BRSV); FI-BRSV formulated with EMULSIGEN® and CpG ODN (FI-BRSV/CpG); or MEM without FI-BRSV (medium). EMULSIGEN® was given at 30% (vol/vol) and CpG ODN 2007 was given at 1 mg per vaccine dose. CpG ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT) is phosphorothioate modified and was synthesized by Qiagen GmbH (Hilden Germany) and diluted in endotoxin-free water (Gibco-BRL). EMULSIGEN®, an oil-in-water adjuvant, was chosen because of its efficacy as a coadjuvant with CpG ODN (Ioannou *et al.*, 2002a). One calf was removed from each of the FI-BRSV and FI-BRSV/CpG groups because of losses of ability to feed properly due to a twisted bowel and damaged leg muscles, respectively. Two weeks after the second vaccination, all groups were challenged as previously described (Tjornehoj *et al.*, 2003). Briefly, calves were given 45 ml of the Iowa strain of BRSV at 10^5 PFU/ml; 15 ml via a nebulizer and breathing mask over a 10 min interval and, following intravenous sedation with Xylazine at 0.2 mg/kg (ROMPUN®, Bayer AG, Germany), an additional 30 ml of virus was administered intratracheally via an endotracheal

tube. Sedation was reversed by intravenous injection of tolazoline hydrochloride at 1.5 mg/kg (TOLAZINE®, Lloyd Inc. of Iowa, Shenandoah, IA). Calves were euthanized one week after challenge, and lungs were removed for macroscopic analysis by a vaccine group-blinded veterinarian and lungs were sampled for virus isolation. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council for Animal Care.

2.3.3 Enzyme-linked immunosorbent assay (ELISA) - Serum was collected prior to vaccination, two weeks after the second vaccination, and one week after challenge, and BRSV-specific IgG1 and IgG2 titers were determined. Ninety-six-well polystyrene IMMULON® 2 microtiter plates (Thermo Electron, OY, Finland) were coated overnight at 37 ^{o}C with BRSV antigen composed of Nonidet-P40 (Sigma-Aldrich, St. Louis, MO)-treated GBK cells previously infected with the Iowa strain of BRSV as described above. Mock-infected GBK cells were used as negative control antigen. Plates were incubated overnight at 4 ^{o}C with serially diluted bovine sera, beginning at 1:10 and continuing in 4-fold dilutions. Bovine IgG1-specific ascites M-23 at a dilution of 1:40,000 and bovine IgG2-specific ascites M-37 at a dilution of 1:8,000, followed by alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a dilution of 1:10,000, were used to detect bound IgG1 and IgG2, respectively. The reaction was visualized with *p*-nitrophenyl phosphate (Sigma-Aldrich). Results were expressed as the reciprocal of the highest dilution resulting in a reading equal to that of undiluted serum tested on negative control antigen.

2.3.4 IFN-γ enzyme-linked immunospot (ELISPOT) assay - Blood with anti-coagulant (EDTA to a final concentration of 0.2%) was collected one week after the second vaccination

and one week after challenge, and peripheral blood mononuclear cells (PBMCs) were isolated by layering on FICOLL-PAQUE® Plus (Amersham Pharmacia Biotech, AB, Sweden) as previously described (Loehr *et al.*, 2000). Broncho-tracheal lymph node cells were isolated one week after challenge as follows. Pieces of lymph node tissue approximately 1 cm^3 in size were excised and placed on ice in wash medium (MEM supplemented with 10 mM HEPES buffer and antibiotic-antimycotic (Gibco-BRL)). Subsequently, the tissue pieces were placed in sterile petri dishes with wash medium, minced with a sterile scalpel, pipetted through a 100 micron filter to isolate single cells, and washed twice in wash medium. Ninety-six-well Multiscreen-HA ELISPOT plates (Millipore, Bedford, MA) were coated overnight at 4 ^oC with bovine IFN-γ-specific monoclonal antibodies (clone 2.2.1) (Raggo *et al.*, 2000) at a dilution of 1:1,500. Unbound antibody was removed by washing with phosphate buffered saline (PBS) with 0.05% (vol/vol) Tween 20 (PBST), with a final wash with PBS. Wells were blocked with MEM for 1 to 2 h at 37 ^oC. PBMCs and lymph node cells were resuspended at 10^6 cells per well in MEM supplemented with 10% heat-inactivated FBS, 0.1 mM non-essential amino acids, 10 mM HEPES buffer, 50 μg/ml gentamicin, 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 1 ng/ml dexamethasone (Sigma-Aldrich), and cultured in triplicate wells in the presence of BRSV-infected or mock-infected cell lysate. Lysates were used at a final protein concentration of 25 μg/ml. Dexamethasone was added to the culture medium to reduce the amount of non-specific cytokine secretion (Raggo *et al.*, 2000). After 20 h of incubation at 37 ^oC in modified atmosphere with 5% CO_2, plates were washed with ddH_2O and PBST and then incubated with rabbit anti-bovine IFN-γ (lot 92-131) (Raggo *et al.*, 2000) diluted 1:1,500 for 1 to 2 h at RT, followed by AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) diluted 1:1,500 for 1 to 2 h at RT. Bound antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitroblue substrate

tablets (Sigma-Aldrich). Plates were washed with ddH_2O and air dried. Spots were counted in a blinded manner with the aid of an inverted microscope. Results are expressed as the difference between the number of IFN-γ secreting cells per 10^6 cells in BRSV-infected lysate-stimulated wells and the number of IFN-γ secreting cells per 10^6 cells in mock-infected lysate-stimulated wells. Concanavalin A (Sigma-Aldrich) at 5 μg/ml was included in positive control wells and resulted in median values of 96 and 79 IFN-γ secreting cells per 10^6 cells in lymph node cells and PBMCs, respectively.

2.3.5 Virus isolation from lung tissue - Production of BRSV in the lungs was assessed after challenge. Portions of lung tissue with approximate dimensions of 0.5 x 0.5 x 1.0 cm were excised from the same area of the lungs of each of the calves and homogenized four times for 45 sec in a tube containing 1.0 mm glass microbeads (Biospec Products, Inc., Bartlesville, OK) and MEM. Ten μg/ml each of aprotinin (Sigma-Aldrich) and leupeptin (Sigma-Aldrich), as well as 0.1 mM EDTA and 1 mM PMSF (Sigma-Aldrich), were added to the medium to prevent enzymatic degradation of the virus. Between homogenizations, tubes containing the lung tissue were kept cold in an ethanol bath chilled by dry ice. Lung homogenates were clarified by centrifugation (10,000 x *g* for 1 min), and virus titers were determined by a micro-isolation plaque assay as described above.

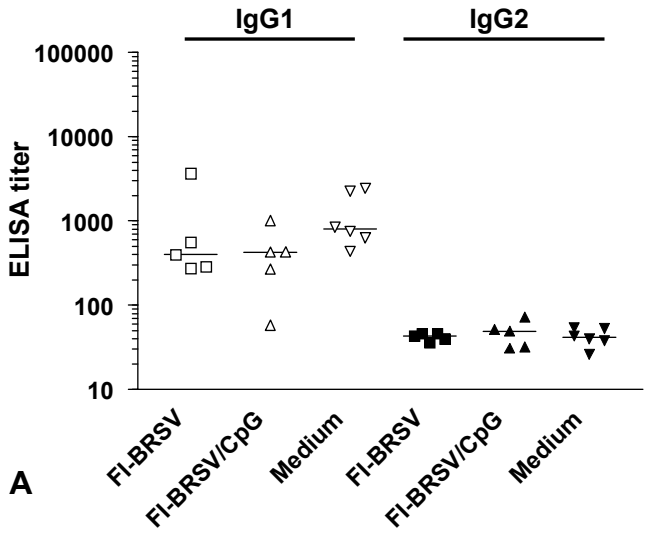
2.3.6 Statistical analysis - All data were analyzed using statistical software (GraphPad Prism Version 3.00). As sample sizes were small (n = 6), outcome variables were assumed not to be normally distributed. Therefore, differences among all groups were examined using the Kruskal-Wallis test. If a significant difference was found among the groups, median ranks

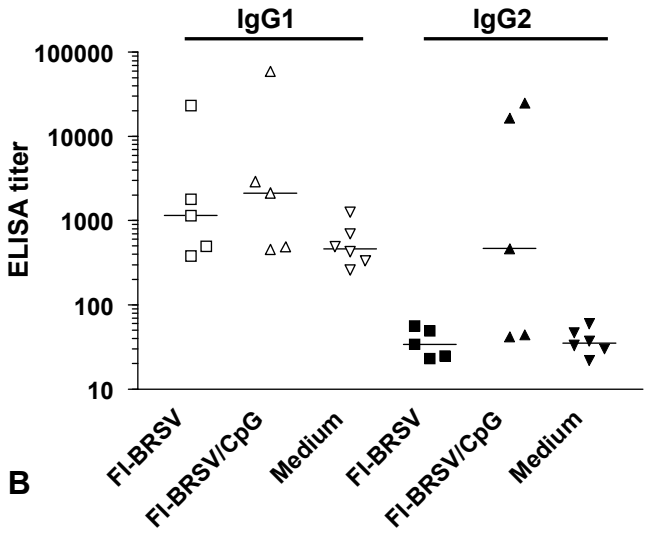
between pairs of groups were compared using the Mann-Whitney U test. Differences were considered significant if $P<0.05$.

2.4 RESULTS

2.4.1 BRSV-specific humoral immune responses - The ability of CpG ODN to modulate the antibody response to FI-BRSV was investigated by measuring BRSV-specific IgG1 and IgG2 titers in serum. Calves were vaccinated with either FI-BRSV formulated with EMULSIGEN® (FI-BRSV), FI-BRSV formulated with EMULSIGEN® and CpG ODN (FI-BRSV/CpG), or culture medium. Prior to vaccination, there were no significant differences among groups in either IgG1 or IgG2 BRSV-specific titers (Fig. 2.1A). As maternal antibodies against BRSV have been determined to be mainly of the IgG1 isotype (Uttenthal *et al.*, 2000), the presence of IgG1 in animals prior to vaccination can be attributed to maternal antibodies passed to the calves during colostrum feeding. Despite increases in IgG1 titers in individual animals vaccinated with FI-BRSV or FI-BRSV/CpG, and decreases in IgG1 titers in individual animals that received the mock vaccination, there were no significant differences among groups after two vaccinations (Fig. 2.1B) or after challenge (Fig. 2.1C). However, the group vaccinated with FI-BRSV/CpG was the only one to have a significant increase in IgG1 following two vaccinations ($P=0.03$). Two weeks after the second immunization, three out of the five animals in the group immunized with FI-BRSV/CpG developed increased IgG2 levels, while none of the animals in the other groups had increased IgG2 titers (Fig. 2.1B). After challenge, the calves immunized with FI-BRSV/CpG continued to produce serum IgG2 and at that time the difference

in IgG2 production between the FI-BRSV and FI-BRSV/CpG vaccinated groups was significant (P=0.008) (Fig. 2.1C).


IgG1
IgG2
100000
10000
1000
100
10
ELISA titer
FI-BRSV
FI-BRSV/CpG
Medium
FI-BRSV
FI-BRSV/CpG
Medium
A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IgG2
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10000
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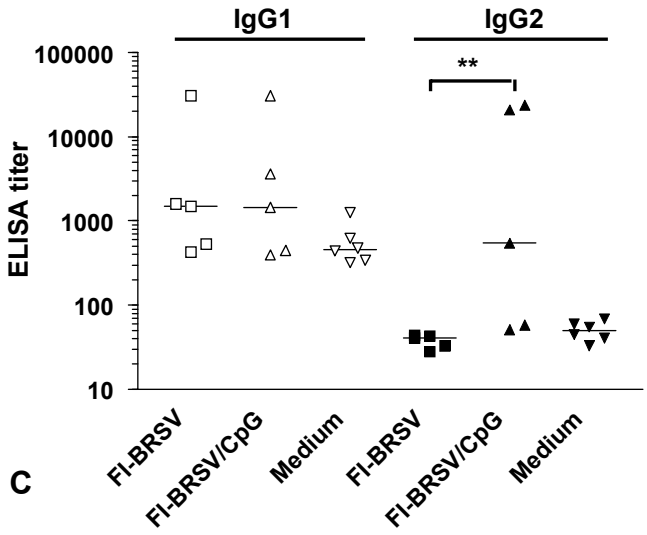
100
10
ELISA titer
FI-BRSV
FI-BRSV/CpG
Medium
FI-BRSV
FI-BRSV/CpG
Medium
B


IgG1
IgG2
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100000
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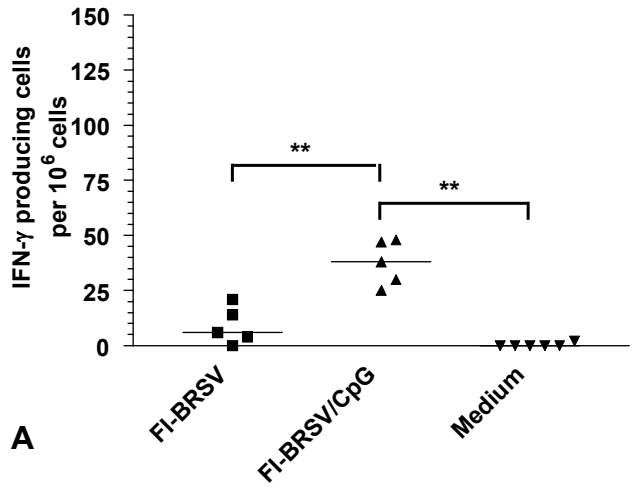
ELISA titer
FI-BRSV
FI-BRSV/CpG
Medium
FI-BRSV
FI-BRSV/CpG
Medium
C

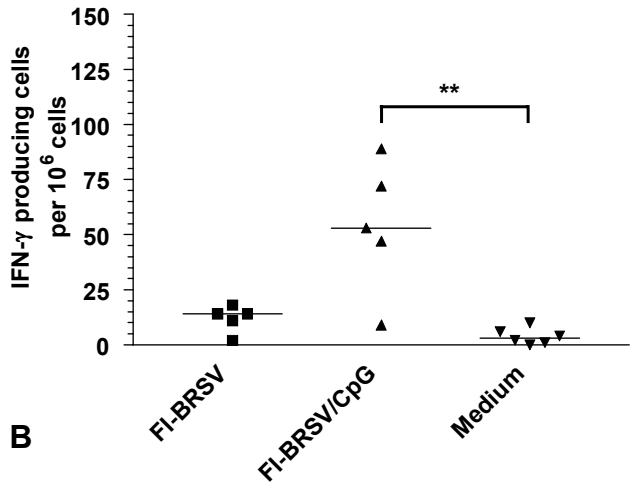
Fig. 2.1

Figure 2.1. BRSV-specific IgG1 and IgG2 in bovine sera prior to vaccination (A), two weeks after the second vaccination (B), and one week after challenge (C). Calves were vaccinated twice at a 3-week interval with either FI-BRSV; FI-BRSV/CpG; or medium. All animals were challenged with BRSV two weeks after the second vaccination and euthanized one week later. Results were expressed as the reciprocal of the highest dilution resulting in a reading equal to that of undiluted serum tested on negative control antigen. Each data point represents an individual animal, and median values are indicated by horizontal bars. **, $P<0.01$.

2.4.2 BRSV-specific cell-mediated immune responses - To further evaluate the effects of formulation with CpG ODNs on the type of immune responses induced, we measured the BRSV-induced secretion of IFN-γ by PBMCs one week after the second vaccination and one week after challenge. No IFN-γ secreting PBMCs were induced by mock vaccination and low numbers of IFN-γ secreting PBMCs were observed after two vaccinations with FI-BRSV. In contrast, significantly higher numbers of IFN-γ secreting PBMCs were detected in the group immunized with FI-BRSV/CpG than in the groups immunized with FI-BRSV (P=0.008) or medium (P=0.004) (Fig. 2.2A). One week after BRSV challenge, FI-BRSV and mock vaccinated calves still had low numbers of IFN-γ secreting PBMCs, whereas FI-BRSV/CpG vaccinated animals continued to have high numbers of IFN-γ secreting cells (Fig. 2.2B), significantly higher than those in the mock-vaccinated group (P=0.009).

To evaluate the local immune responses, the numbers of IFN-γ secreting cells in the broncho-tracheal lymph nodes were determined. The calves immunized with FI-BRSV or medium developed low numbers of IFN-γ secreting lymph node cells, whereas all FI-BRSV/CpG vaccinated animals had high numbers of IFN-γ secreting lymph node cells (Fig. 2.2C). The number of IFN-γ secreting lymph node cells induced in the FI-BRSV/CpG vaccinated group was significantly higher than that in the groups vaccinated with FI-BRSV (P=0.008) or medium (P=0.004). The increase in BRSV-induced secretion of IFN-γ by PBMCs and lymph node cells of the group vaccinated with FI-BRSV/CpG suggests that this group developed a stronger cell-mediated immune response against BRSV.


IFN-γ producing cells per 10^6 cells
150
125
100
75
50
25
0
**
**
FI-BRSV
FI-BRSV/CpG
Medium
A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125
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FI-BRSV
FI-BRSV/CpG
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B

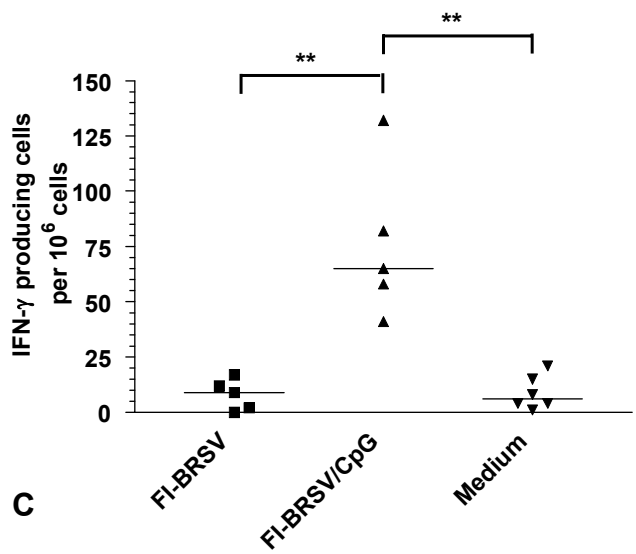

IFN-γ producing cells per 10^6 cells
150
125
100
75
50
25
0
**
**
FI-BRSV
FI-BRSV/CpG
Medium
C

Fig. 2.2

Figure 2.2. Numbers of IFN-γ secreting peripheral blood mononuclear cells (A and B) and broncho-tracheal lymph node cells (C) in response to *in vitro* restimulation with BRSV-positive and -negative cell lysates. Calves were vaccinated twice at a 3-week interval with either FI-BRSV; FI-BRSV/CpG; or medium. All animals were challenged with BRSV two weeks after the second vaccination and euthanized one week later. Peripheral blood mononuclear cells were isolated one week after the second vaccination (A), and one week after challenge (B). Broncho-tracheal lymph node cells were isolated one week after challenge (C). Results are the difference between the number of IFN-γ secreting cells per 10^6 cells in BRSV-stimulated wells and the number of IFN-γ secreting cells per 10^6 cells in non-stimulated wells. Each data point represents an individual animal, and median values are indicated by horizontal bars. **, $P<0.01$.

2.4.3 Gross lung pathology - Based on the observation that formulation with CpG ODN resulted in increased cell-mediated immune responses, we investigated the ability of the CpG ODN-formulated vaccine to reduce gross lung pathology. One week after challenge, animals were euthanized and the percentage of lung surface with visible lesions was determined (Fig. 2.3). Statistically, there were no differences between groups (P=0.06), but the five animals in the group vaccinated with FI-BRSV/CpG had 11% of the lung or less covered in visible lesions (median of 8% and range of 2-11%). In contrast, the group vaccinated with FI-BRSV had a median of 22% (range of 4-38%) with four out of the five animals at 18% or higher, and the mock vaccinated group had a median of 18% (range of 4-45%) with five out of six animals at 11% or higher.

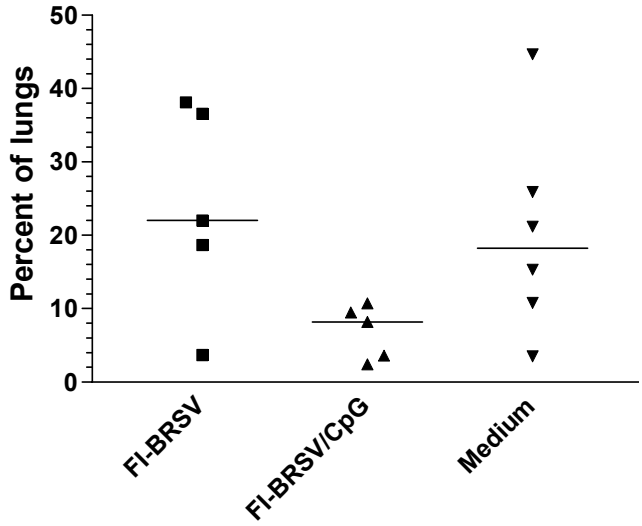


Fig. 2.3

Figure 2.3. Gross lung pathology following two vaccinations and challenge with BRSV. Calves were vaccinated twice at a 3-week interval with either FI-BRSV; FI-BRSV/CpG; or medium. All animals were challenged with BRSV two weeks after the second vaccination and euthanized one week later. Results are presented as the percent of the lung with visible lesions as scored by a vaccine group-blinded veterinarian. Each data point represents an individual animal, and median values are indicated by horizontal bars.

2.4.4 Virus isolation from lung tissue - To further assess the ability of CpG ODNs to enhance protection from infection, the level of virus replication in the lungs was determined. After the animals were euthanized, lung tissue was excised and processed for BRSV isolation. The median titers of virus isolated from the groups vaccinated with FI-BRSV, FI-BRSV/CpG, or medium were 1817, 383 and 1500 PFU/ml, respectively. The amount of virus isolated from the group immunized with FI-BRSV and from the group that received the mock vaccination ranged from 667 to 3500 and 417 to 6950 PFU/ml, respectively. In contrast, the amount of virus isolated from the group vaccinated with FI-BRSV/CpG ranged from 235 to 500 PFU/ml. Indeed, the group vaccinated with FI-BRSV/CpG had a significantly lower virus load in the lung than the groups vaccinated with FI-BRSV (P=0.008) or medium (P=0.03) (Fig. 2.4). The reduction in the amount of virus isolated from the group immunized with FI-BRSV/CpG demonstrates that this group experienced a reduction in viral replication in the lungs.

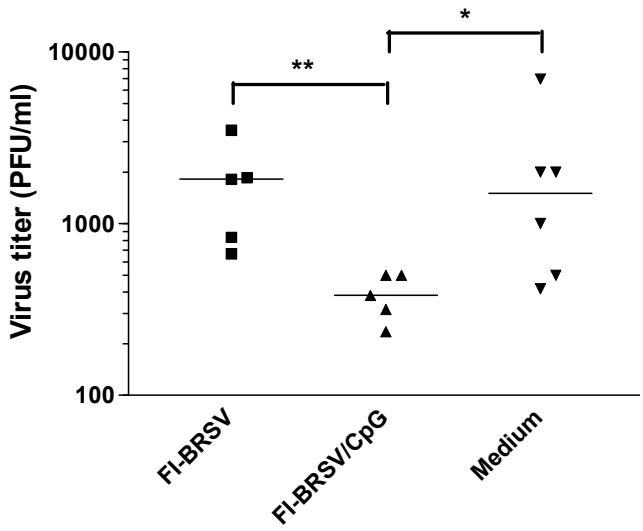


Fig. 2.4

Figure 2.4. Virus isolation from lung tissue following two vaccinations and challenge with BRSV. Calves were vaccinated twice at a 3-week interval with FI-BRSV; FI-BRSV/CpG; or medium. All animals were challenged with BRSV two weeks after the second vaccination and euthanized one week later. Viral titers are expressed as PFU/ml. Each data point represents an individual animal, and median values are indicated by horizontal bars. *, $P<0.05$; **, $P<0.01$.

2.5 DISCUSSION

Newborns are not only prone to infections, they also develop weak immune responses to most conventional vaccines, and they specifically appear to have difficulty mounting a cellular immune response, due to the impaired ability of their T cells to secrete IL-2 and IFN-γ (Melvin *et al.*, 1995). This impairment is compounded by the fact the neonatal macrophages are hypo-responsive to activation by IFN-γ (Marodi *et al.*, 1994; Marodi *et al.*, 2000). Another problem with vaccination of neonates is the possible interference of existing maternal antibodies. While there is evidence that DNA vaccines can elicit protective immune responses in neonates (Hassett *et al.*, 1997; Van Drunen Littel-van den Hurk *et al.*, 1999; Zhang *et al.*, 2002), conventional vaccines are often unable to induce protection in the presence of maternal antibodies (Crowe, 2001; Siegrist, 1997; 2003). Thus, the challenge for any vaccine is to overcome the immaturity of the infant's immune system and the interference of maternal antibodies. Since CpG ODNs are strong Th1-promoting adjuvants, (Chu *et al.*, 1997; Davis *et al.*, 1998; Ioannou *et al.*, 2002a; Jakob *et al.*, 1998; Oumouna *et al.*, 2005), we hypothesized that, in newborn calves, formulation of an inactivated BRSV vaccine with CpG ODN would enhance the BRSV-specific cellular immune response in neonatal calves.

In cattle, production of antigen-specific IgG2 accompanies the induction of cellular immune responses (Estes & Brown, 2002). Formulation of a BHV-1 subunit vaccine with CpG ODNs has been shown to increase BHV-1-specific IgG2 (Ioannou *et al.*, 2002b). In this study, we measured BRSV-specific IgG1 and IgG2 titers in serum prior to vaccination, after two vaccinations, and after challenge. BRSV-specific IgG1 levels were high prior to vaccination presumably due to the presence of maternal antibodies passed to the calves during colostrum

feeding. Maternal antibodies against BRSV have been determined to be mainly of the IgG1 isotype (Uttenthal *et al.*, 2000) and the presence of maternal antibodies can lead to the suppression of antibody production (Kimman *et al.*, 1989), which may account for the lack of a significant increase in IgG1 in the FI-BRSV vaccinated group. In contrast, the group vaccinated with FI-BRSV/CpG experienced an increase in IgG1 (~5-fold), but this was less significant than the increase in IgG2 (~10-fold). This agrees with a previous report for mice (Oumouna *et al.*, 2005), which developed more IgG2a when CpG ODN was added to the vaccine. The presence of both BRSV-specific IgG2 and IgG1 in sera from the group vaccinated with FI-BRSV/CpG suggests a more balanced immune response.

Antigen-induced IFN-γ secretion is an established sign of a cellular immune response in cattle (Estes & Brown, 2002). According to a previous report, IFN-γ production was relatively low in calves vaccinated with FI-BRSV (Woolums *et al.*, 1999). Formulation of a BHV-1 subunit vaccine with CpG ODNs increased antigen-stimulated IFN-γ secretion in cattle (Ioannou *et al.*, 2002b). Similarly, inactivated BRSV vaccines formulated with CpG ODNs induced enhanced antigen-stimulated IFN-γ secretion in mice (Oumouna *et al.*, 2005). In the present study, the FI-BRSV vaccinated group developed low numbers of IFN-γ secreting cells, whereas the frequency of IFN-γ secreting cells was significantly enhanced when CpG ODN was added to the vaccine, again indicating that formulation of FI-BRSV with CpG ODN results in an increase in cell-mediated immunity against BRSV. Indeed, the effect on IFN-γ production may have been one of the most important determinants for the enhanced protection observed with the CpG ODN-formulated vaccine.

Although results were not significant, the gross lung pathology tended to be reduced when CpG ODNs were added to the vaccine. Furthermore, viral replication was significantly

decreased in neonatal calves when CpG ODNs were added to an inactivated BRSV vaccine. This agrees with previously reported studies, where formulation of HRSV F protein and FI-BRSV vaccines with CpG ODNs led to a reduction in virus replication in the lungs of mice (Hancock *et al.*, 2001; Oumouna *et al.*, 2005).

In summary, we demonstrated that in newborn calves with maternal antibodies, FI-BRSV formulated with CpG ODN induces a cellular immune response against BRSV, characterized by production of IFN-γ following *in vitro* restimulation of PBMCs and broncho-tracheal lymph node cells, as well as production of BRSV-specific serum IgG2. In addition, calves that received the vaccine formulated with CpG ODN experienced a reduction in the amount of BRSV isolated from lung tissue. The fact that not all calves responded equally to the vaccine may be due to the outbred nature of the animals as well as the presence of varying levels of maternal antibodies. Based on these observations, we conclude that CpG ODN appears to be a suitable candidate adjuvant for inactivated BRSV vaccines. Since HRSV and BRSV cause very similar clinical disease, this study also provides a vaccination strategy that might be transferable to humans.

3.0 Intranasal immunization of mice with a formalin-inactivated bovine respiratory syncytial virus vaccine co-formulated with CpG oligodeoxynucleotides and polyphosphazenes results in enhanced protection

Published in the *Journal of General Virology* (Mapletoft *et al.*, 2008).

3.1 ABSTRACT

Since respiratory syncytial virus (RSV) targets the mucosal surfaces of the respiratory tract, induction of both systemic and mucosal immunity will be critical for optimal protection. In this study we evaluated the ability of an intranasally delivered formalin-inactivated bovine-RSV (FI-BRSV) vaccine co-formulated with CpG oligodeoxynucleotides (ODN) and polyphosphazenes (PP) to induce systemic and mucosal immunity, as well as protection from BRSV challenge. Intranasal immunization of mice with FI-BRSV formulated with CpG ODN and PP resulted in both humoral and cell-mediated immunity, characterized by enhanced production of BRSV-specific serum IgG, as well as increased IFN-γ and decreased IL-5 production by *in vitro* re-stimulated splenocytes. These mice also developed mucosal immune responses, as evident from increased production of BRSV-specific IgG and IgA in lung fragment cultures. Indeed, the increases in serum and mucosal IgG, and in particular mucosal IgA and virus neutralizing antibodies, were the most critical differences observed between FI-BRSV formulated with both CpG ODN and PP in comparison to formulations with CpG ODN, non-

CpG ODN or PP individually. Finally, FI-BRSV/CpG/PP was the only formulation that resulted in a significant reduction in viral replication following BRSV challenge. Co-formulation of CpG ODN and PP is a promising new vaccine platform technology that may have application in mucosal immunization of humans.

3.2 INTRODUCTION

Human respiratory syncytial virus (HRSV) is a leading cause of respiratory disease in infants and young children worldwide (Heilman, 1990) and is responsible for significant economic loss; in 2000 alone, there were 86,000 HRSV infection-related hospitalizations in the United States, costing a total of $394,000,000 USD (Paramore *et al.*, 2004). Nearly 98% of these hospitalizations occurred in children less than 5 years old. Between 1997 and 2000, HRSV bronchiolitis was the leading cause of infant hospitalization, and in 1999, an estimated 360 HRSV-associated postneonatal (28-365 days old) deaths occurred in the United States (Leader & Kohlhase, 2003). Like HRSV, bovine respiratory syncytial virus (BRSV) is an enveloped, non-segmented, single-stranded RNA *Pneumovirus* of the family *Paramyxoviridae* and order *Mononegavirales*. BRSV is responsible for significant economic loss to the cattle industry (Stott & Taylor, 1985), and is one of the four known viral components of bovine shipping fever. HRSV and BRSV have similar clinical outcomes in their respective host species, ranging from asymptomatic infection, to bronchiolitis and pneumonia, and sometimes death (Philippou *et al.*, 2000). The distribution of both viruses is worldwide.

While there are several commercial BRSV vaccines currently available for immunizing cattle, better vaccines that are more efficacious in the face of maternal antibodies and induce longer lasting protection would be desirable. Also, there is currently no safe and effective vaccine against HRSV available for use in humans. Several studies using parenterally-delivered formalin-inactivated (FI)-HRSV vaccines were carried out in children in the 1960s (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kim *et al.*, 1969; Weibel *et al.*, 1966). Not only did FI-HRSV

vaccines fail to protect upon natural infection, but in most cases, disease was enhanced. In one study, 80% of vaccinated children were hospitalized and two of them died (Kim *et al.*, 1969).

It has been reasonably well established that the failure of FI-RSV vaccines and other vaccines including recombinant vaccinia virus (rVV) encoding the RSV G protein (Openshaw *et al.*, 1992) in mice is due to a Th2-biased immune response (Connors *et al.*, 1994; Connors *et al.*, 1992; Waris *et al.*, 1997; Waris *et al.*, 1996). An adjuvant that induces Th1-type or balanced immune responses would therefore be a candidate component of a successful vaccine formulation. CpG oligodexynucleotides (ODN) are short pieces of DNA that contain unmethylated CG dinucleotides flanked by two 5' purines and two 3' pyrimidines. CpG ODN bind toll-like receptor 9 (TLR9), inducing production of IL-1, IL-6, IL-12, and tumor necrosis factor-α by dendritic cells and macrophages, as well as production of IFN-γ, IL-6, and IL-10 by natural killer cells (Ballas *et al.*, 1996; Hartmann & Krieg, 2000; Hartmann *et al.*, 2000). CpG ODN generally induce an overall Th1-type immune response characterized by production of IFN-γ and antigen-specific IgG2a in mice (Chu *et al.*, 1997; Davis *et al.*, 1998; Ioannou *et al.*, 2002a; Jakob *et al.*, 1998). We have previously demonstrated the ability of CpG ODN to shift the immune response induced by parenteral immunization with FI-BRSV from a Th2-biased response to a more balanced response in mice (Oumouna *et al.*, 2005) and calves (Mapletoft *et al.*, 2006).

An alternate or complementary approach to avoid enhanced disease while maintaining or increasing protection is to change the route of immunization. Several intranasal immunization strategies have been employed to protect rodents from RSV challenge, including recombinant F protein adjuvanted with CpG ODN (Prince *et al.*, 2003), cholera toxin (Tebbey *et al.*, 2000; Walsh, 1993), or caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate

(Tebbey *et al.*, 1999), as well as live viral (Kahn *et al.*, 2001; Matsuoka *et al.*, 2002; Stott *et al.*, 1987) or bacterial (Cano *et al.*, 2000; Falcone *et al.*, 2006) vectors expressing whole RSV proteins or peptides. One of the challenges of intranasal immunization is delivering the vaccine components in such a manner that they are not degraded or removed prior to the initiation of the immune response. Polyphosphazenes (PP) are synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms and organic side groups attached at each phosphorus atom (Payne & Andrianov, 1998). PP form non-covalent complexes when mixed with compounds of interest, i.e. antigens and other adjuvants, increasing their stability and allowing for multimeric presentation. Delivery of antigens formulated with PP has been successful in enhancing murine antibody responses to influenza (Mutwiri *et al.*, 2007; Payne *et al.*, 1995; Payne *et al.*, 1998), rotavirus (McNeal *et al.*, 1999) and cholera (Wu *et al.*, 2001). Protection was also enhanced in the latter two models.

We have previously shown the adjuvant effects of CpG ODN on parenteral FI-BRSV vaccines in mice (Oumouna *et al.*, 2005) and calves (Mapletoft *et al.*, 2006). Here we report on adjuvant effects of CpG ODN and PP, individually and as co-adjuvants, on an intranasal FI-BRSV vaccine in mice.

3.3 MATERIALS AND METHODS

3.3.1 Cells and virus - The 375 strain of BRSV (American Type Culture Collection) was propagated in Bovine Turbinate (BT) cells (American Type Culture Collection), maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco/Invitrogen) supplemented with 1% heat-inactivated fetal bovine serum of New Zealand origin (FBS; Gibco/Invitrogen), 25 mM HEPES

(EMD Biosciences Inc.), 44 mM sodium bicarbonate (EMD Biosciences Inc.) and 50 μg/ml gentamicin (Gibco/Invitrogen). Infected cells were incubated at 37 ^{o}C in modified atmosphere with 5% CO_2. Four to seven days after infection, infected cells were collected by scraping and either frozen at -70 ^{o}C until vaccine or challenge virus preparation, or used at a dilution of 1:10 to infect more cells for up to two infection cycles. Virus titers were determined by a plaque assay on BT cells. Briefly, ten-fold serial dilutions of virus in DMEM with 1% FBS were added to 70-80% confluent BT monolayers in 96-well tissue culture plates (Corning Incorporated). Cells were incubated for seven days at 37 ^{o}C in modified atmosphere with 5% CO_2, and BRSV plaques were visualized by immunostaining. Cells were fixed with 80% acetone in 0.1M PBS (38 mM sodium phosphate monobasic, 61 mM sodium phosphate dibasic heptahydrate, 0.15 M sodium chloride, pH 7.2). Polyclonal goat anti-BRSV IgG (VMRD, Inc.) at a dilution of 1:5,000, followed by biotinylated rabbit anti-goat IgG (Vector Laboratories, Inc.) at a dilution of 1:750 were used to detect BRSV plaques. Vectastain Avidin-Biotin Complex horseradish peroxidase (Vector Laboratories, Inc.), followed by diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories, Inc.) were used to visualize BRSV plaques. Viral titers were expressed as PFU/ml.

FI-BRSV was prepared as previously described (Kim *et al.*, 1969). Briefly, infected cell lysate was clarified by centrifugation for 15 min at 550 x *g*. One part of 37% formalin (Sigma-Aldrich) was incubated with 4,000 parts of clarified lysate at 2 x 10^6 PFU per ml for three days at 37^{o}C and pelleted by ultracentrifugation for 1 h at 50,000 x *g*. The resulting pellet was resuspended in 1/25 of the original volume in serum-free DMEM (Gibco/Invitrogen) and assayed for protein concentration. The final vaccine protein concentration was 150 μg/ml, and 1.875 μg were given per immunization.

Challenge virus was prepared as follows. Infected cell lysate was centrifuged for 30 min at 1,940 x *g* at 4°C. The resulting pellet was resuspended in 1/100 of the original volume in serum-free DMEM (Gibco/Invitrogen), and disrupted using a cuphorn sonicator until the pellet was visibly homogenized. Challenge virus titers were determined as indicated above.

3.3.2 Immunization and challenge - Six to eight week-old female BALB/c mice (Charles River) were randomly allocated into seven groups of 10 animals and immunized intranasally three times with a total volume of 25 μl (12.5 μl in each nostril) as indicated in Table 3.1. CpG ODN 1826 (TCC ATG ACG TTC CTG ACG TT) was provided by Merial Limited. Non-CpG ODN (TCC ATG AGC TTC CTG AGC TT) was synthesized by Operon Biotechnologies Inc. Two mice died during the course of the study and were excluded from all analyses. All ODNs were phosphorothioate-modified during synthesis to enhance nuclease resistance and were given at 20 μg per immunization. Polyphosphazene Polymer 6 was synthesized by John Klaehn (Idaho National Laboratory) according to a previously published method (Andrianov *et al.*, 2004) and was given at 25 μg per immunization. The components of each vaccine were mixed prior to immunization and were given in a total volume of 25 μl as a single administration. Two weeks after the third immunization, all groups were challenged. Following sedation with ketamine and xylazine (60 mg/kg; Butler Co.), 10^7 PFU/ml of BRSV strain 375 in a final volume of 50 μl was applied to the nostrils. Five mice per group were euthanized four days after challenge, for detection of viral RNA, and the remaining mice were euthanized six days after challenge, for lung fragment cultures and enzyme-linked immunospot assays. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Table 3.1. Immunization protocol.

Group	**Immunizations** (days 0, 21, 42)	**Challenge** (day 56)
1	FI-BRSV	BRSV
2	FI-BRSV/CpG	BRSV
3	FI-BRSV/non-CpG	BRSV
4	FI-BRSV/PP	BRSV
5	FI-BRSV/CpG/PP	BRSV
6	Saline	BRSV
7	Saline	Saline

3.3.3 Lung fragment culture supernatants - Lung fragment cultures were prepared as previously described (Etchart *et al.*, 2006; Logan *et al.*, 1991), with a few modifications. Six days after challenge, mice were euthanized and lungs were lavaged and removed to tubes containing RPMI 1640 medium (Gibco/Invitrogen) supplemented with 10% FBS (Gibco/Invitrogen), 10 mM HEPES buffer, 0.1 mM non-essential amino acids (Gibco/Invitrogen), 1 mM sodium pyruvate (Gibco/Invitrogen), 50 μg/ml gentamicin (Gibco/Invitrogen), and 1X antibiotic/antimycotic (Gibco/Invitrogen), on ice. Under sterile conditions, lungs were cut into four pieces of roughly equal size, and deposited into 48-well plates (1 piece of lung per well) containing 500 μl per well of RPMI 1640 medium supplemented as indicated above. Following 5 days of incubation at 37 ^{o}C in modified atmosphere with 5% CO_2, supernatants were collected, pooled for each individual mouse, clarified by centrifugation for 1 min at 10,000 x *g*, and stored at -80 ^{o}C until they could be assayed for IgG and IgA, as detailed below.

3.3.4 Enzyme-linked immunosorbent assay (ELISA) – Sera and lung fragment culture supernatants were assayed for BRSV-specific IgG and IgA. Ninety-six-well polystyrene IMMULON® 2 microtiter plates (Thermo Electron) were coated overnight at 37 ^{o}C with BRSV antigen composed of Nonidet-P40 (Sigma-Aldrich)-treated BT cells previously infected with the 375 strain of BRSV as described above and were frozen at -20 ^{o}C until use. Mock-infected BT cells were used as negative control antigen. Plates were washed, then incubated overnight at 4 ^{o}C with serially diluted samples, beginning at 1:10 (lung fragment culture supernatants) or 1:40 (sera), and continuing in 4-fold dilutions. Alkaline phosphatase (AP)-labeled goat anti-mouse IgG or IgA (Kirkegaard & Perry Laboratories) at dilutions of 1:5,000 and 1:2,500 were used to

detect bound IgG and IgA, respectively. Reactions were visualized with *p*-nitrophenyl phosphate (Sigma-Aldrich). Results were expressed as the reciprocal of the highest dilution resulting in a reading equal to that of undiluted serum tested on negative control antigen.

3.3.5 Virus neutralization assay – BT cells were cultured overnight in 96-well tissue culture plates (Corning Incorporated) to achieve 70-80% confluent monolayers. Samples were diluted in 96-well round-bottom culture plates (Corning Incorporated), beginning at 1:2 (lung fragment culture supernatants) or 1:20 (pooled sera), and continuing in 2-fold dilutions. Lung fragment culture supernatants were heat-inactivated at 56 ^{o}C for 30 min prior to dilution. Five hundred pfu/well of BRSV strain 375 were added to each sample dilution and plates were incubated for 1 h at 37 ^{o}C. Sample-virus mixtures were then added to duplicate BT cell cultures and incubated at 37 ^{o}C in modified atmosphere with 5% CO_2 for 6 days. BRSV-specific cytopathic effects were visualized by immunostaining as described above. Virus neutralizing titers are expressed as the highest dilution of sample that resulted in less than 50% of cells displaying cytopathic effects.

3.3.6 IFN-γ and IL-5 enzyme-linked immunospot (ELISPOT) assays – Splenocytes were isolated as previously described (Baca-Estrada *et al.*, 1996), with a few modifications. Six days after challenge, mice were euthanized and spleens were removed to tubes containing minimal essential medium (MEM; Gibco/Invitrogen) supplemented with 50 μg/ml gentamicin and 10 mM HEPES buffer (Gibco/Invitrogen), on ice. Following removal of excess fat, spleens were cut into pieces and gently pushed through sterile 100 μm cell strainers (BD Biosciences) into Petri dishes containing MEM. Splenocytes were centrifuged for 10 min at 310 x *g* at 4 ^{o}C

and resuspended in 1 ml of ammonium chloride lysis buffer (0.14 M ammonium chloride, 17 mM Tris, pH 7.2). Thirty seconds later, 10 ml of MEM was added. Splenocytes were washed twice with MEM and then resuspended in culture medium (AIM-V medium (Gibco/Invitrogen) supplemented with 0.1 mM non-essential amino acids (Gibco/Invitrogen), 10 mM HEPES buffer, 1 mM sodium pyruvate (Gibco/Invitrogen) and 50 μM 2-mercaptoethanol (Sigma-Aldrich)).

Ninety-six-well Multiscreen-HA ELISPOT plates (Millipore) were coated overnight at 4 ^{o}C with murine IFN-γ or IL-5-specific monoclonal antibodies (BD PharMingen) at a concentration of 2 μg/ml. On the day the splenocytes were isolated, plates were washed with sterile phosphate buffered saline (PBS; pH 7.4, Gibco/Invitrogen) and blocked with culture medium for 1 to 2 h at 37 ^{o}C. Splenocytes were resuspended in culture medium and cultured at 10^6 cells per well in triplicate wells in the presence of BRSV-infected or mock-infected cell lysate. Lysates were used at a final protein concentration of 25 μg/ml. After approximately 20 h of incubation at 37 ^{o}C in modified atmosphere with 5% CO_2, plates were washed with double distilled (dd)H_2O and PBS with 0.05% Tween 20 (Sigma-Aldrich) and then incubated with biotinylated anti-mouse IFN-γ or IL-5 (BD PharMingen) at a concentration of 2 μg/ml in PBS with 1% bovine serum albumin (BSA) for 1 to 2 h at RT. Subsequently, the plates were incubated with AP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:1,000 in PBS with 1% bovine serum albumin (Sigma-Aldrich) for 1 to 2 h at RT. Bound antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitroblue substrate tablets (Sigma-Aldrich). Plates were washed with ddH_2O and air dried. Spots were counted in a blinded manner with the aid of an inverted microscope. Results are expressed as the difference between the number of cytokine secreting cells per 10^6 cells in BRSV-infected lysate-stimulated

wells and the number of cytokine secreting cells per 10^6 cells in mock-infected lysate-stimulated wells.

3.3.7 IFN-γ and IL-5 ELISAs on lung homogenate supernatants - Four days after challenge, mice were euthanized and lungs were removed to 2 ml screw-cap tubes (VWR International) containing 2.4 mm zirconia microbeads (Biospec Products, Inc.) and 1 ml of DMEM (Gibco/Invitrogen) supplemented with 25 mM HEPES (EMD Biosciences Inc.), 44 mM sodium bicarbonate (EMD Biosciences Inc.), 50 μg/ml gentamicin (Gibco/Invitrogen), 10 μg/ml aprotinin (Sigma-Aldrich), 10 μg/ml leupeptin (Sigma-Aldrich), 0.1 mM EDTA, 1 mM PMSF (Sigma-Aldrich), and 1X antibiotic-antimycotic (Gibco/Invitrogen). Lungs were homogenized in a mini-beadbeater (BioSpec Products, Inc.) for 10 sec, clarified by centrifugation for 1 min at 10,000 x *g*, and stored at -80 ^{o}C. Lung homogenate supernatants were assayed for the presence of IFN-γ and IL-5 using Quantikine Mouse Immunoassay Kits (R&D Systems) as per the manufacturer's instructions.

3.3.8 Detection of viral RNA – Four days after challenge, mice were euthanized and lungs were removed to 2 ml screw-cap tubes (VWR International) containing 2.4 mm zirconia microbeads (Biospec Products, Inc.) and 1 ml of Trizol Reagent (Invitrogen), and were homogenized in a mini-beadbeater (BioSpec Products, Inc.) for 10 sec. RNA was isolated from lung homogenates using the Trizol Reagent method, as per manufacturer's instructions. DNA removal and cDNA synthesis were performed using the QuantiTect Reverse Transcription Kit (Qiagen), as per manufacturer's instructions. Real-time quantitative PCR (qPCR) reactions were prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), as per manufacturer's

instructions, in iCycler iQ PCR Plates (Bio-Rad Laboratories, Inc.) sealed with iCycler iQ Optical Tape (Bio-Rad Laboratories, Inc.). Primers to the BRSV F gene (primer A: 5'-AACCGGCCTCCTTCAGTAGA-3', primer B: 5'-TGGACACTGCTACACCACTT-3') were designed using primer design software for personal computers (Clone Manager Version 6.00) from a consensus sequence generated from 27 different BRSV F gene sequences using the MultAlin multiple sequence alignment tool available online at **http://bioinfo.genopole-toulouse.prd.fr/multalin/** (Corpet, 1988). qPCR was performed on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboritories, Inc.) for 45 cycles using the following parameters: denaturation for 15 s at 95 ^{o}C, annealing for 30 sec at 60 ^{o}C, and extension for 30 sec at 72 ^{o}C. Serial dilutions of a plasmid that contains a truncated version of the BRSV F gene used in qPCR reactions carried out under the same conditions as those outlined above allowed construction of a standard curve that enabled the determination of gene copy number. Results are expressed as viral RNA copies per ml of lung homogenate.

3.3.9 Statistical analysis - All data were analyzed using statistical software (GraphPad Prism Version 3.00). As sample sizes were small (n = 5 or 10), outcome variables were assumed not to be normally distributed. Therefore, differences among all groups were examined using the Kruskal-Wallis test. If a significant difference was found among the groups, median ranks between pairs of groups were compared using the Mann-Whitney U test. Differences were considered significant if $P < 0.05$.

3.4 RESULTS

3.4.1 BRSV-specific humoral immune responses – The humoral immune responses induced by the various vaccine formulations were examined by measuring the BRSV-specific IgG titers in the serum after each immunization and after challenge. After the final immunization, significantly increased IgG production ($P = 0.0002$) was observed when PP was added to the FI-BRSV vaccine (Fig. 3.1A). This response was further increased by addition of CpG ODN to the FI-BRSV/PP vaccine. Indeed, the levels of IgG produced by the FI-BRSV/CpG/PP group were significantly higher than those in all other groups ($P = 0.0003$, when compared to the FI-BRSV/PP group; $P < 0.0001$, compared to all other groups). Examination of the kinetics of the BRSV-specific serum IgG response (Fig. 3.1B) revealed that only two intranasal immunizations of the FI-BRSV/CpG/PP vaccine were required to induce a robust serum antibody response.

The additive or synergistic effect due to co-formulation with CpG ODN and PP in terms of humoral immunity against BRSV was further confirmed upon quantification of virus neutralizing antibodies in pooled sera; immunization with FI-BRSV/CpG/PP resulted in the highest virus neutralizing titers before and after challenge (Fig. 3.1C).

In addition, BRSV-specific IgA was measured after challenge (Fig 3.1D). All adjuvanted vaccine groups performed significantly better than FI-BRSV alone ($P = 0.006$, compared to FI-BRSV/CpG; $P < 0.0001$, compared to FI-BRSV/non-CpG; $P = 0.0002$, compared to FI-BRSV/PP; $P = 0.0006$, compared to FI-BRSV/CpG/PP). The only significant difference observed between adjuvanted vaccine groups, however, was that between FI-BRSV/CpG and FI-BRSV/PP ($P = 0.02$).

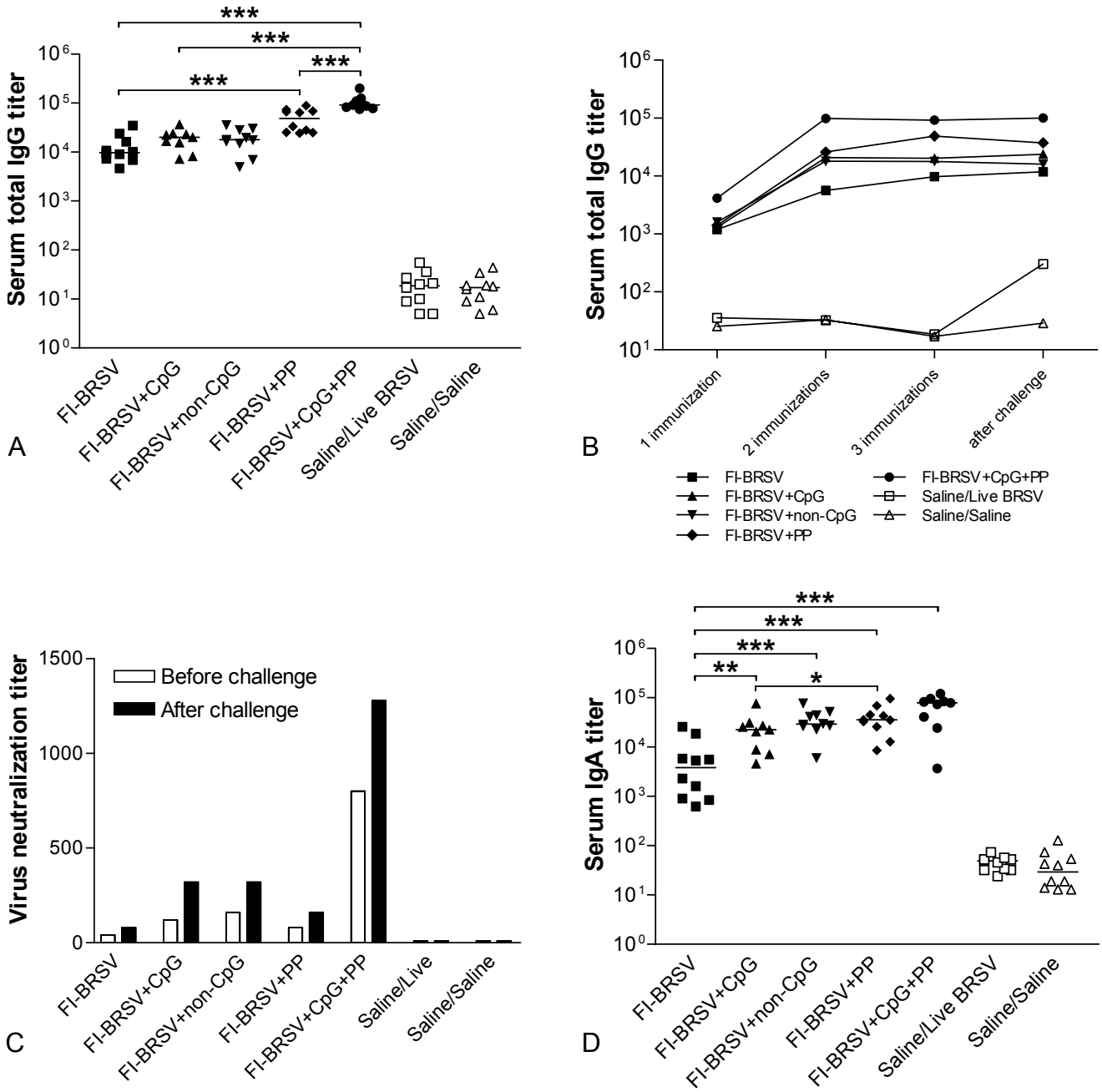
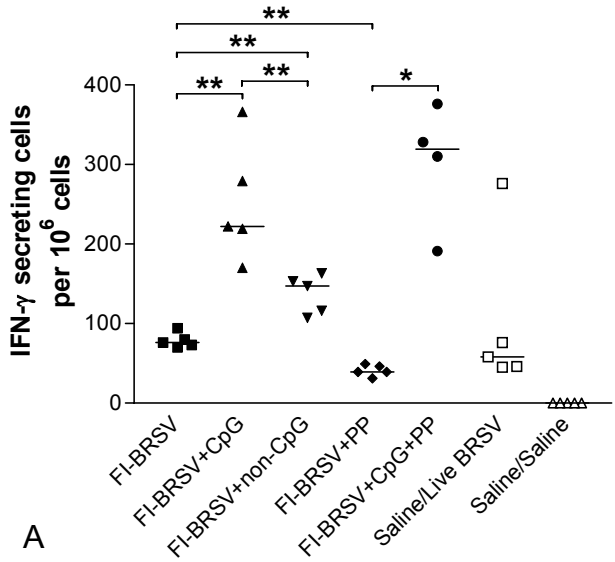

***
***
***
***
Serum total IgG titer
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
A
Serum total IgG titer
1 immunization
2 immunizations
3 immunizations
after challenge
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
B
Virus neutralization titer
1500
1000
500
0
Before challenge
After challenge
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live
Saline/Saline
C
***
***
***
**
*
Serum IgA titer
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
D

Fig. 3.1

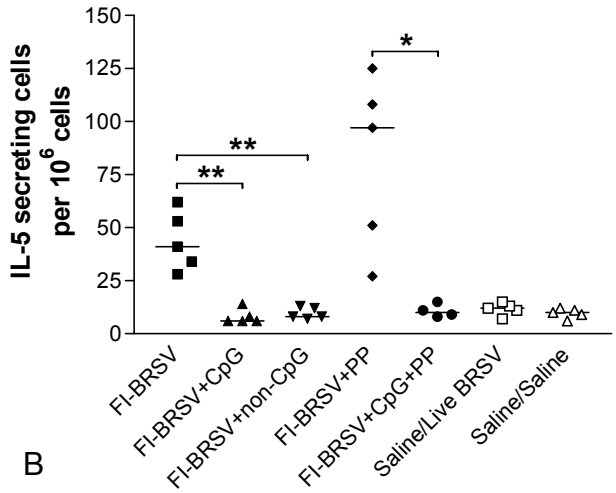
Figure 3.1. BRSV-specific IgG in sera after three immunizations (A). Kinetics of the BRSV-specific serum IgG response (B). Virus neutralizing antibodies in sera before and after challenge (C). BRSV-specific IgA in sera after challenge (D). Mice were immunized intranasally with FI-BRSV, FI-BRSV/CpG, FI-BRSV/non-CpG, FI-BRSV/PP, FI-BRSV/CpG/PP, or saline. CpG and non-CpG ODN were given at 20 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV 2 weeks after the final immunization (except for the Saline/Saline group). For (A) and (D), each data point represents an individual animal, and median values are indicated by horizontal bars. For (B), median values are indicated by data points. For (C), bars indicate values obtained by assaying pooled sera. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

3.4.2 BRSV-specific cell-mediated immune responses – To further evaluate the type of immune responses induced by the various vaccine formulations, the BRSV-induced secretion of IFN-γ and IL-5 by splenocytes was measured 6 days after challenge. IFN-γ secreting cells were induced in the groups that received FI-BRSV or FI-BRSV/PP (Fig. 3.2A). Addition of CpG ODN to these vaccines significantly increased IFN-γ secretion in the FI-BRSV/CpG and FI-BRSV/CpG/PP groups ($P = 0.008$ and $P = 0.02$, respectively). Although there was an increase in IFN-γ production in the FI-BRSV/non-CpG group in comparison with the FI-BRSV group ($P = 0.008$), suggesting a phosphorothioate backbone effect, there was a further increase in the FI-BRSV/CpG group in comparison with the FI-BRSV/non-CpG group ($P = 0.008$). In contrast to the CpG ODN effects, addition of PP to the FI-BRSV vaccine significantly decreased IFN-γ secretion ($P = 0.008$).

High numbers of IL-5 secreting cells were induced in the groups that received FI-BRSV or FI-BRSV/PP (Fig. 3.2B). Addition of CpG ODN to these vaccines significantly decreased IL-5 secretion in the FI-BRSV/CpG and FI-BRSV/CpG/PP groups ($P = 0.008$ and $P = 0.02$, respectively). Non-CpG ODN also decreased the amount of IL-5 secretion when compared to FI-BRSV alone ($P = 0.008$). There were no significant differences between the FI-BRSV/CpG and FI-BRSV/CpG/PP groups in terms of IFN-γ or IL-5 secretion by splenocytes. These results indicate that the addition of CpG ODN, and to an extent non-CpG ODN, to the vaccine formulations shifted the cell-mediated immune response from a Th2-type response, characterized by high levels of IL-5 secretion, to a Th1-type response, characterized by high levels of IFN-γ secretion, but it was less clear as to exactly what effect PP had on the cell-mediated immune response.


**
**
**
**
*
400
300
200
100
0
IFN-γ secreting cells per 10^6 cells
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline

A


150
125
100
75
50
25
0
**
**
*
IL-5 secreting cells per 10^6 cells
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline

B

Fig. 3.2

Figure 3.2. Numbers of IFN-γ (A) or IL-5 (B) secreting splenocytes in response to *in vitro* restimulation with BRSV-infected cell lysates. Mice were immunized intranasally with FI-BRSV, FI-BRSV/CpG, FI-BRSV/non-CpG, FI-BRSV/PP, FI-BRSV/CpG/PP, or saline. CpG and non-CpG ODN were given at 20 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV 2 weeks after the final immunization (except for the Saline/Saline group) and euthanized 6 days later. Results are the difference between the number of cytokine secreting cells per 10^6 cells in BRSV-infected lysate-stimulated wells and the number of cytokine secreting cells per 10^6 cells in mock-infected lysate-stimulated wells. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

3.4.3 BRSV-specific mucosal immune responses – To evaluate the mucosal immune responses induced by the various vaccine formulations, the secretion of IgG and IgA in lung culture fragment supernatants was measured. Low levels of IgG were produced in the group that received FI-BRSV (Fig. 3.3A). Addition of CpG or PP to the vaccine significantly increased IgG production ($P = 0.008$ and $P = 0.03$, respectively), whereas there was no significant effect of non-CpG. The group that received FI-BRSV/CpG/PP produced significantly higher levels of IgG than any other group ($P = 0.03$, compared to FI-BRSV/PP; $P = 0.02$, compared to all other groups). Low levels of IgA were produced in the group that received FI-BRSV (Fig. 3.3B). Addition of CpG, but not non-CpG ODN or PP, to the vaccine significantly increased production of IgA ($P = 0.008$). The group that received FI-BRSV/CpG/PP produced significantly higher levels of IgA than any other group ($P = 0.02$). These results indicate that the addition of CpG ODN and PP to FI-BRSV enhanced the mucosal immune response.

To further evaluate the biological effectiveness of the antibodies produced in the lungs, virus neutralizing titers were determined. Neutralizing antibody titers were found in all immunized mice, but the group that received CpG ODN and PP performed significantly better than all other groups ($P = 0.03$, compared to FI-BRSV/CpG; $P = 0.02$, compared to all other groups) (Fig. 3.3C). There were no significant differences among the other immunized groups.

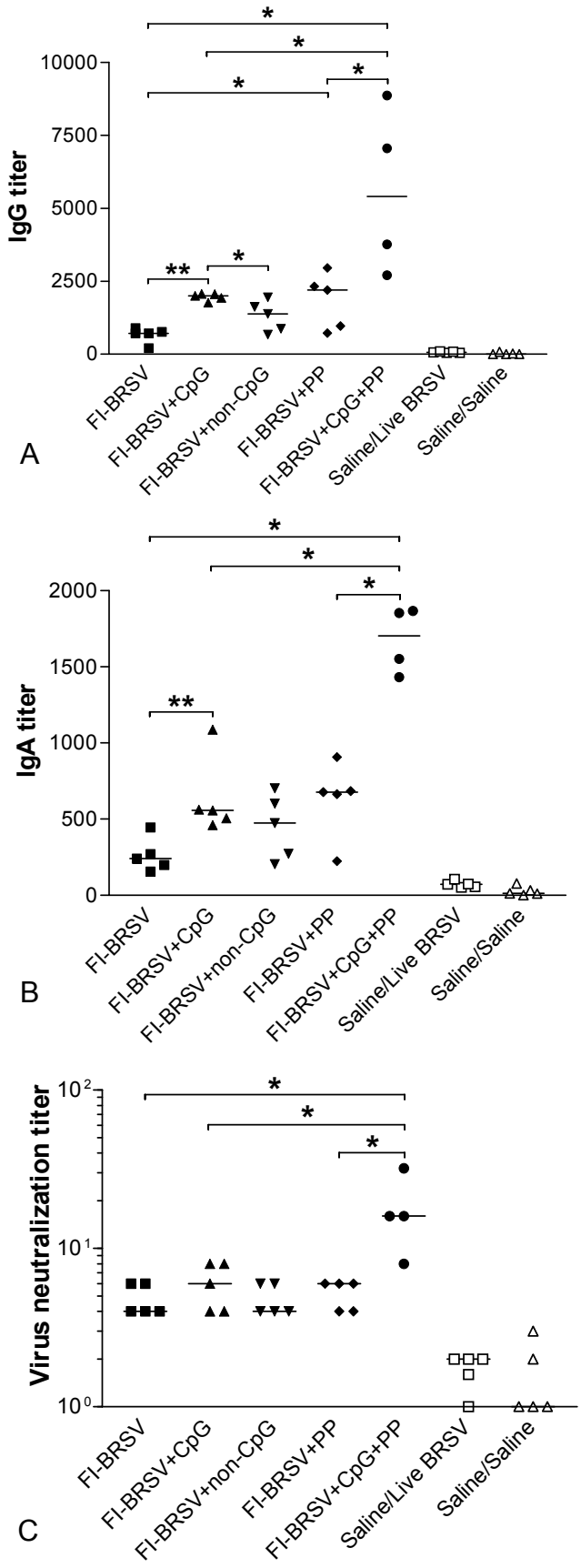

IgG titer
10000
7500
5000
2500
0
*
*
*
*
**
*
FI-BRSV
FI-BRSV+CpG
FI-BRSV+non-CpG
FI-BRSV+PP
FI-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
A
IgA titer
2000
1500
1000
500
0
*
*
*
**
FI-BRSV
FI-BRSV+CpG
FI-BRSV+non-CpG
FI-BRSV+PP
FI-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
B
Virus neutralization titer
10^2
10^1
10^0
*
*
*
FI-BRSV
FI-BRSV+CpG
FI-BRSV+non-CpG
FI-BRSV+PP
FI-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
C

Fig. 3.3

Figure 3.3. BRSV-specific IgG (A), IgA (B) and virus neutralizing antibodies (C) in lung fragment culture supernatants. Mice were immunized intranasally with FI-BRSV, FI-BRSV/CpG, FI-BRSV/non-CpG, FI-BRSV/PP, FI-BRSV/CpG/PP, or saline. CpG and non-CpG ODN were given at 20 μg per immunization, and PP was given at 25 μg per immunization. All animals (except for the Saline/Saline group) were challenged with BRSV 2 weeks after the final immunization and euthanized 4 days later. Virus neutralization titers are expressed as the highest dilution of lung fragment culture supernatant that resulted in less than 50% of cells displaying cytopathic effects. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

3.4.4 IFN-γ and IL-5 production in the lungs – The effects of the various vaccine formulations on the type of immune response induced was further examined by measuring the amounts of IFN-γ and IL-5 produced in the lungs. Mice that received FI-BRSV/PP or FI-BRSV/CpG/PP produced significantly lower amounts of IFN-γ compared to those that received FI-BRSV ($P = 0.008$) or FI-BRSV/CpG ($P = 0.008$), respectively (Fig. 3.4A), indicating the possibility for PP to reduce lung IFN-γ production. This agrees somewhat with our observations with *in vitro* re-stimulated splenocytes, in which addition of PP to the FI-BRSV vaccine significantly decreased the number of IFN-γ secreting cells. Addition of CpG ODN to the FI-BRSV/PP did, however, significantly increase lung IFN-γ production ($P = 0.05$). Mice that received FI-BRSV/CpG, FI-BRSV/non-CpG, or FI-BRSV/CpG/PP produced significantly lower amounts of IL-5 compared to those that received FI-BRSV ($P = 0.03$, 0.008, or 0.008, respectively) (Fig. 3.4B), indicating a role for CpG and non-CpG ODN in the reduction of lung IL-5 production. Incidently, there was no significant difference between the FI-BRSV/CpG and FI-BRSV/CpG/PP groups in terms of lung IL-5 production, demonstrating that PP does not interfere with the ability of CpG ODN to reduce IL-5 production.

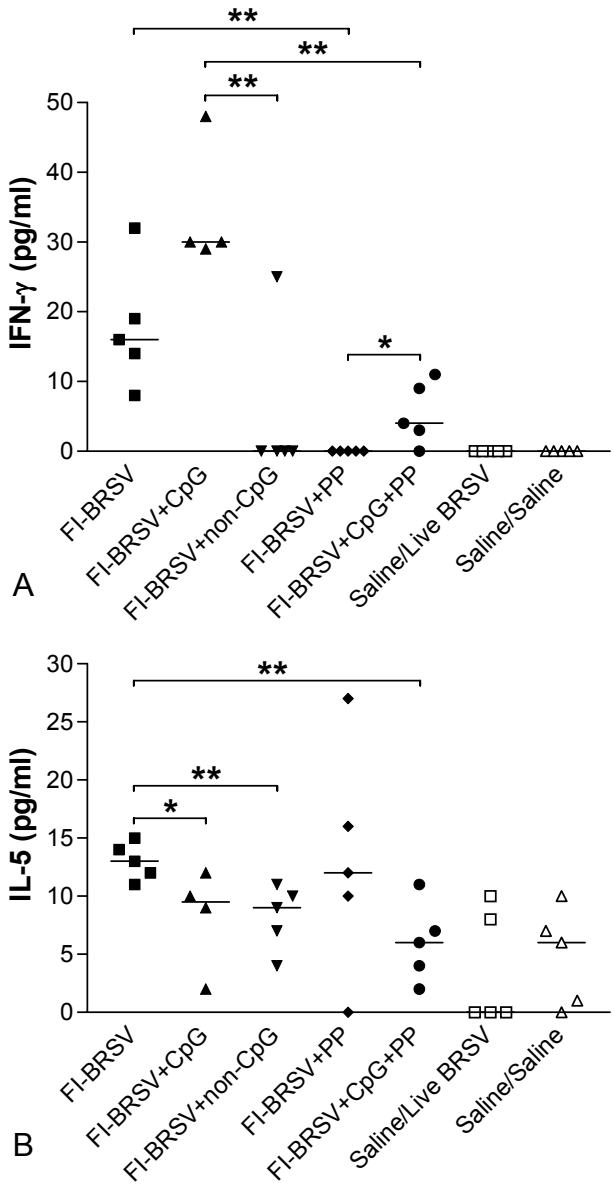

**
**
**
*
IFN-γ (pg/ml)
50
40
30
20
10
0
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
A
**
**
*
IL-5 (pg/ml)
30
25
20
15
10
5
0
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline
B

Fig. 3.4

Figure 3.4. IFN-γ (A) and IL-5 (B) production in lung homogenate supernatants. Mice were immunized intranasally with FI-BRSV, FI-BRSV/CpG, FI-BRSV/non-CpG, FI-BRSV/PP, FI-BRSV/CpG/PP, or saline. CpG and non-CpG ODN were given at 20 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV 2 weeks after the final immunization (except for the Saline/Saline group) and euthanized 6 days later. Results are expressed in pg/ml. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

3.4.5 Detection of viral RNA in the lung tissue - To assess the ability of CpG ODN and PP to enhance protection from infection, the level of virus replication in the lungs was determined by the detection of viral RNA. The unvaccinated group (Saline/BRSV) displayed the highest level of viral replication (Fig. 3.5). Compared to the Saline/BRSV group, all the groups that received a vaccine displayed some level of reduction in viral replication, but the largest significant reduction in viral replication was observed in the group that received FI-BRSV/CpG/PP ($P = 0.008$). The FI-BRSV/CpG/PP group also displayed significantly less viral replication than all other vaccine groups ($P = 0.008$). The small number of viral RNA copies observed in the uninfected mice was due to the formation of non-specific PCR products synthesized in the absence of viral RNA. These results indicated a synergy between CpG ODN and PP in terms of enhancing protection against BRSV.

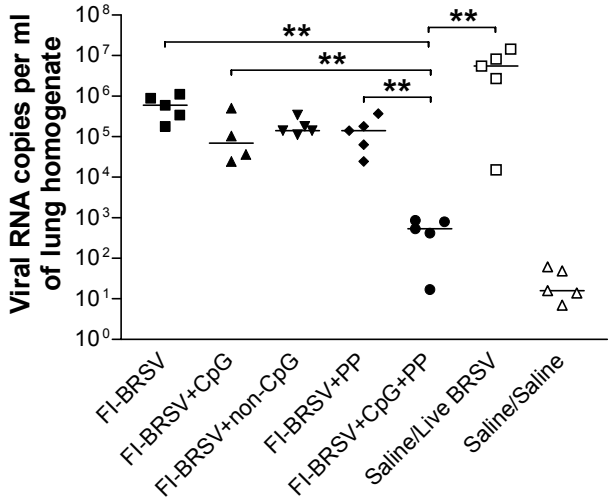

Viral RNA copies per ml of lung homogenate
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
**
**
**
**
Fl-BRSV
Fl-BRSV+CpG
Fl-BRSV+non-CpG
Fl-BRSV+PP
Fl-BRSV+CpG+PP
Saline/Live BRSV
Saline/Saline

Fig. 3.5

Figure 3.5. Detection of viral RNA in lung tissue. Mice were immunized intranasally with FI-BRSV, FI-BRSV/CpG, FI-BRSV/non-CpG, FI-BRSV/PP, FI-BRSV/CpG/PP, or saline. CpG and non-CpG ODN were given at 20 μg per immunization, and PP was given at 25 μg per immunization. All animals (except for the Saline/Saline group) were challenged with BRSV 2 weeks after the final immunization and euthanized 4 days later. Results are expressed as viral RNA copies per ml of lung homogenate. Each data point represents an individual animal, and median values are indicated by horizontal bars. $^{**}P < 0.01$.

3.5 DISCUSSION

Like many pathogens**,** RSV targets the mucosal surfaces of the respiratory tract, so the induction of both systemic and mucosal immune responses is expected to be critical for optimal disease protection. In order to achieve effective mucosal immunity by intranasal vaccination, non-replicating vaccines need to be formulated with effective adjuvants and/or delivery vehicles. In this study we demonstrated the ability of an intranasally delivered FI-BRSV vaccine co-formulated with CpG ODN and PP to induce systemic and mucosal immunity, as well as protection from BRSV challenge in mice.

Humoral immunity, though not the only component of a successful immune response, especially in the case of a BRSV infection, is a traditional measure of the success of an immunization protocol. Here we found that co-formulation of FI-BRSV with CpG ODN and PP resulted in significantly higher levels of BRSV-specific IgG in the serum compared to all other vaccine formulations, indicating a synergy between CpG ODN and PP. It is well known, however, that humoral immunity, in the absence of cell-mediated immunity, is not sufficient to combat RSV infections, whereas cell-mediated immunity is universally recognized to be an important factor in the resolution of RSV infections. Addition of CpG ODN to the FI-BRSV and FI-BRSV/PP vaccines resulted in significant increases in IFN-γ secretion. This agrees with an earlier report in which high levels of IFN-γ were induced by parental immunization of mice with FI-BRSV formulated with CpG ODN (Oumouna *et al.*, 2005). In contrast, IL-5 secretion was significantly reduced to virtually nothing when CpG ODN was added to the FI-BRSV and FI-BRSV/PP vaccines. Although non-CpG ODN also enhanced IFN-γ and reduced IL-5 levels, suggesting some phosphorothioate backbone effect, the increase in IFN-γ induced by CpG ODN

was greater in magnitude in comparison to that induced by non-CpG ODN. It was less clear, however, as to what the exact effects of PP were on the type of immune response induced in *in vitro* re-stimulated splenocytes or in the lungs, but this is not surprising given an earlier study that concluded that parenteral immunization of mice with influenza virus antigens formulated with a similar PP results in mixed Th1/Th2 immune responses (Mutwiri *et al.*, 2007).

While humoral and cell-mediated immunity are traditionally considered to be important correlates of protection, in BRSV disease the first encounter between the host and pathogen takes place in the lung mucosa. Addition of CpG ODN to the FI-BRSV and FI-BRSV/PP vaccines resulted in significant increases in production of both IgG and IgA, whereas there was no effect of non-CpG ODN. Furthermore, the group co-formulated with CpG ODN and PP produced significantly higher levels of IgG and IgA compared to all other groups. These findings correspond to those in a previous study in which intranasal immunization of mice with hepatitis B surface antigen with CpG ODN induced humoral and cell-mediated systemic immune responses, as well as a mucosal (IgA) response in the lungs (McCluskie & Davis, 1998). Similarly, intranasal immunization of mice with *Streptococcus pyogenes* M6 protein with CpG ODN induced production of serum IgG and lung IgA (Teloni *et al.*, 2004). The effects of CpG ODN were further validated by the observation that the presence of CpG ODN appeared to shift the IFN-γ and IL-5 levels in the lungs, as vaccine groups that received CpG ODN produced significantly less IL-5 in the lungs than the FI-BRSV group, whereas addition of CpG ODN, but not non-CpG ODN, increased IFN-γ production.

There is evidence that phosphorothioate modified non-CpG ODN can also have immune modulatory effects via TLR9 (Roberts *et al.*, 2005; Vollmer *et al.*, 2002), particularly when used as an adjuvant at mucosal sites (McCluskie & Davis, 2000). Although in this study we indeed

observed some effects of the non-CpG ODN on IFN-γ and IL-5 production in splenocytes and lungs, the IFN-γ production was more enhanced by CpG ODN. Furthermore, the non-CpG ODN did not have any influence on lung IgG or IgA production, which confirms the immune modulatory effects of the CpG motifs, not only in the FI-BRSV/CpG group but also in the FI-BRSV/CpG/PP group.

Signs of inflammation, such as production of BRSV-specific serum IgE and enhanced eosinophilia, have been reported following the challenge of BALB/c mice that had been parenterally immunized with FI-BRSV (Oumouna *et al.*, 2005). In this study, however, no BRSV-specific IgE was detected in the sera and no increases in the number of eosinophils were found in bronchioalveolar lavages of any of the mice (data not shown). This is not necessarily surprising, though, as the classic model of RSV disease enhancement in mice tends to occur following parenteral immunization with RSV vaccines, not mucosal immunization. In fact, while scarification with recombinant vaccinia virus (rVV) expressing the RSV G protein primes mice for enhanced lung immunopathology (Openshaw *et al.*, 1992), intranasal and intraperitoneal immunization with rVV-G results in protection with no lung lesions (Stott *et al.*, 1986). Another study, featuring rVV expressing the RSV F protein, demonstrated that, while intranasal immunization induced some pathological lung inflammation upon challenge with live BRSV, it was milder than that induced following intradermal immunization (Matsuoka *et al.*, 2002).

The most important quality offered by a successful BRSV vaccine formulation is protection upon viral challenge. Because of the labile nature of BRSV, as well as its limited growth in experimental animals and cell culture, reverse transcription (RT)-PCR assays have been developed to detect the viral RNA in the lungs of mice, as an alternative to the isolation of live virus (Almeida *et al.*, 2004). We have increased the sensitivity of these assays via the use of

real-time quantitative RT-PCR reagents and detection systems. Despite reductions in the median viral RNA copy number in all vaccinated groups, the only group that experienced a significant reduction in viral replication was the group that received FI-BRSV/CpG/PP. This group also demonstrated the largest reduction in median viral RNA copy number. This agrees with an earlier report in which intranasal immunization of cotton rats with RSV F protein plus CpG ODN reduced viral production upon challenge (Prince *et al.*, 2003). They, however, required higher amounts of purified antigen and/or CpG ODN than those used in the present study to achieve this level of protection. Furthermore, intranasal delivery of CpG co-formulated vaccines consisting of human immunodeficiency virus (HIV) immunogen (Dumais *et al.*, 2002), or herpes simplex virus (HSV) recombinant glycoprotein B (Gallichan *et al.*, 2001), induced protection of mice from intravaginal challenge with rVV expressing HIV-1 *gag* or HSV-2, respectively.

Because of their ability to form non-covalent complexes with antigens and other adjuvants, PP is attractive for use in mucosal immunization, a situation in which constant secretion of mucous membrane fluids and high turnover of epithelial cells threatens the stability and uptake of vaccine components. We demonstrated that intranasal immunization of mice with FI-BRSV co-formulated with CpG ODN and PP induced both humoral and cell-mediated immunity. In addition, mice immunized intranasally with FI-BRSV/CpG/PP developed enhanced mucosal immunity, characterized by increased production of BRSV-specific IgG and IgA in lung fragment cultures. Indeed, the production of enhanced serum and lung IgG, and in particular lung IgA and virus neutralizing antibodies, are probably the most important characteristics of this formulation in comparison with the individual components. Finally, the FI-BRSV/CpG/PP formulation induced a reduction in viral replication upon BRSV challenge, which correlates with the enhanced mucosal IgG and IgA. Based on these results, we conclude that FI-BRSV co-

formulated with CpG ODN and PP, delivered intranasally, is a good candidate vaccine formulation for protection against BRSV. Since BRSV and HRSV cause similar clinical disease in their respective host species, an intranasally delivered RSV vaccine co-formulated with CpG ODN and PP might also be efficacious in humans.

4.0 Intranasal immunization of mice with bovine respiratory syncytial virus vaccines induces superior immunity and protection in comparison to subcutaneous delivery or combinations of intranasal and subcutaneous prime-boost strategies

In preparation for publication.

4.1 ABSTRACT

Bovine respiratory syncytial virus (BRSV) infects cells of the respiratory mucosa, so a vaccine that induces mucosal immune responses against BRSV would be desirable. We examined the immune responses and protection induced by formalin-inactivated (FI)-BRSV and BRSV fusion (F) protein vaccines co-formulated with CpG oligodeoxynucleotides (ODN) and polyphosphazenes (PP) using various delivery protocols. Intranasal delivery of FI-BRSV co-formulated with CpG ODN and PP was superior to subcutaneous delivery, and generally superior to combinations of intranasal and subcutaneous prime-boost strategies in terms of humoral, cell-mediated and mucosal immunity, as well as protection against BRSV challenge. Furthermore, intranasal delivery of BRSV F protein, co-formulated with CpG ODN and PP, resulted in stronger immune responses and protection when compared to subcutaneous delivery. Finally, lower levels of viral replication were detected in the lungs of mice immunized with FI-BRSV than in the animals that received recombinant F protein when co-formulated with CpG ODN and PP and delivered intranasally. These results suggest that intranasal delivery of BRSV vaccines

formulated with CpG ODN and PP is an effective protocol in terms of induction of humoral, cell-mediated, and mucosal immunity, as well as protection against BRSV in mice. Because of the similarities between BRSV and human RSV (HRSV), formulation with CpG ODN and PP could prove important in the development of a mucosal vaccine that induces protective immunity against HRSV in humans.

4.2 INTRODUCTION

Human respiratory syncytial virus (HRSV) is the most important cause of lower respiratory tract infection in infants and young children worldwide (Smyth & Openshaw, 2006), and is responsible for significant mortality. In 1999, an estimated 360 HRSV-associated postneonatal (four to 52 weeks old) deaths occurred in the United States (Leader & Kohlhase, 2003). HRSV is also of economic importance. In 2000, there were 86,000 HRSV-related hospitalizations in the United States, costing a total of $394 million USD (Paramore *et al.*, 2004). Like HRSV, bovine respiratory syncytial virus (BRSV) is an enveloped, non-segmented, single-stranded RNA *Pneumovirus* of the family *Paramyxoviridae* and order *Mononegavirales*. BRSV is responsible for significant economic loss to the cattle industry (Stott & Taylor, 1985), and is one of the four known viral components of bovine shipping fever. It has been estimated that BRSV is responsible for more than 60% of epizootic respiratory disease in dairy herds, and up to 70% in beef herds, with typical mortality levels ranging from two to three percent, but ranging up to 20% in some cases (Meyer *et al.*, 2008). HRSV and BRSV have similar clinical outcomes in their respective host species, ranging from asymptomatic infection, to bronchiolitis and pneumonia, and sometimes death (Philippou *et al.*, 2000), and the pathogenesis of both viruses is directly related to the host immune response (Gershwin, 2007).

While several commercial BRSV vaccines are currently available for use in cattle, vaccines that induce longer lasting protection and are more efficacious in the face of maternal antibodies are desirable. Also, there currently is no approved HRSV vaccine for use in humans. In the 1960s, studies using parenterally delivered formalin-inactivated (FI)-HRSV vaccines were carried out in children (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kim *et al.*, 1969; Weibel *et al.*,

1966). Not only did these vaccines fail to protect children from natural infection, in most cases, disease was enhanced. In one study, 80% of vaccinated children were hospitalized and two of them died (Kim *et al.*, 1969).

Once FI-RSV started to be investigated in mouse models, it became generally accepted that the disease enhancement caused by FI-RSV vaccines is due to the induction of a Th2-biased immune response (Connors *et al.*, 1994; Connors *et al.*, 1992; Waris *et al.*, 1997; Waris *et al.*, 1996). An adjuvant that promotes Th1-type or balanced immune responses would therefore be a desirable component of candidate vaccine formulations. CpG oligodexynucleotides (ODN) are short stretches of DNA that feature unmethylated CG dinucleotides flanked by two 5' purines and two 3' pyrimidines. CpG ODN are ligands of toll-like receptor 9, and induce production of IL-1, IL-6, IL-12, and tumor necrosis factor-α by dendritic cells and macrophages, as well as production of IFN-γ, IL-6, and IL-10 by natural killer cells (Ballas *et al.*, 1996; Hartmann & Krieg, 2000; Hartmann *et al.*, 2000). CpG ODN generally induce an overall Th1-type immune response characterized by production of IFN-γ and antigen-specific IgG2a in mice (Chu *et al.*, 1997; Davis *et al.*, 1998; Ioannou *et al.*, 2002a; Jakob *et al.*, 1998). We have previously used CpG ODN to shift the immune response induced by immunization with FI-BRSV from a Th2-biased response to a more balanced response in mice (Mapletoft *et al.*, 2008; Oumouna *et al.*, 2005) and calves (Mapletoft *et al.*, 2006).

Another approach that can be used to avoid the enhancement of disease while maintaining immunity is to alter the route of immunization. For example, while scarification of mice with live recombinant vaccinia virus expressing the HRSV G protein (rVV-G) results in enhanced lung immunopathology upon subsequent challenge (Openshaw *et al.*, 1992), intranasal (IN) and intraperitoneal immunization with rVV-G results in protection and no lung lesions

(Stott *et al.*, 1986). Similarly, IN immunization with rVV expressing the HRSV F protein results in lung inflammation upon challenge that is milder than that induced by intradermal immunization (Matsuoka *et al.*, 2002). In addition, several IN immunization protocols have been successfully used to protect rodents from RSV, including recombinant F protein adjuvanted with CpG ODN (Prince *et al.*, 2003), cholera toxin (Tebbey *et al.*, 2000; Walsh, 1993), or caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate (Tebbey *et al.*, 1999), as well as live viral (hYu *et al.*, 2008; Kahn *et al.*, 2001; Matsuoka *et al.*, 2002; Stott *et al.*, 1987) or bacterial (Cano *et al.*, 2000; Falcone *et al.*, 2006) vectors expressing whole RSV proteins or peptides. Two novel IN vaccine strategies that have also been demonstrated to protect rodents against RSV include the use of chimeras featuring portions of the RSV F protein fused with the ctxA_2B gene of cholera toxin (Singh *et al.*, 2007a; Singh *et al.*, 2007b), as well as the use of a subunit RSV antigen enriched for the F and G proteins and formulated with the proteosome-based adjuvant Protollin (meningococcal outer membrane proteins mixed with LPS from *Shigella flexner*) (Cyr *et al.*, 2007a; Cyr *et al.*, 2007b).

One of the challenges of IN immunization is delivering the vaccine components in such a way that they are not degraded before an immune response can be initiated. Polyphosphazenes (PP) are synthetic polymers that feature a backbone of alternating phosphorus and nitrogen atoms, with organic side groups attached at each phosphorus atom (Payne & Andrianov, 1998). PP form non-covalent complexes when formulated with antigens and/or other adjuvants, enhancing their stability and allowing for multimeric presentation. Immunization with antigens formulated with PP has been shown to induce robust immune responses against rotavirus (McNeal *et al.*, 1999), cholera (Wu *et al.*, 2001), influenza (Mutwiri *et al.*, 2007; Payne *et al.*,

1995; Payne *et al.*, 1998), hepatitis B (Mutwiri *et al.*, 2008), and BRSV (Mapletoft *et al.*, 2008). The latter two studies featured co-formulation of PP and CpG ODN.

We have previously demonstrated the adjuvant effects of CpG ODN in parenterally delivered FI-BRSV vaccines in mice (Oumouna *et al.*, 2005) and calves (Mapletoft *et al.*, 2006), as well as the adjuvant effects of CpG ODN and PP, individually and as co-adjuvants, on an IN-delivered FI-BRSV vaccine in mice (Mapletoft *et al.*, 2008). Here we present two studies that further examine the potential of BRSV vaccines co-formulated with CpG ODN and PP. As route of immunization can affect not only the magnitude and location of immune responses, but also their Th1/Th2 bias, the purpose of these studies was to determine the effects of route of delivery on humoral, mucosal and cell-mediated immunity, as well as protection against BRSV. In the first study, we compared IN and subcutaneous (SC) delivery of FI-BRSV formulated with CpG ODN and/or PP. In the second study, we introduced IN prime/SC boost (IN/SC) and SC prime/IN boost (SC/IN) immunization protocols for FI-BRSV formulated with CpG ODN and PP, as well as a comparison of IN and SC delivery of the BRSV F protein formulated with CpG ODN and PP.

4.3 MATERIALS AND METHODS

4.3.1 Cells and virus - The 375 strain of BRSV (American Type Culture Collection) was propagated in Bovine Turbinate (BT) cells (American Type Culture Collection), and virus titers were determined, as previously described (Mapletoft *et al.*, 2008). FI-BRSV was prepared as previously described (Kim *et al.*, 1969) with modifications (Mapletoft *et al.*, 2008). The final

vaccine protein concentration was 120 μg/ml, and 1.5 μg were given per immunization. Challenge virus was prepared as previously described (Mapletoft *et al.*, 2008). HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen) supplemented with 10% fetal bovine serum (Gibco/Invitrogen), 10 mM HEPES (Gibco/Invitrogen), 0.1 mM non-essential amino acids (Gibco/Invitrogen), and 50 μg/mL gentamycin (Gibco/Invitrogen).

4.3.2 Construction of the truncated BRSV F protein expression plasmid - The truncated F gene (ΔF; encoding amino acids 1 to 522 of the BRSV F protein) was amplified from pMASIA-ΔF with the primers 5'-TCGGATCCATATGACGTCGACGCGTCTG-3' and 5'-GCGGATCCATTTCCCACATCGACGCTGTGAA 3'. The product was then digested with *Hind* III and *Bam* HI (New England Biolabs) and the resulting fragment was inserted into the pcDNA 6/V5-His expression plasmid (Invitrogen). The resulting vector was digested with *Not* I (New England Biolabs) and dephosphorylated using Antarctic Phosphatase (New England Biolabs). A synthetic oligonucleotide (Sigma-Genosys) encoding ten repeating serine-glycine (Ser/Gly) residues, followed by ten histidine (His) residues, was inserted into pcDNA-ΔF, resulting in the pcDNA-ΔF-10His expression plasmid.

4.3.3 Expression and purification of the truncated F protein - Using Lipofectamine Plus Reagent (Invitrogen) in Opti-MEM (Gibco/Invitrogen) as per the manufacturer's instructions, HEK 293 cells were transfected with the pcDNA-ΔF-10His expression plasmid. Forty-eight hours later, medium was harvested and replaced. Medium was harvested twice more at 48 h intervals. Harvested medium was clarified by centrifugation and concentrated by ultrafiltration using a YM 10 membrane (MWCO 10kDa) (Millipore). ProBond nickel-chelating

resin (Invitrogen) was used to purify ΔF under native conditions. Washes with 50 mM monobasic sodium phosphate, 500 mM sodium chloride, pH 8.0, containing 20 mM and 30 mM imidazole (Sigma-Aldrich), respectively, were used to remove impurities. A final wash with 50 mM monobasic sodium phosphate, 500 mM sodium chloride, pH 8.0, containing 300 mM imidazole was used to elute the bound protein. Purified protein was dialyzed against sterile 1X phosphate buffered saline, pH 7.4 (Gibco/Invitrogen) to remove the imidazole. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to confirm molecular mass and purity. A bicinchoninic acid (BCA) assay (Pierce) was used, as per the manufacturer's instructions, to determine protein concentration.

4.3.4 Immunization and challenge - Six to eight week-old female BALB/c mice (Charles River) were randomly allocated into eight groups of 10 animals and immunized with a total volume of 25 μl (for IN immunizations), or 50 μl (for SC immunizations) as indicated in Tables 4.1 and 4.2. FI-BRSV was given at 1.5 μg per immunization, and the BRSV F protein was given at 0.5 μg per immunization. CpG oligodeoxynucleotide (ODN) 1826 (TCC ATG ACG TTC CTG ACG TT) provided by Merial Limited, was phosphorothioate-modified during synthesis to enhance nuclease resistance, and was given at 4 μg per immunization. PP, a 90% substituted poly(di-p-dicarboxylatophenoxy)-phosphazene (PCPP)/10% hydroxylate (90:10 PCPP/OH), was synthesized by John Klaehn (Idaho National Laboratory) according to a previously published method (Andrianov *et al.*, 2004) and was given at 25 μg per immunization. The components of each vaccine were mixed prior to immunization and were given as a single administration. Two weeks after the second immunization, all groups were challenged with BRSV as previously described (Mapletoft *et al.*, 2008). Five mice per group were euthanized

four days after challenge for detection of lung viral RNA, and the remaining mice were euthanized six days after challenge, for bronchoalveolar lavages, lung fragment cultures, lung homogenate supernatants, and isolation of splenocytes for enzyme-linked immunospot assays. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Table 4.1. Immunization protocol for the comparison of intranasal and subcutaneous delivery of FI-BRSV with CpG ODN and PP, individually and as co-adjuvants.

Group	**1st immunization (Day 0)**	**2nd immunization (Day 21)**	**Challenge (Day 35)**
FI-BRSV/CpG (IN)	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
FI-BRSV/PP (IN)	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
FI-BRSV/CpG/PP (IN)	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
FI-BRSV/CpG (SC)	Subcutaneous - 50 μl	Subcutaneous - 50 μl	BRSV
FI-BRSV/PP (SC)	Subcutaneous - 50 μl	Subcutaneous - 50 μl	BRSV
FI-BRSV/CpG/PP (SC)	Subcutaneous - 50 μl	Subcutaneous - 50 μl	BRSV
Saline/BRSV challenge	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
Saline/Saline	Intranasal - 25 μl	Intranasal - 25 μl	Saline

Table 4.2. Immunization protocol for the comparison of combinations of intranasal and subcutaneous prime-boost strategies for the delivery of FI-BRSV or BRSV F protein co-adjuvanted with CpG ODN and PP.

Group	**1st immunization (Day 0)**	**2nd immunization (Day 21)**	**Challenge (Day 35)**
FI-BRSV/CpG/PP (IN/IN)	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
FI-BRSV/CpG/PP (IN/SC)	Intranasal - 25 μl	Subcutan. - 50 μl	BRSV
FI-BRSV/CpG/PP (SC/IN)	Subcutan. - 50 μl	Intranasal - 25 μl	BRSV
FI-BRSV/CpG/PP (SC/SC)	Subcutan. - 50 μl	Subcutan. - 50 μl	BRSV
F/CpG/PP (IN/IN)	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
F/CpG/PP (SC/SC)	Subcutan. - 50 μl	Subcutan. - 50 μl	BRSV
Saline/BRSV challenge	Intranasal - 25 μl	Intranasal - 25 μl	BRSV
Saline/Saline	Intranasal - 25 μl	Intranasal - 25 μl	Saline

4.3.5 Lung fragment culture supernatants - Lung fragment cultures were prepared as previously described (Etchart *et al.*, 2006; Logan *et al.*, 1991), with modifications (Mapletoft *et al.*, 2008), and supernatants were stored at -80 ^{o}C until they could be assayed for IgG and IgA.

4.3.6 Enzyme-linked immunosorbent assay (ELISA) – Sera and lung fragment culture supernatants were assayed for BRSV-specific IgG and IgA, as previously described (Mapletoft *et al.*, 2008).

4.3.7 Virus neutralization assay – Sera and lung fragment culture supernatants were assayed for BRSV neutralizing antibodies as previously described (Mapletoft *et al.*, 2008). Virus neutralizing titers were expressed as the highest dilution of sample that resulted in less than 50% of cells displaying cytopathic effects.

4.3.8 IFN-γ and IL-5 enzyme-linked immunospot (ELISPOT) assays – Splenocytes were isolated as previously described (Baca-Estrada *et al.*, 1996), with modifications (Mapletoft *et al.*, 2008). Splenocytes were restimulated *in vitro* with BRSV antigen and the numbers of IFN-γ and IL-5 secreting cells were determined by ELISPOT assays as previously described (Mapletoft *et al.*, 2008). Spots were counted in a blinded manner with the aid of an inverted microscope. Results are expressed as the difference between the number of cytokine secreting cells per 10^6 cells in BRSV-infected lysate-stimulated wells and the number of cytokine secreting cells per 10^6 cells in mock-infected lysate-stimulated wells.

4.3.9 Eotaxin and IL-5 ELISAs on lung homogenate supernatants - Four days after challenge, mice were euthanized and lungs were removed to 2 ml screw-cap tubes (VWR International) containing 2.4 mm zirconia microbeads (Biospec Products) and 1 ml of DMEM (Gibco/Invitrogen) supplemented with 10 mM HEPES buffer, 0.1 mM non-essential amino acids (Gibco/Invitrogen), 1 mM sodium pyruvate (Gibco/Invitrogen), 50 μg/ml gentamicin (Gibco/Invitrogen), 10 μg/ml aprotinin (Sigma-Aldrich), 10 μg/ml leupeptin (Sigma-Aldrich), 0.1 mM EDTA, 1 mM PMSF (Sigma-Aldrich), and 1X antibiotic-antimycotic (Gibco/Invitrogen). Lungs were homogenized in a mini-beadbeater (BioSpec Products) for 10 sec, clarified by centrifugation for 1 min at 10,000 x *g*, and stored at -80 ^{o}C. Lung homogenate supernatants were assayed for the presence of eotaxin and IL-5 using Quantikine Mouse Immunoassay Kits (R&D Systems), as per the manufacturer's instructions.

4.3.10 Eosinophils in bronchoalveolar lavages – Six days after challenge, mice were euthanized and bronchoalveaolar lavages (BALs) were collected and pooled from each vaccine group. Cytospin slides were prepared using 1 x 10^{5} and 5 x 10^{4} cells, and stained with Wright-Giemsa stain (Bayer HealthCare). The percentage of eosinophils for each vaccine group was determined by examination of at least 200 cells.

4.3.11 Detection of viral RNA – Four days after challenge, mice were euthanized and lungs were removed to 2 ml screw-cap tubes (VWR International) containing 2.4 mm zirconia microbeads (Biospec Products) and 1 ml of Trizol Reagent (Invitrogen), and were homogenized in a mini-beadbeater (BioSpec Products) for 10 sec. RNA was isolated from lung homogenates using the Trizol Reagent method, as per manufacturer's instructions. DNA removal and cDNA

synthesis were performed using the QuantiTect Reverse Transcription Kit (Qiagen), as per manufacturer's instructions. Real-time quantitative PCR (qPCR) reactions were prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), as per the manufacturer's instructions, in iCycler iQ PCR Plates (Bio-Rad) sealed with iCycler iQ Optical Tape (Bio-Rad). Primers amplifying a 168 base pair fragment of the BRSV F gene (primer A: 5'-AACCGGCCTCCTTCAGTAGA-3', primer B: 5'-TGGACACTGCTACACCACTT-3') were designed using primer design software for personal computers (Clone Manager Version 6.00) from a consensus sequence generated from 27 different BRSV F gene sequences using the MultAlin multiple sequence alignment tool available online at **http://bioinfo.genopole-toulouse.prd.fr/multalin/** (Corpet, 1988). qPCR was performed on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad) as previously described (Mapletoft *et al.*, 2008). Serial dilutions of a plasmid that contains a truncated version of the BRSV F gene used in qPCR reactions carried out under the same conditions as those outlined above allowed construction of a standard curve that enabled the determination of gene copy number. Results are expressed as viral RNA copies per ml of lung homogenate.

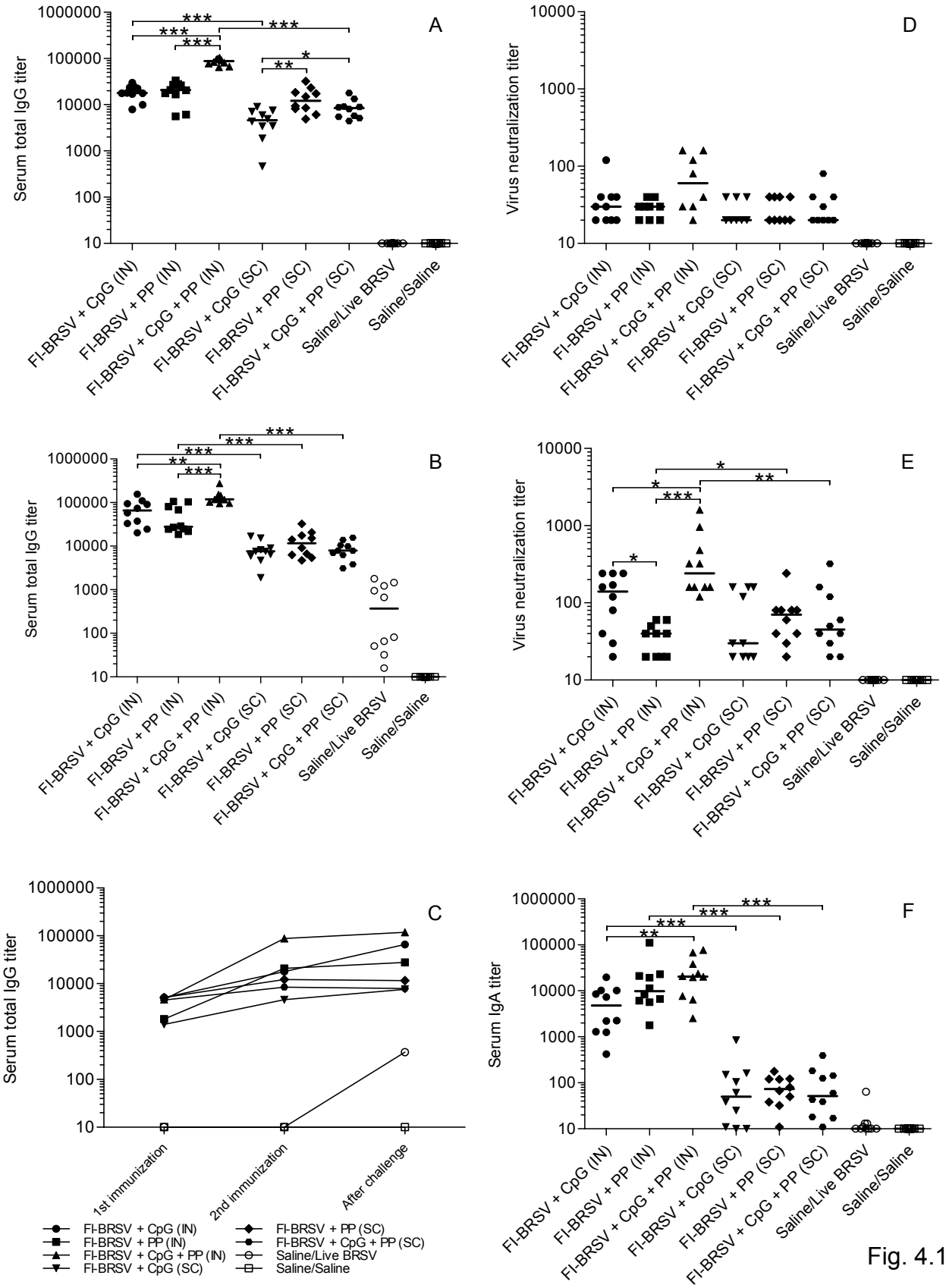
4.3.12 Statistical analysis - All data were analyzed using statistical software (GraphPad Prism Version 3.00). As sample sizes were small (n = 5 or 10), outcome variables were assumed not to be normally distributed. Therefore, differences among all groups were examined using the Kruskal-Wallis test. If a significant difference was found among the groups, median ranks between pairs of groups were compared using the Mann-Whitney U test. Differences were considered significant if $P < 0.05$.

4.4 RESULTS

4.4.1 COMPARISON OF INTRANASAL AND SUBCUTANEOUS DELIVERY OF FI-BRSV WITH CPG ODN AND PP, INDIVIDUALLY AND AS CO-ADJUVANTS

4.4.1.1 BRSV-specific humoral immune responses – The humoral immune responses induced by the various vaccine formulations were examined by measuring the BRSV-specific IgG titers in the serum after two immunizations and after challenge. Following the second immunization, FI-BRSV/CpG/PP(IN) resulted in higher levels of serum IgG production than any other vaccine formulation, and IN delivery of FI-BRSV/CpG resulted in higher levels of IgG than SC delivery (Fig 4.1A). Immunization with either FI-BRSV/PP(SC) or FI-BRSV/CpG/PP(SC) resulted in higher levels of IgG than FI-BRSV/CpG(SC).

Only the IN groups experienced increases in IgG after challenge with BRSV. Immunization with FI-BRSV/CpG/PP(IN) continued to result in more IgG production than any other formulation (Fig. 4.1B). Immunization with FI-BRSV/CpG(IN) or FI-BRSV/CpG/PP(IN) also resulted in higher levels of IgG than the corresponding SC-delivered vaccines. These results demonstrate that, in terms of induction of anti-BRSV serum IgG production, IN inoculation appears to be the preferable route of delivery, with the formulation of FI-BRSV/CpG/PP resulting in higher levels of IgG than the other formulations after two immunizations and after challenge (Fig. 4.1C).


A
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1000000
100000
10000
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100
10
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FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline
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Serum total IgG titer
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FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline
C
Serum total IgG titer
1000000
100000
10000
1000
100
10
1st immunization
2nd immunization
After challenge
FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline
D
Virus neutralization titer
10000
1000
100
10
FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline
E
Virus neutralization titer
10000
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10
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FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline
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Serum IgA titer
1000000
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FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline

Fig. 4.1

Figure 4.1. BRSV-specific IgG in sera after two immunizations (A) and after challenge (B). Kinetics of the BRSV-specific serum IgG response (C). Virus neutralizing antibodies in sera after two immunizations (D) and after challenge (E). BRSV-specific IgA in sera after challenge (F). Mice were immunized with FI-BRSV/CpG(IN), FI-BRSV/PP(IN), FI-BRSV/CpG/PP(IN), FI-BRSV/CpG(SC), FI-BRSV/PP(SC), FI-BRSV/CpG/PP(SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Virus neutralization titers are expressed as the highest dilution of serum that resulted in less than 50% of cells displaying cytopathic effects. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

To evaluate the biological effectiveness of the antibodies produced in the sera, virus neutralizing titers were determined after two immunizations and after challenge. After two immunizations, mice immunized IN with FI-BRSV/CpG/PP tended to have higher levels of neutralizing antibodies than mice that received any other formulation, although the differences were not significant (Fig. 4.1D). Only three formulations resulted in increases in neutralizing antibodies after challenge: FI-BRSV/CpG(IN), FI-BRSV/CpG/PP(IN), and FI-BRSV/PP(SC). FI-BRSV/CpG/PP(IN) was the only formulation that resulted in higher levels of neutralizing antibodies than its corresponding SC-delivered vaccine after challenge (Fig. 4.1E). Conversely, SC delivery of FI-BRSV/PP resulted in higher levels of neutralizing antibodies than IN delivery. Among the IN-delivered vaccines, FI-BRSV/CpG/PP induced higher levels of neutralizing antibodies than either FI-BRSV/CpG or FI-BRSV/PP, and FI-BRSV/CpG induced higher levels of neutralizing antibodies than FI-BRSV/PP. Thus, in terms of virus neutralizing antibodies in the serum, IN delivery of FI-BRSV co-formulated with CpG ODN and PP tended to perform better than any other formulation.

In addition, BRSV-specific serum IgA was measured after challenge. Immunization with any of the three IN formulations resulted in higher levels of IgA than immunization with the corresponding SC-delivered vaccines, with FI-BRSV/CpG/PP(IN) inducing higher levels of IgA than FI-BRSV/CpG(IN) (Fig. 4.1F).

4.4.1.2 BRSV-specific cell-mediated immune responses – To further evaluate the type of immune responses induced by the various vaccine formulations, the BRSV-induced secretion of IFN-γ and IL-5 by *in vitro* restimulated splenocytes was measured six days after challenge. Immunization with any of the three IN formulations resulted in higher levels of IFN-γ secretion

than immunization with the corresponding SC-delivered vaccines (Fig. 4.2A). Among the IN-delivered vaccines, FI-BRSV/CpG/PP elicited higher levels of IFN-γ secretion than FI-BRSV/CpG or FI-BRSV/PP, and FI-BRSV/CpG induced higher levels of IFN-γ secretion than FI-BRSV/PP. Similarly, among the SC-delivered vaccines, FI-BRSV/CpG/PP induced higher levels of IFN-γ secretion than FI-BRSV/CpG or FI-BRSV/PP, though the numbers of IFN-γ secreting cells induced by these vaccines was much lower than those elicited by the IN vaccines.

In terms of IL-5 secretion, all three IN-delivered vaccines induced lower levels of IL-5 than the corresponding SC-delivered vaccines (Fig. 4.2B). FI-BRSV/PP induced the highest levels of IL-5 secretion, regardless of the route of delivery. In general, the vaccines formulated with CpG ODN induced a Th1-biased immune response characterized by high levels of IFN-γ secretion and low levels of IL-5 secretion, with the IN-delivered vaccines inducing more IFN-γ secretion and less IL-5 secretion than the corresponding SC-delivered vaccines. These results suggest that FI-BRSV/CpG/PP(IN) and FI-BRSV/CpG(IN) induced a Th1-biased immune response, in contrast to the other strategies, which either resulted in balanced or Th2-type immune responses.

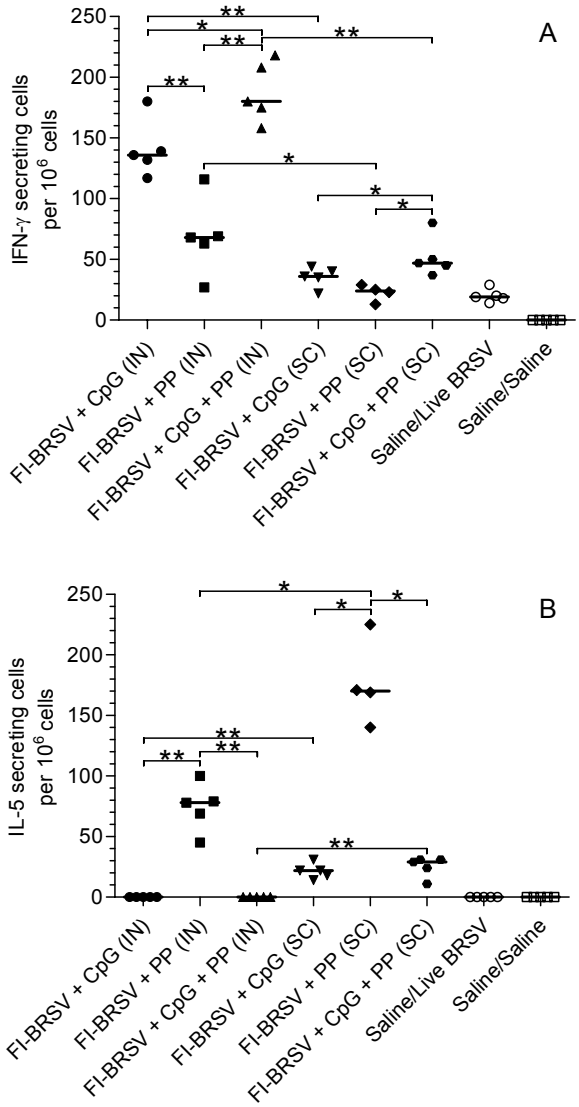

A
IFN-γ secreting cells per 10^6 cells
250
200
150
100
50
0
**
*
**
**
**
*
*
*
FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline
B
IL-5 secreting cells per 10^6 cells
250
200
150
100
50
0
*
*
*
**
**
**
FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline

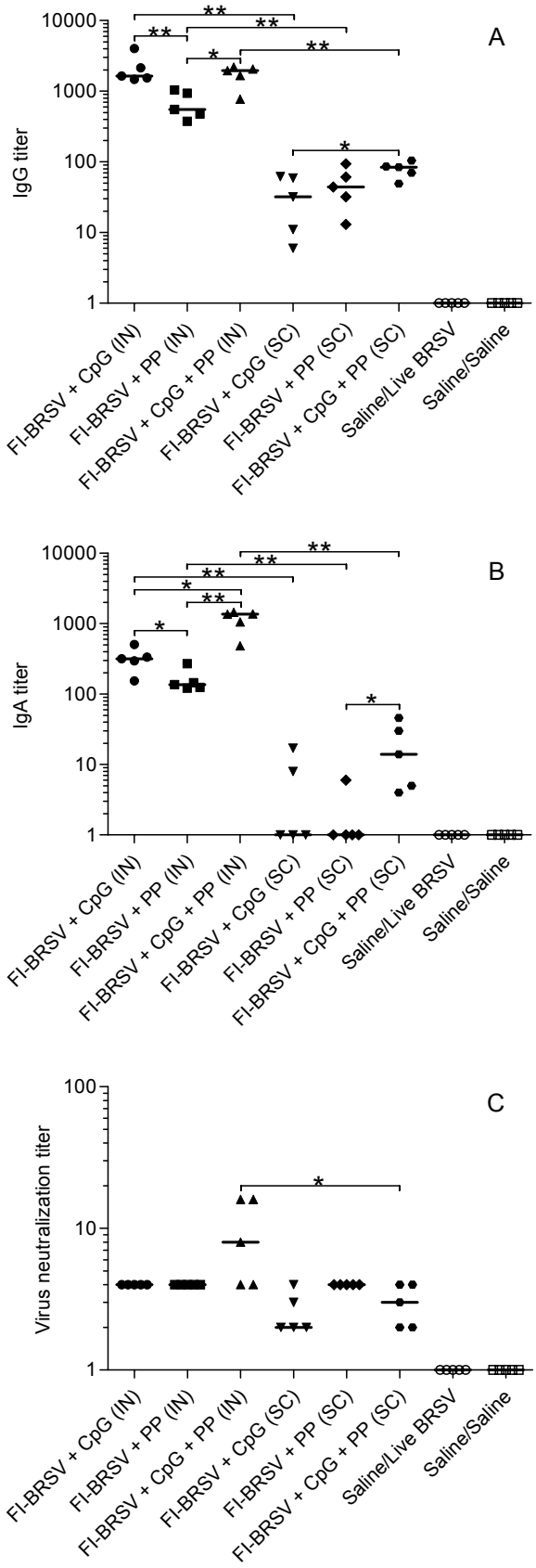
Fig. 4.2

Figure 4.2. Numbers of IFN-γ (A) and IL-5 (B) secreting splenocytes in response to *in vitro* restimulation with BRSV-infected cell lysates. Mice were immunized with FI-BRSV/CpG(IN), FI-BRSV/PP(IN), FI-BRSV/CpG/PP(IN), FI-BRSV/CpG(SC), FI-BRSV/PP(SC), FI-BRSV/CpG/PP(SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

4.4.1.3 BRSV-specific mucosal immune responses – To evaluate the mucosal immune responses induced by the various vaccine formulations, the secretion of BRSV-specific IgG and IgA in lung fragment culture supernatants was measured. In terms of inducing IgG production, all three IN delivered vaccines outperformed the corresponding SC delivered vaccines (Fig. 4.3A). Among the IN-delivered vaccines, both FI-BRSV/CpG and FI-BRSV/CpG/PP induced higher IgG levels than FI-BRSV/PP. Whereas among the SC-delivered vaccines, FI-BRSV/CpG/PP induced higher IgG levels than FI-BRSV/CpG, but overall, IgG levels were much lower than those elicited by the IN-delivered vaccines.

As was seen with IgG production, all three IN delivered vaccines induced higher IgA titers than the corresponding SC-delivered vaccines (Fig. 4.3B). Among the IN-delivered vaccines, FI-BRSV/CpG/PP induced higher IgA titers than FI-BRSV/CpG or FI-BRSV/ PP, and FI-BRSV/CpG induced higher IgA levels than FI-BRSV/PP. Among the SC delivered vaccines, FI-BRSV/CpG/PP induced higher IgA levels than FI-BRSV/PP. Similar to what was observed with IgG, the IgA amounts induced by the SC delivered vaccines were overall much lower than those induced by the IN delivered vaccines. As expected, in terms of induction of anti-BRSV IgG and IgA production in lung fragment cultures, IN inoculation was the preferable route of delivery, with IN formulations that contain CpG ODN outperforming FI-BRSV formulated with PP alone.

To evaluate the biological effectiveness of the antibodies produced in the lungs, virus neutralizing titers were determined. While there were no significant differences among the IN or SC groups, IN delivery of FI-BRSV/CpG/PP resulted in significantly higher virus neutralizing antibody levels than SC delivery (Fig 4.3C).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1000
100
10
1
B
IgA titer
C
Virus neutralization titer
100
10
1
FI-BRSV + CpG (IN)
FI-BRSV + PP (IN)
FI-BRSV + CpG + PP (IN)
FI-BRSV + CpG (SC)
FI-BRSV + PP (SC)
FI-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline

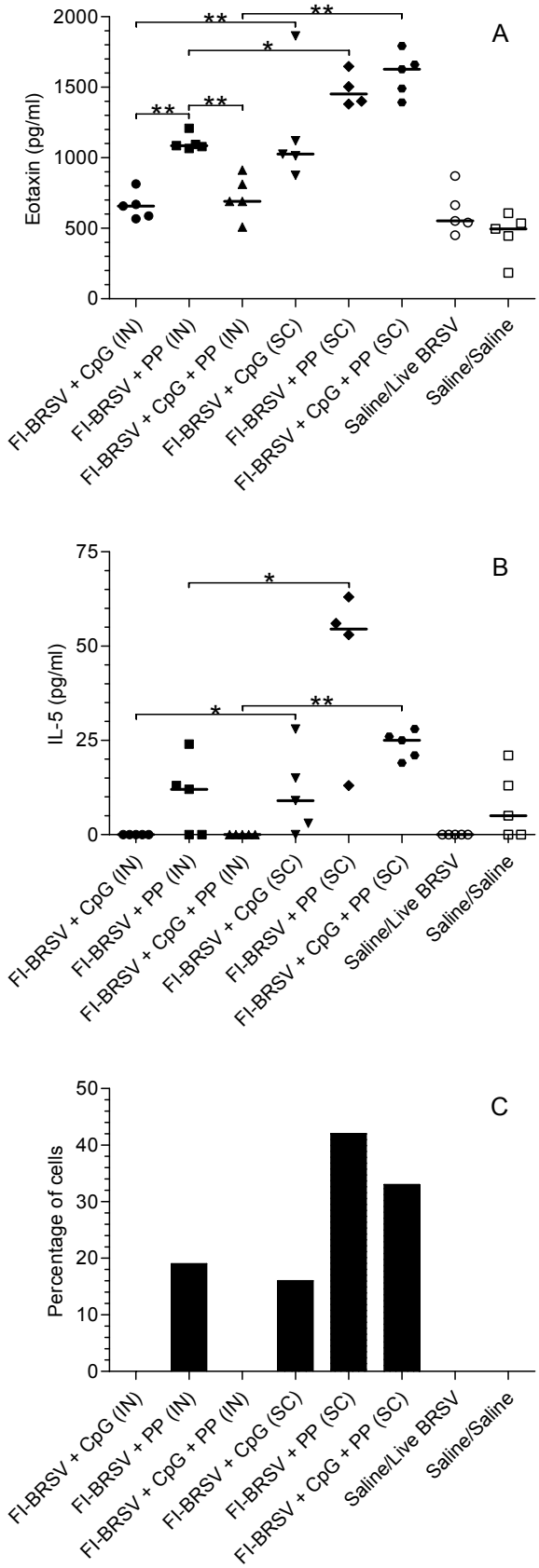
Fig. 4.3

Figure 4.3. BRSV-specific IgG (A), IgA (B) and virus neutralizing antibodies (C) in lung fragment culture supernatants. Mice were immunized with FI-BRSV/CpG(IN), FI-BRSV/PP(IN), FI-BRSV/CpG/PP(IN), FI-BRSV/CpG(SC), FI-BRSV/PP(SC), FI-BRSV/CpG/PP(SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Virus neutralization titers are expressed as the highest dilution of lung fragment culture supernatant that resulted in less than 50% of cells displaying cytopathic effects. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

4.4.1.4 Eotaxin and IL-5 production and eosinophilia in the lungs – The effects of the various vaccine formulations on the type of immune response induced was further examined by measuring the amounts of eotaxin and IL-5 produced in the lungs. Each of the three SC-delivered vaccines induced production of more eotaxin than the corresponding IN-delivered vaccines (Fig. 4.4A). Among the IN-delivered vaccines, FI-BRSV/PP induced higher levels of eotaxin production than FI-BRSV/CpG or FI-BRSV/CpG/PP.

All three SC groups produced more IL-5 than the corresponding IN groups (Fig. 4.4B). In terms of eotaxin and IL-5 production in the lungs, the IN-delivered vaccines induced lower levels than the corresponding SC-delivered vaccines. Furthermore, vaccines that were formulated with CpG ODN tended to induce lower levels of eotaxin and IL-5 than vaccines formulated with PP alone.

The presence or absence of pulmonary eosinophilia was also determined six days after challenge. Eosinophils were found in the lungs of all groups that received SC delivered vaccines (Fig. 4.4C). Among IN groups, however, eosinophils were only found in the group that received FI-BRSV/PP. These data show that IN delivered FI-BRSV vaccines that contain CpG ODN do not result in the induction of pulmonary eosinophilia upon subsequent challenge with BRSV. As these determinations were performed on BALs pooled from each vaccine group, no statistical analyses were possible.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Eotaxin (pg/ml)
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1500
1000
500
0
**
**
**
*
**
B
IL-5 (pg/ml)
75
50
25
0
*
*
**
C
Percentage of cells
50
40
30
20
10
0
Fl-BRSV + CpG (IN)
Fl-BRSV + PP (IN)
Fl-BRSV + CpG + PP (IN)
Fl-BRSV + CpG (SC)
Fl-BRSV + PP (SC)
Fl-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline

Fig. 4.4

Figure 4.4. Eotaxin (A) and IL-5 (B) production in lung homogenate supernatants, and percentage of eosinophils in bronchoalveolar lavages (C). Mice were immunized with FI-BRSV/CpG(IN), FI-BRSV/PP(IN), FI-BRSV/CpG/PP(IN), FI-BRSV/CpG(SC), FI-BRSV/PP(SC), FI-BRSV/CpG/PP(SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). For (A) and (B), each data point represents an individual animal, and median values are indicated by horizontal bars. For (C), bars represent the percentage of eosinophils in pooled bronchoalveolar lavage fluids per minimum of 200 cells. *$P < 0.05$; **$P < 0.01$.

4.4.1.5 Detection of viral RNA in the lung tissue - To assess the ability of the various vaccine formulations to protect mice from infection, virus replication in the lungs was examined by detection of viral RNA. With the exception of the group that received FI-BRSV/CpG/PP(SC), all vaccinated groups experienced decreases in viral replication, when compared to the mock-vaccinated, virus-challenged control group (Fig. 4.5). Among IN formulations, vaccination with FI-BRSV/CpG or FI-BRSV/CpG/PP resulted in lower viral replication than immunization with FI-BRSV/PP. IN delivery of FI-BRSV/CpG resulted in lower viral replication than SC delivery. Immunization with FI-BRSV/PP(IN) or FI-BRSV/CpG/PP(IN), however, did not result in significant decreases compared to immunization with the corresponding SC-delivered vaccines. Of all the formulations tested, FI-BRSV/CpG/PP(IN) resulted in the lowest median viral RNA copy number. The low viral RNA copy numbers observed in the mock-vaccinated, mock-challenged mice were due to the formation of non-specific PCR products synthesized in the absence of viral RNA. Although five out of six of the vaccinated groups experienced decreases in viral RNA levels, compared to the mock-vaccinated, live-challenged control group, there was an overall trend for IN-delivered vaccines formulated with CpG to result in additional decreases in viral replication.

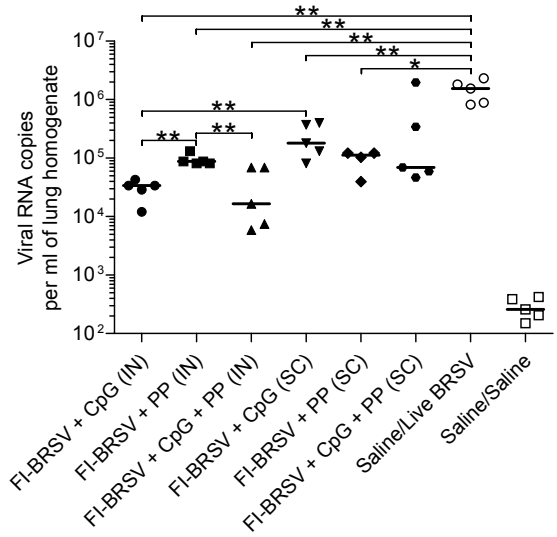

Viral RNA copies
per ml of lung homogenate
10^7
10^6
10^5
10^4
10^3
10^2
**
**
**
**
*
**
**
**
Fl-BRSV + CpG (IN)
Fl-BRSV + PP (IN)
Fl-BRSV + CpG + PP (IN)
Fl-BRSV + CpG (SC)
Fl-BRSV + PP (SC)
Fl-BRSV + CpG + PP (SC)
Saline/Live BRSV
Saline/Saline

Fig. 4.5

Figure 4.5. Detection of viral RNA in lung tissue. Mice were immunized with FI-BRSV/CpG(IN), FI-BRSV/PP(IN), FI-BRSV/CpG/PP(IN), FI-BRSV/CpG(SC), FI-BRSV/PP(SC), FI-BRSV/CpG/PP(SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Results are expressed as viral RNA copies per ml of lung homogenate. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

4.4.2 COMPARISON OF COMBINATIONS OF INTRANASAL AND SUBCUTANEOUS PRIME-BOOST STRATEGIES FOR THE DELIVERY OF FI-BRSV OR BRSV F PROTEIN CO-ADJUVANTED WITH CpG ODN AND PP

4.4.2.1 BRSV-specific humoral immune responses – To determine which immunization strategy induced the highest levels of humoral immunity, BRSV-specific serum IgG titers were determined after two immunizations and after BRSV challenge. FI-BRSV/CpG/PP(IN/IN) induced higher levels of serum IgG production than any other FI-BRSV formulation but the same as those elicited by F/CpG/PP(IN/IN) (Fig. 4.6A). Both IN/SC and SC/IN delivery of the FI-BRSV formulation resulted in higher antibody responses than SC/SC delivery. Mice that received F/CpG/PP(SC/SC) developed higher IgG titers than mice that were given FI-BRSV/CpG/PP(SC/SC) and the same as animals that received F/CpG/PP(IN/IN).

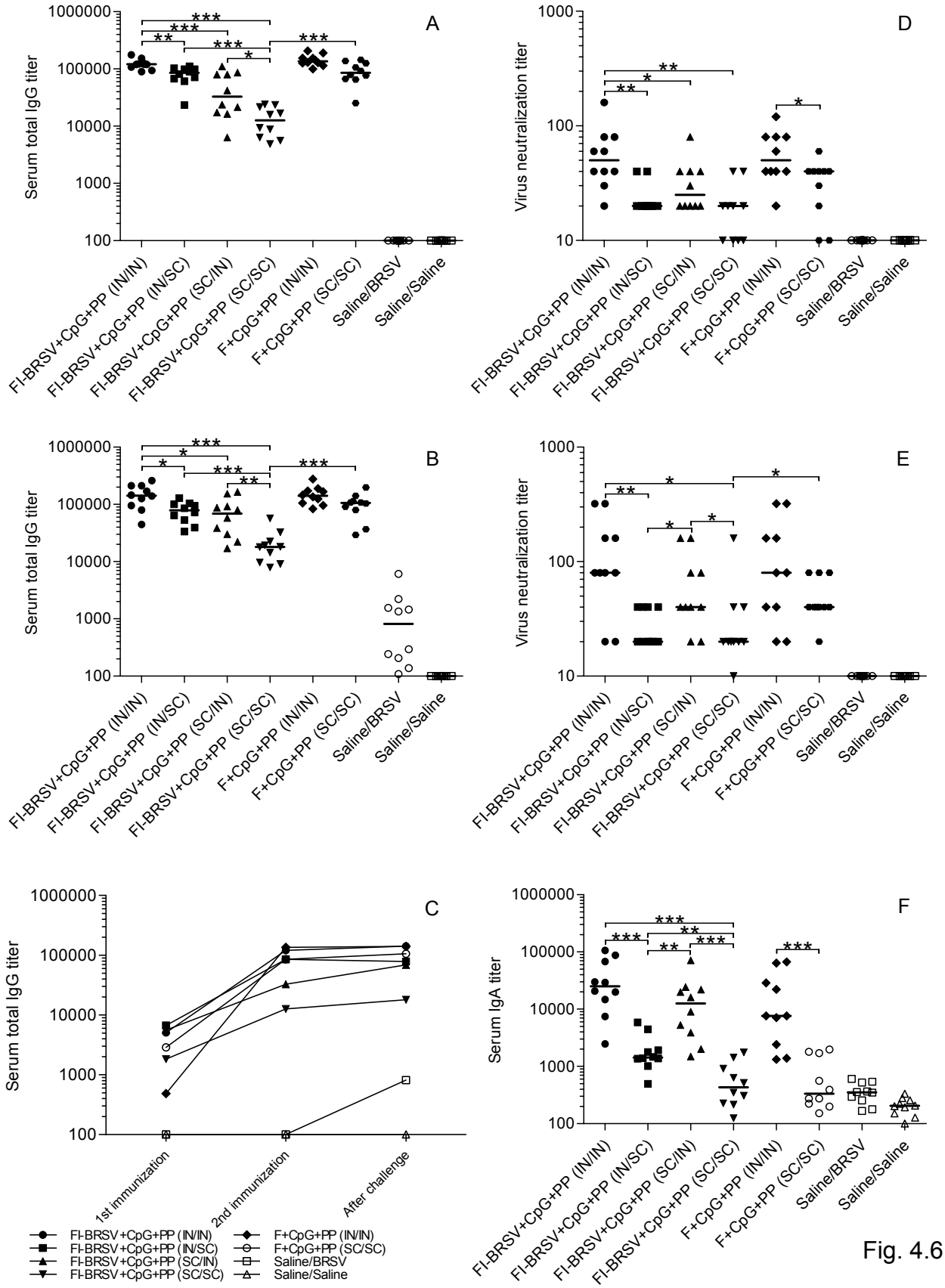

A
B
C
D
E
F
Serum total IgG titer
Virus neutralization titer
Serum IgA titer
1000000
100000
10000
1000
100
10
***
**
*
Fl-BRSV+CpG+PP (IN/IN)
Fl-BRSV+CpG+PP (IN/SC)
Fl-BRSV+CpG+PP (SC/IN)
Fl-BRSV+CpG+PP (SC/SC)
F+CpG+PP (IN/IN)
F+CpG+PP (SC/SC)
Saline/BRSV
Saline/Saline
1st immunization
2nd immunization
After challenge

Fig. 4.6

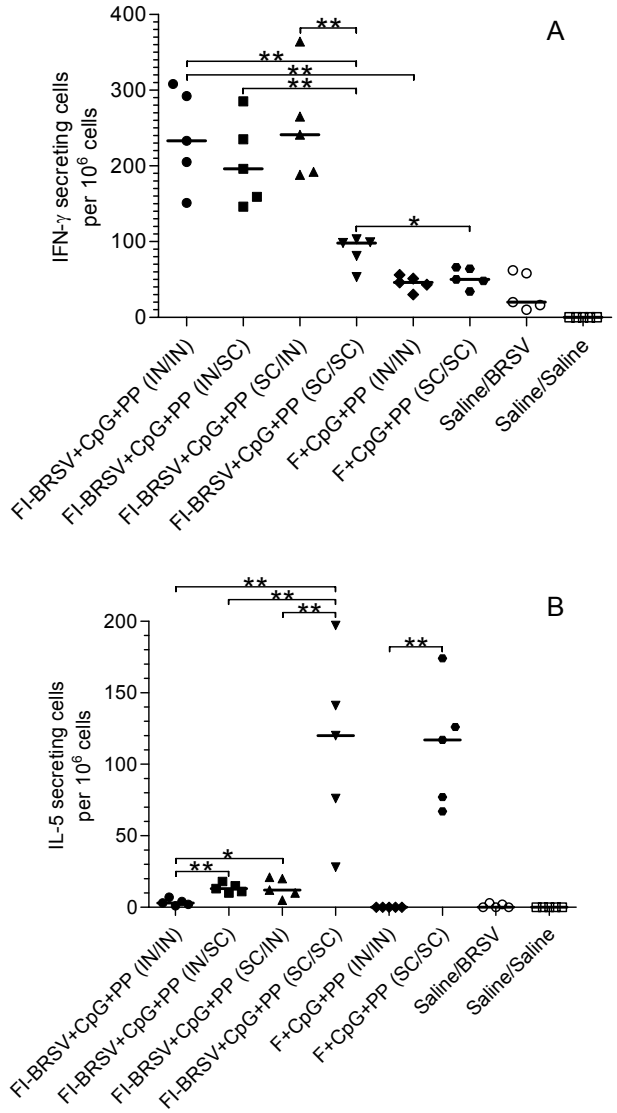
Figure 4.6. BRSV-specific IgG in sera after two immunizations (A) and after challenge (B). Kinetics of the BRSV-specific serum IgG response (C). Virus neutralizing antibodies in sera after two immunizations (D) and after challenge (E). BRSV-specific IgA in sera after challenge (F). Mice were immunized with FI-BRSV/CpG/PP(IN/IN), FI-BRSV/CpG/PP(IN/SC), FI-BRSV/CpG/PP(SC/IN), FI-BRSV/CpG/PP(SC/SC), F/CpG/PP(IN/IN), F/CpG/PP(SC/SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Virus neutralization titers are expressed as the highest dilution of serum that resulted in less than 50% of cells displaying cytopathic effects. Each data point represents an individual animal, and median values are indicated by horizontal bars. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

No increases in IgG production were observed after challenge; the immunized groups displayed a trend that was very similar to that seen after the second immunization. Animals that were given FI-BRSV/CpG/PP(IN/IN) continued to display higher IgG titers than mice that received any other FI-BRSV formulation and the same as animals that were given F/CpG/PP(IN/IN) (Fig 4.6B). Both IN/SC and SC/IN delivery of FI-BRSV continued to result in higher IgG levels than SC/SC delivery. Finally, F/CpG/PP(SC/SC) continued to induce IgG levels that were higher than those induced by FI-BRSV/CpG/PP(SC/SC) and the same as those elicited by F/CpG/PP(IN/IN). These results suggest, that in terms of induction of anti-BRSV serum IgG production, the route of delivery is critical for FI-BRSV/CpG/PP vaccines, with IN/IN performing better, and SC/SC performing worse than any of the other routes. The route of delivery appears to be of less importance, however, when immunizing with F/CpG/PP as there were no differences observed between IN/IN and SC/SC delivery after the second immunization or after challenge.

The biological activity of the antibodies produced in the sera was evaluated by determining virus neutralizing titers after two immunizations and after challenge. After the second immunization, FI-BRSV/CpG/PP(IN/IN) and F/CpG/PP(IN/IN) induced higher neutralizing antibody titers than the corresponding vaccines delivered via different routes (Fig. 4.6D). After challenge, both IN/IN and SC/IN delivery of FI-BRSV/CpG/PP resulted in higher neutralizing antibody levels than IN/SC or SC/SC delivery, and F/CpG/PP(SC/SC) elicited higher neutralizing antibody titers than FI-BRSV/CpG/PP(SC/SC) (Fig. 4.6E). Overall, FI-BRSV/CpG/PP delivered IN tended to be the superior formulation and route, in terms of the induction of virus neutralizing antibody production in the serum, both after two immunizations and after challenge.

In addition, BRSV-specific serum IgA was measured after challenge. IN/IN delivery of FI-BRSV/CpG/PP resulted in IgA levels that were higher than those resulting from IN/SC or SC/SC delivery but the same as those induced by FI-BRSV/CpG/PP(SC/IN) or F/CpG/PP(IN/IN) (Fig 4.6F). Both IN/SC and SC/IN delivery of the FI-BRSV resulted in higher IgA levels than those induced by SC/SC delivery, and the SC/IN delivered vaccine performed better than the IN/SC delivered vaccine. Furthermore, recipients of F/CpG/PP(IN/IN) experienced stronger IgA responses than recipients of the corresponding SC/SC delivered vaccine. In contrast to IgG production, IN/IN or combinations of IN and SC delivery resulted in much higher IgA titers than SC/SC delivery. The route of delivery, therefore appears to be critical in determining the amounts of serum IgA induced by FI-BRSV/CpG/PP and F/CpG/PP vaccines.

4.4.2.2 BRSV-specific cell-mediated immune responses – BRSV-induced secretion of IFN-γ and IL-5 by *in vitro* restimulated splenocytes was determined six days after challenge as an indicator of cell-mediated immunity. IN/IN, IN/SC and SC/IN delivery of FI-BRSV/CpG/PP resulted in higher levels of IFN-γ secretion than SC/SC delivery (Fig 4.7A). Mice that were given FI-BRSV/CpG/PP(IN/IN) experienced greater levels of IFN-γ secretion than animals that received F/CpG/PP(IN/IN), and FI-BRSV/CpG/PP(SC/SC) elicited higher levels of IFN-γ secretion than F/CpG/PP(SC/SC).


A
IFN-γ secreting cells per 10^6 cells
400
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Fl-BRSV+CpG+PP (IN/IN)
Fl-BRSV+CpG+PP (IN/SC)
Fl-BRSV+CpG+PP (SC/IN)
Fl-BRSV+CpG+PP (SC/SC)
F+CpG+PP (IN/IN)
F+CpG+PP (SC/SC)
Saline/BRSV
Saline/Saline
B
IL-5 secreting cells per 10^6 cells
200
150
100
50
0
**
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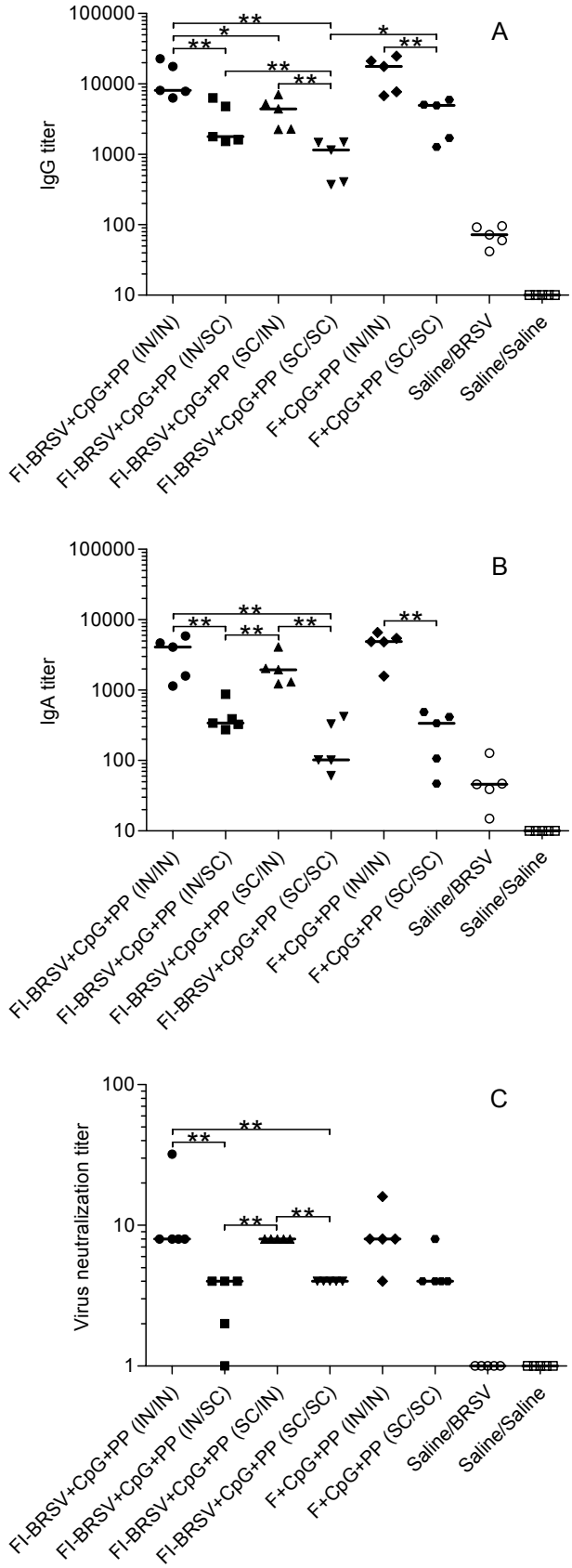
*
Fl-BRSV+CpG+PP (IN/IN)
Fl-BRSV+CpG+PP (IN/SC)
Fl-BRSV+CpG+PP (SC/IN)
Fl-BRSV+CpG+PP (SC/SC)
F+CpG+PP (IN/IN)
F+CpG+PP (SC/SC)
Saline/BRSV
Saline/Saline

Fig. 4.7

Figure 4.7. Numbers of IFN-γ (A) and IL-5 (B) secreting splenocytes in response to *in vitro* restimulation with BRSV-infected cell lysates. Mice were immunized with FI-BRSV/CpG/PP(IN/IN), FI-BRSV/CpG/PP(IN/SC), FI-BRSV/CpG/PP(SC/IN), FI-BRSV/CpG/PP(SC/SC), F/CpG/PP(IN/IN), F/CpG/PP(SC/SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

The groups that received FI-BRSV/CpG/PP(SC/SC) or F/CpG/PP(SC/SC) exhibited the highest numbers of IL-5 secreting cells, and IN/IN delivery of FI-BRSV/CpG/PP resulted in lower levels of IL-5 production than IN/SC or SC/IN delivery (Fig 4.7B). SC/SC delivery of FI-BRSV/CpG/PP gave rise to much lower levels of IFN-γ secretion, and much higher levels of IL-5 secretion than the other delivery routes. Furthermore, SC/SC delivery of F/CpG/PP led to much higher levels of IL-5 secretion than IN/IN delivery, again indicating that IN delivery is preferable to SC delivery with regards to the balance between IFN-γ and IL-5 secretion by *in vitro* restimulated splenocytes.

4.4.2.3 BRSV-specific mucosal immune responses – The secretion of BRSV-specific IgG and IgA in lung fragment cultures was determined as an indicator of mucosal immunity. In terms of IgG production, IN/IN delivery of FI-BRSV/CpG/PP gave rise to stronger responses than those resulting from IN/SC, SC/IN or SC/SC delivery, but the same as those induced by F/CpG/PP(IN/IN) (Fig 4.8A). IN/SC and SC/IN delivery of FI-BRSV/CpG/PP led to higher IgG titers than SC/SC delivery. Furthermore, IN/IN delivery of F/CpG/PP resulted in higher IgG levels than SC/SC delivery, and recipients of F/CpG/PP(SC/SC) showed higher IgG titers than recipients of FI-BRSV/CpG/PP(SC/SC).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10000
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10
C
Virus neutralization titer
100
10
1
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*
Fl-BRSV+CpG+PP (IN/IN)
Fl-BRSV+CpG+PP (IN/SC)
Fl-BRSV+CpG+PP (SC/IN)
Fl-BRSV+CpG+PP (SC/SC)
F+CpG+PP (IN/IN)
F+CpG+PP (SC/SC)
Saline/BRSV
Saline/Saline

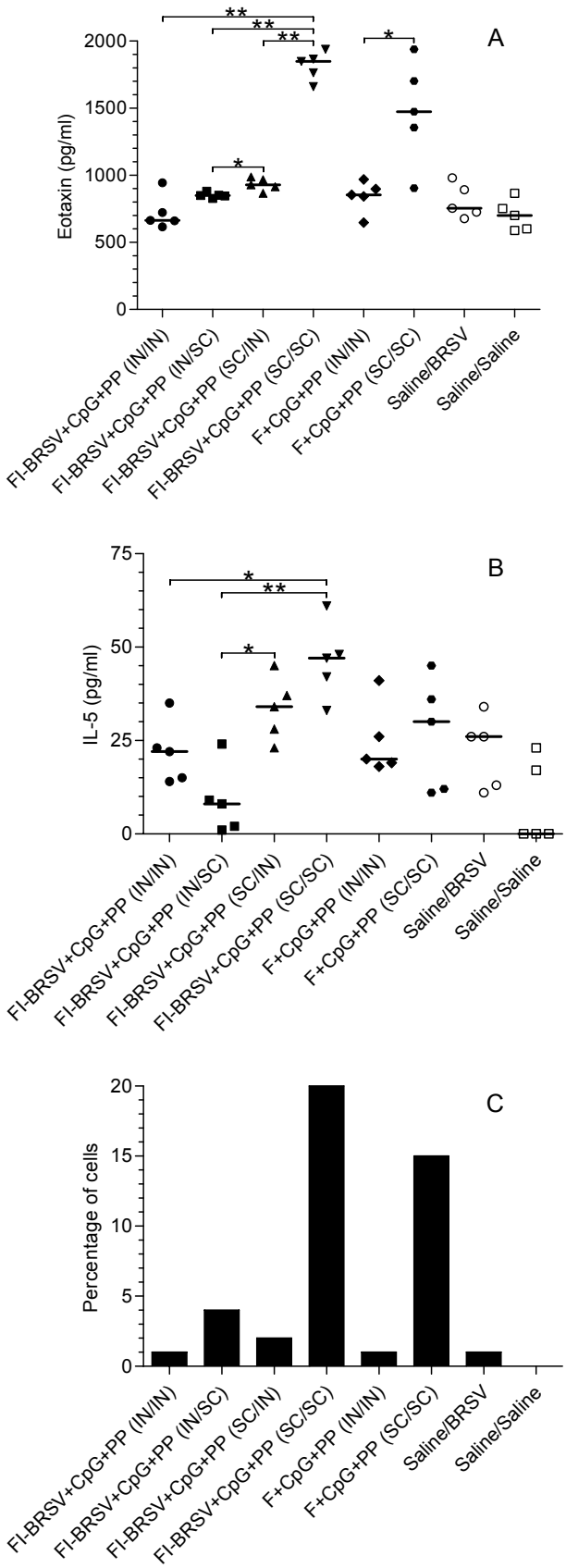
Fig. 4.8

Figure 4.8. BRSV-specific IgG (A), IgA (B) and virus neutralizing antibodies (C) in lung fragment culture supernatants. Mice were immunized with FI-BRSV/CpG/PP(IN/IN), FI-BRSV/CpG/PP(IN/SC), FI-BRSV/CpG/PP(SC/IN), FI-BRSV/CpG/PP(SC/SC), F/CpG/PP(IN/IN), F/CpG/PP(SC/SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Virus neutralization titers are expressed as the highest dilution of lung fragment culture supernatant that resulted in less than 50% of cells displaying cytopathic effects. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

The determination of IgA production in lung fragment culture supernatants revealed a pattern similar to that observed for IgG production. IN/IN delivery of FI-BRSV/CpG/PP gave rise to higher IgA titers than those resulting from IN/SC or SC/SC delivery, and the same as those induced by FI-BRSV/CpG/PP(SC/IN) or F/CpG/PP(IN/IN) (Fig 4.8B). SC/IN delivery of FI-BRSV/CpG/PP led to higher IgA titers than IN/SC or SC/SC delivery, and IN/IN delivery of F/CpG/PP resulted in higher IgA levels than SC/SC delivery. With respect to IgG and IgA production in lung fragment culture supernatants, both FI-BRSV/CpG/PP and F/CpG/PP vaccines induced the highest antibody levels when given IN/IN, and the lowest antibody levels when given SC/SC.

The biological effectiveness of the antibodies produced in the lungs was evaluated by determining the virus neutralizing titers. Both IN/IN and SC/IN delivery of FI-BRSV/CpG/PP resulted in higher neutralizing antibody levels than IN/SC or SC/SC delivery (Fig. 4.8C).

4.4.2.4 Eotaxin and IL-5 production and eosinophilia in the lungs – The amounts of eotaxin and IL-5 produced in the lungs were determined in order to further examine the effects of the various routes of delivery on the type of immune response induced. With the exception of FI-BRSV/CpG/PP(SC/SC) and F/CpG/PP(SC/SC), all other vaccines elicited eotaxin levels that were comparable to those induced by the control groups; mice that received FI-BRSV/CpG/PP(SC/SC) produced much higher amounts of eotaxin than animals that were given FI-BRSV by any other route, and SC/SC delivery of F/CpG/PP led to much higher eotaxin levels than IN/IN delivery (Fig. 4.9A). As well, SC/IN delivery of FI-BRSV/CpG/PP resulted in higher eotaxin levels than IN/SC delivery.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1000
500
0
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Fl-BRSV+CpG+PP (IN/IN)
Fl-BRSV+CpG+PP (IN/SC)
Fl-BRSV+CpG+PP (SC/IN)
Fl-BRSV+CpG+PP (SC/SC)
F+CpG+PP (IN/IN)
F+CpG+PP (SC/SC)
Saline/BRSV
Saline/Saline
B
IL-5 (pg/ml)
75
50
25
0
*
**
*
C
Percentage of cells
20
15
10
5
0

Fig. 4.9

Figure 4.9. Eotaxin (A) and IL-5 (B) production in lung homogenate supernatants, and percentage of eosinophils in bronchoalveolar lavages (C). Mice were immunized with FI-BRSV/CpG/PP(IN/IN), FI-BRSV/CpG/PP(IN/SC), FI-BRSV/CpG/PP(SC/IN), FI-BRSV/CpG/PP(SC/SC), F/CpG/PP(IN/IN), F/CpG/PP(SC/SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). For (A) and (B), each data point represents an individual animal, and median values are indicated by horizontal bars. For (C), bars represent the percentage of eosinophils in pooled bronchoalveolar lavage fluids per minimum of 200 cells. *$P < 0.05$; **$P < 0.01$.

SC/SC delivery of FI-BRSV/CpG/PP elicited higher lung IL-5 levels than IN/IN or IN/SC delivery, and SC/IN delivery gave rise to higher IL-5 levels than IN/SC delivery (Fig 4.9B). In terms of lung IL-5 production, there were no other differences among or between FI-BRSV and F protein vaccines.

The presence or absence of pulmonary eosinophilia was determined six days after challenge. The only groups that displayed relatively high numbers of eosinophils were the groups that received FI-BRSV/CpG(SC/SC) and F/CpG/PP(SC/SC); none of the IN/IN or combination delivery methods resulted in eosinophilia (Fig. 4.9C). As these determinations were performed on BALs pooled from each group, no statistical analyses were possible. These data suggest that the route of delivery is critical in determining whether or not FI-BRSV/CpG/PP and F/CpG/PP vaccines result in the induction of lung eotaxin production and lung eosinophilia.

4.4.2.5 Detection of viral RNA in the lung tissue - The level of virus replication in the lungs was determined in order to assess the ability of the various vaccine formulations to protect from BRSV infection. All vaccinated groups experienced decreases in viral replication, compared to the mock-vaccinated, virus-challenged control group. In terms of reducing viral replication, FI-BRSV/CpG/PP(IN/IN) performed better than FI-BRSV/CpG/PP(SC/SC) or F/CpG/PP(IN/IN), and the same as FI-BRSV/CpG/PP(IN/SC) and FI-BRSV/CpG/PP(SC/IN) (Fig 4.10). IN/IN delivery of F/CpG/PP, however, did result in lower levels of viral replication than SC/SC delivery. These data suggest that, in terms of reducing viral replication, IN/IN delivery of both FI-BRSV/CpG/PP and F/CpG/PP vaccines is superior to SC/SC delivery, and that FI-BRSV/CpG/PP(IN/IN) is a better vaccine formulation than F/CpG/PP(IN/IN).

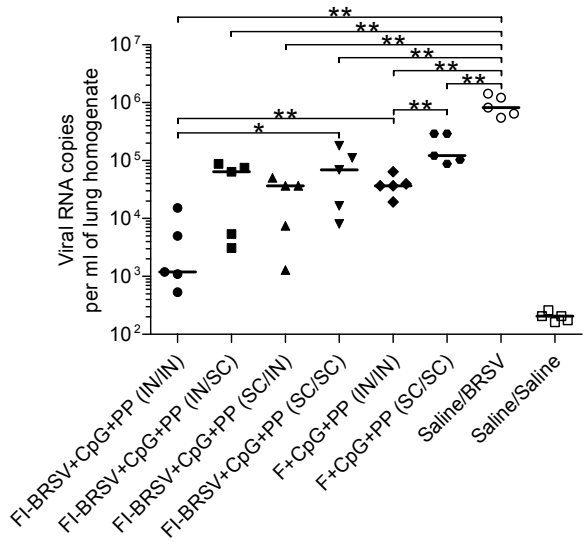
Viral RNA copies
per ml of lung homogenate
10^7
10^6
10^5
10^4
10^3
10^2
**
**
**
**
**
**
**
*
**
Fl-BRSV+CpG+PP (IN/IN)
Fl-BRSV+CpG+PP (IN/SC)
Fl-BRSV+CpG+PP (SC/IN)
Fl-BRSV+CpG+PP (SC/SC)
F+CpG+PP (IN/IN)
F+CpG+PP (SC/SC)
Saline/BRSV
Saline/Saline

Fig. 4.10

Figure 4.10. Detection of viral RNA in lung tissue. Mice were immunized with FI-BRSV/CpG/PP(IN/IN), FI-BRSV/CpG/PP(IN/SC), FI-BRSV/CpG/PP(SC/IN), FI-BRSV/CpG/PP(SC/SC), F/CpG/PP(IN/IN), F/CpG/PP(SC/SC), or saline. CpG ODN were given at 4 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV two weeks after the second immunization (except for the Saline/Saline group). Results are expressed as viral RNA copies per ml of lung homogenate. Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$; **$P < 0.01$.

4.5 DISCUSSION

The first encounter between BRSV and a prospective host occurs in the lung mucosa. In addition to systemic immunity, mucosal immunity is therefore expected to be important for protection from BRSV. In these studies, we compared several BRSV vaccination protocols in terms of induction of systemic and mucosal immunity, as well as protection from BRSV infection in mice.

Although it is not the only component of a protective immune response, humoral immunity is a classical indicator of how well a vaccine has performed. Here, we have confirmed that co-formulation of IN delivered FI-BRSV with both CpG ODN and PP outperforms IN-delivered FI-BRSV formulated with CpG ODN or PP alone, in terms of the induction of IgG, IgA and virus neutralizing antibodies in the serum, agreeing with our previously published study (Mapletoft *et al.*, 2008). It was also observed that IN/IN delivery of FI-BRSV/CpG/PP was generally preferable to IN/SC, SC/IN, or SC/SC delivery, in terms of the above humoral immunity indicators. IN delivery of BRSV F protein co-formulated with CpG ODN and PP induced serum IgG, IgA and virus neutralizing antibody levels that were statistically the same as those induced by IN delivery of FI-BRSV/CpG/PP, and superior to those induced by SC delivery of F/CpG/PP.

A successful response against BRSV, however, requires more than humoral immunity alone; cell mediated immunity is required, as well. In terms of the balance between IFN-γ and IL-5 secretion by *in vitro* restimulated splenocytes, IN delivery of FI-BRSV formulated with both CpG ODN and PP was confirmed to be preferable to IN delivery of FI-BRSV adjuvanted with CpG ODN or PP alone. In addition, IN delivery of FI-BRSV co-formulated with CpG ODN

and PP was preferable to SC delivery, but IN/SC or SC/IN delivery was efficacious as well. This high IFN-γ, low IL-5 cell-mediated immunity profile is characteristic of a Th1-type immune response, which is necessary to avoid RSV vaccine-enhanced disease. While, in contrast to SC delivered protein, IN-delivered BRSV F protein did not elicit any IL-5 secretion, it did not appear to induce IFN-γ secretion to levels as high those induced by IN delivery of FI-BRSV/CpG/PP. This may have been due to the fact that the splenocytes were restimulated with whole inactivated BRSV, rather than recombinant F protein.

While the induction of both humoral and cell-mediated immunity is important for protection against BRSV, the first line of defense is found in the lung mucosa, where the host encounters the virus. IgG, IgA and virus neutralizing antibodies in lung fragment cultures were assayed as indicators of mucosal immunity. Secretion of mucosal IgA, in particular, has been observed following IN immunization with CpG ODN-adjuvanted vaccines against hepatitis B virus (McCluskie & Davis, 1998), *Steptococcus pyogenes* (Teloni *et al.*, 2004), and BRSV (Mapletoft *et al.*, 2008). IN delivery of FI-BRSV/CpG/PP was generally better than any other delivery route, but SC/IN delivery induced levels of IgA and virus neutralizing antibodies that were as high as those induced following IN delivery. IN delivered F/CpG/PP vaccine induced stonger responses than SC delivered F/CpG/PP, and as good as those induced by IN delivered FI-BRSV/CpG/PP. Lung eotaxin, IL-5 and eosinophilia levels were also measured as potential indicators of immunopathological responses. The implementation of any of the SC vaccine delivery protocols resulted in eotaxin and eosinophilia levels that were above those observed in mock-vaccincated control groups, whereas the implementation of IN, IN/SC or SC/IN delivery protocols, regardless of whether FI-BRSV or purified F protein was used, did not result in increases in eotaxin and eosinophilia, as long as CpG ODN were used in the vaccine. In contrast,

IN delivered FI-BRSV adjuvanted with PP alone resulted in increases in eotaxin and eosinophilia, but these increases were abrogated when CpG ODN were included in the vaccine formulation. With respect to IL-5 production, implementation of IN delivery protocols resulted in lower IL-5 levels that implementation of SC delivery protocols.

The most important hallmark of a successful vaccine is the demonstrable ability to protect from experimental challenge. Mucosally delivered vaccines, when formulated with CpG ODN, have been demonstrated to confer protection; IN delivery of herpes simplex virus (HSV) recombinant glycoprotein B (Gallichan *et al.*, 2001) or human immunodeficiency virus (HIV) immunogen (Dumais *et al.*, 2002) resulted in protection of mice from intravaginal challenge with HSV or live recombinant vaccinia virus expressing HIV *gag*, respectively. Because of BRSV's lability and limited growth in mice, RT-PCR assays have been developed to detect viral RNA as an alternative to live virus isolation (Almeida *et al.*, 2004). Using quantitative PCR reagents and detection systems, we have increased the sensitivity of these assays, allowing us to quantitate the amount of viral RNA in the lungs of mice experimentally infected with BRSV (Mapletoft *et al.*, 2008). Generally, mice treated with any of the immunization protocols that we tested experienced significant reductions in viral replication, compared to a mock-immunized, virus-challenged control group. IN-delivered FI-BRSV co-formulated with CpG ODN and PP, however, significantly reduced viral replication compared to SC delivered vaccine, and tended to reduce viral replication compared to all other FI-BRSV vaccines. While, compared to SC delivered vaccine, IN delivered F/CpG/PP vaccine resulted in a reduction in viral replication, it was not able to reduce viral replication to the same extent as the IN-delivered FI-BRSV/CpG/PP vaccine.

As could be expected, IN/SC and SC/IN delivery of FI-BRSV/CpG/PP for the most part resulted in responses ranging between those induced following IN/IN and SC/SC delivery. In several instances, however, the results were surprising. After challenge, in both the serum and lung fragment cultures, SC/IN delivery resulted in identical IgA production to that induced after IN/IN delivery. Furthermore, both IN/SC and SC/IN delivery resulted in the induction of cell-mediated immunity profiles that were virtually the same as those induced by IN/IN delivery (i.e. high levels of IFN-γ secretion and low levels of IL-5 secretion by *in vitro* restimulated splenocytes). Finally, IN/SC and SC/IN delivery resulted in low levels of lung eotaxin and eosinophilia, similar to what was observed following IN/IN delivery.

We have already demonstrated the potential of an IN delivered FI-BRSV vaccine co-formulated with CpG ODN and PP, in terms of induction of humoral, cell-mediated and mucosal immunity, as well as protection against BRSV in mice (Mapletoft *et al.*, 2008). Here, we have shown conclusively that IN delivery of FI-BRSV/CpG/PP is superior to SC delivery, and generally superior to IN/SC and SC/IN delivery protocols, as well. We have also demonstrated that IN delivery of the BRSV F protein, co-formulated with CpG ODN and PP, is generally superior to SC delivery. IN delivered FI-BRSV/CpG/PP was observed to perform better than IN delivered F/CpG/PP, in terms of increasing IFN-γ secretion by *in vitro* restimulated splenocytes, and reducing viral replication in the lungs. These two vaccines, however, performed virtually identically in every other assay that was performed (IgG and IgA, in both the serum and in lung fragment cultures, splenocyte IL-5 secretion, lung eotaxin and IL-5, and lung eosinophilia). The differences in IFN-γ secretion and viral replication can be readily explained. The difference observed between FI-BRSV/CpG/PP and F/CpG/PP in IFN-γ secretion can be ascribed to the antigen used to restimulate the splenocytes. While whole inactivated virus may be the most

appropriate restimulation antigen for approximating a real life BRSV infection, F protein alone may be the most appropriate antigen in terms of measuring the response to the F/CpG/PP vaccine. The difference in viral replication can be ascribed to the fact that the formulation of F/CpG/PP has yet to be optimized. The doses of CpG ODN and PP were chosen because they were optimal for the FI-BRSV vaccine, and the dose of 0.5 μg per immunization was chosen as a suboptimal dose for another adjuvant study (Kovacs-Nolan *et al.*, in preparation). While 0.25 μg of purified HRSV F protein given IN has been demonstrated to confer protection from HRSV in cotton rats, this F protein was adjuvanted with 20 μg of CpG ODN (five times more CpG ODN than was used in our study), and 1.25 μg of purified F protein adjuvanted with 20 or 100 μg of CpG ODN conferred even higher levels of protection (Prince *et al.*, 2003). Furthermore, IN immunization with 3 μg of purified HRSV F protein adjuvanted with cholera holotoxin was able to confer protection from HRSV in mice (Tebbey *et al.*, 2000). Therefore, higher doses of recombinant BRSV F protein, adjuvanted with CpG ODN and PP, may be able to induce levels of immunity and protection that are as high, or higher, than those induced by the optimized FI-BRSV vaccine formulation. IN delivery of BRSV vaccines formulated with CpG ODN and PP is an effective strategy in terms of its ability to induce humoral, cell-mediated, and mucosal immunity, as well as protection against BRSV in mice. Because of the similarities between BRSV and HRSV, and the similarities between their interactions with their respective hosts, formulation of IN-delivered HRSV vaccines with CpG ODN and PP could prove important in the development of a vaccine that induces safe and protective immunity against HRSV in humans.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Since the failure of FI-RSV in the 1960s, scientists have been attempting to develop a safe, effective vaccine against RSV. Research in the intervening years has led to the belief that the enhanced disease observed upon infection of the infants that were previously immunized with FI-RSV was due to the induction of an improper Th2-biased immune response. In terms of inducing protective immune responses, we have demonstrated the value of CpG ODN as a Th1-promoting adjuvant in a parenterally-delivered FI-BRSV vaccine in calves, and in mucosally-delivered FI-BRSV and recombinant BRSV F protein vaccines in mice. We have also shown the superiority of mucosally delivered BRSV vaccines co-formulated with CpG ODN and PP, as compared to parenterally-delivered vaccines.

Our work was initially built upon a study completed by Oumouna *et al.* in mice (Oumouna *et al.*, 2005). Because parenteral delivery of inactivated whole virus RSV vaccines typically induces Th2-type immune responses in BALB/c mice, Oumouna *et al*. sought to alter the vaccine-induced immune profile by formulating an FI-BRSV vaccine with CpG ODN, an adjuvant know for its ability to enhance Th1-type immune responses in BALB/c mice. They found that addition of CpG ODN to parenterally delivered FI-BRSV or commercially available multivalent killed BRSV vaccine (Triangle 4, Fort Dodge Laboratories Inc.) resulted in increased serum IgG2a and BRSV-neutralizing antibodies, and decreased serum IgE. Addition of CpG ODN to these vaccines also resulted in enhanced IFN-γ secretion by *in vitro* restimulated splenocytes. These events were accompanied by decreased lung IL-5, eotaxin and eosinophilia,

as well as reduced viral replication in the lungs. Based on these observations, Oumouna *et al.* concluded that the Th2-type immune response typically induced in BALB/c mice following parenteral immunization with inactivated whole virus RSV vaccines was converted to a more balanced or Th1-biased immune response by the addition of CpG ODN.

Our first step was to examine whether the findings of Oumouna *et al*. could be replicated in cattle, a large outbred animal species whose interaction with BRSV closely resembles that between humans and HRSV. Specifically, we wished to use four week-old calves as model for HRSV in human newborn infants. Working with calves, however, posed several challenges that are not present when working with mice. First of all, because of the cost of both purchasing and caring for the calves, the total number of animals involved in our experiment was kept to a minimum. This meant fewer experimental groups and fewer animals per group. Secondly, the scope of immunological reagents available for cattle is much more limited than that available for mice. As a result, we were not able to examine the production of cyto/chemokines in the lungs of the calves. Despite those limitations, our observations in calves were analogous to those observed by Oumouna *et al*. in mice.

It had previously been demonstrated that exacerbation of disease characterized by decreases in IFN-γ production occurs upon subsequent challenge of calves previously immunized parenterally with FI-BRSV (Woolums *et al.*, 1999). Our hypothesis, therefore, was that BRSV-specific cellular immune responses characterized by production of IFN-γ and BRSV-specific IgG2, could offer protection against subsequent infections. As was done by Oumouna *et al.* in mice, we aimed to induce such cellular immune responses by formulating FI-BRSV with CpG ODN. We demonstrated that, indeed, formulation of FI-BRSV with CpG ODN, followed by parenteral delivery, resulted in the induction of cellular immune responses characterized by the

production of IgG2 in the serum, as well as the production of IFN-γ by *in vitro* restimulated PBMCs and broncho-tracheal lymph node cells. The increases in IgG2 production in the serum and IFN-γ secretion by *in vitro* restimulated cells agreed with the work of Oumouna *et al.* in mice, as well as another study in which a BHV-1 subunit vaccine was formulated with CpG ODN in cattle (Ioannou *et al.*, 2002b). Furthermore, we demonstrated that parenteral vaccination with FI-BRSV formulated with CpG ODN results in a trend for reduced gross lung pathology as well as a significant reduction in viral replication in lungs, upon subsequent challenge with live BRSV. The reduction in viral replication in the lungs agreed with the work of Oumouna *et al.*, as well as another study in which recombinant HRSV F protein was formulated with CpG ODN (Hancock *et al.*, 2001). Overall, these results agreed with our hypothesis that formulation of FI-BRSV with CpG ODN would result in the induction of protective BRSV-specific cellular immune responses. As Oumouna *et al*. had shown in mice, CpG ODN appeared to be a suitable candidate adjuvant for BRSV vaccines in cattle.

Thus far, we had only investigated parenterally-delivered vaccines against BRSV. As BRSV infects the respiratory mucosa, a vaccine that induces mucosal immunity might be desirable. Whereas parenterally-delivered vaccines typically induce systemic immunity with weak or no mucosal immune responses, mucosally-delivered vaccines results in the induction of both mucosal and systemic immune responses (Lamm, 1997; Levine, 2000). Parenteral immunization, therefore, induces immunity that resolves established infections of the mucosa, rather than immunity that prevents the initial interactions at the mucosal surface. Once a pathogen has invaded past the mucosal surface, the host-pathogen interaction has often developed in favor of the pathogen. Furthermore, mucosal epithelial cells express low levels of MHC class II molecules (Ryan *et al.*, 2001). These levels are increased during inflammation,

allowing epithelial cells to present antigen to both $CD4^+$ and $CD8^+$ T cells. Mucosal immunization also offers several logistical advantages, such as ease of administration, the potential for self-administration, removal of the possibility of reusing infected needles, and minimization of adverse effects due to systemic dispersion of potentially toxic vaccine components. For these reasons we wished to examine mucosal immunization and its effect on the type of immune response induced against BRSV. It was necessary, therefore, to return to mice to test whether or not mucosal immunization with BRSV vaccines formulated with CpG ODN was A) feasible, in terms of inducing a protective immune response against BRSV without enhancing immunopathology, and B) superior to parenteral immunization in these same respects. Thus, in our subsequent studies we focused on the feasibility of mucosal immunization against BRSV.

Several studies had already been completed by others with intranasally delivered HRSV vaccines in rodents. A study with recombinant HRSV F protein adjuvanted with CpG ODN resulted in an increase in virus neutralizing antibodies and a decrease in virus production in cotton rats (Prince *et al.*, 2003). In two studies with recombinant F protein adjuvanted with cholera toxin in mice, increases in mucosal IgA and protection from live RSV challenge were observed (Tebbey *et al.*, 2000; Walsh, 1993). In another study with the F protein, this time adjuvanted with caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate, increases in serum IgG, IgG1, IgG2b, and IgA, as well as mucosal IgA were observed (Tebbey *et al.*, 1999). These antibodies were found to be HRSV-neutralizing, and the mice were protected from a live viral challenge. Live viral (hYu *et al.*, 2008; Kahn *et al.*, 2001; Matsuoka *et al.*, 2002; Stott *et al.*, 1987) and bacterial (Cano *et al.*, 2000; Falcone *et al.*, 2006) vectors expressing whole RSV proteins or peptides have also been explored.

In two more recent studies, a novel intranasal vaccine strategy based on chimeras of portions of the RSV F protein fused with the $ctxA_2B$ gene of cholera toxin was evaluated (Singh *et al.*, 2007a; Singh *et al.*, 2007b). In these studies, they observed increases in systemic and mucosal antibodies against HRSV, as well as enhanced protection from live viral challenge. In two additional recent studies, one carried out in BALB/c mice (Cyr *et al.*, 2007a) and another in C57Bl/6 mice (Cyr *et al.*, 2007b), a subunit HRSV antigen enriched for the F and G proteins and formulated with the adjuvant Protollin (meningococcal outer membrane proteins mixed with LPS from *Shigella flexner*) was examined. Here, increases in systemic and mucosal HRSV-specific antibodies and full protection from live viral challenge were observed, without induction of lung eosinophilic pulmonary pathology.

CpG ODN had also been used as a mucosal adjuvant in several vaccines delivered intranasally to mice. When hepatitis B surface antigen was formulated with CpG ODN it resulted in increases in humoral and cell-mediated systemic immune responses, as well as increases in IgA in the lungs and feces (McCluskie & Davis, 1998). Intranasal delivery of recombinant herpes simplex virus (HSV)-1 glycoprotein formulated with CpG ODN resulted in increases in IgA in the genital tract, as well as protection from intravaginal challenge with HSV-2 (Gallichan *et al.*, 2001). Furthermore, an intranasally delivered vaccine consisting of glycoprotein 120-depleted human immunodeficiency virus (HIV)-1 immunogen formulated with CpG ODN induced increases in IgG and IgA in serum and vaginal washes, as well as protection from intravaginal challenge with rVV expressing the HIV-1 *gag* protein (Dumais *et al.*, 2002).

One of the challenges of intranasal immunization is delivering the vaccine components in such a manner that they are not degraded or removed prior to the initiation of the immune response. We thus sought to test the ability of PP to enhance the efficacy of intranasal

immunization against BRSV in mice. PP form non-covalent complexes when mixed with compounds of interest, i.e. antigens and other adjuvants, increasing their stability and allowing for multimeric presentation. Delivery of antigens formulated with PP had been successful in enhancing antibody responses to influenza (Mutwiri *et al.*, 2007; Payne *et al.*, 1995; Payne *et al.*, 1998), rotavirus (McNeal *et al.*, 1999) and cholera (Wu *et al.*, 2001). Protection was also enhanced in the latter two models. Recently, PP has also been co-formulated with CpG ODN as part of a vaccine against hepatitis B in mice (Mutwiri *et al.*, 2008). Other than those completed by us and our collaborators, no studies using PP as an intranasal vaccine adjuvant in mice have been published yet.

Thus we sought to examine the adjuvant effects of CpG ODN and PP, individually and as co-adjuvants, on an intranasally delivered FI-BRSV vaccine in mice. Our hypothesis was that a combination of CpG ODN and PP would be an appropriate adjuvant formulation for intranasal delivery of FI-BRSV, in terms of inducing immune responses and protection against BRSV in mice. Intranasal immunization of mice with FI-BRSV co-formulated with CpG ODN and PP resulted in the induction of both humoral immunity, characterized by production of BRSV-specific serum IgG, IgA and virus neutralizing antibodies, and cell-mediated immunity, characterized by high levels of IFN-γ secretion and low levels of IL-5 secretion by *in vitro* restimulated splenocytes. Again, the increase in IFN-γ secretion agreed with the findings of Oumouna *et al.* in mice, as well as ours (Mapletoft *et al.*, 2006) and those of Ioannou *et al.* (Ioannou *et al.*, 2002b) following parenteral delivery of vaccines formulated with CpG ODN in cattle, despite the difference in route of administration. The mice immunized intranasally with FI-BRSV co-formulated with CpG ODN and PP also developed mucosal immune responses, as was evident from increases in production of BRSV-specific IgG, IgA and virus neutralizing

antibodies in lung fragment cultures. The increase in antigen-specific IgA production in the lungs agreed with two previous studies in which hepatitis B surface antigen (McCluskie & Davis, 1998) or *Streptococcus pyogenes* M6 protein (Teloni *et al.*, 2004) were formulated with CpG ODN and given intranasally to mice. Overall, the observed increases in serum and mucosal IgG and virus neutralizing antibodies, as well as mucosal IgA, were the most critical differences observed between FI-BRSV formulated with both CpG ODN and PP and FI-BRSV formulated with CpG ODN or PP individually. Finally, FI-BRSV co-formulated with CpG ODN and PP was the only formulation that resulted in a significant reduction in lung viral replication following challenge with live BRSV, demonstrating that CpG ODN and PP together make up the optimal formulation. The contribution of the CpG ODN to the reduction in viral replication agreed with an earlier report in which intranasal immunization of cotton rats with recombinant HRSV F protein formulated with CpG ODN also resulted in a reduction in lung viral replication following challenge with live RSV (Prince *et al.*, 2003), as well as other reports in which intranasal delivery of CpG ODN formulated vaccines consisting of herpes simplex virus (HSV) recombinant glycoprotein B (Gallichan *et al.*, 2001) or human immunodeficiency virus (HIV) immunogen (Dumais *et al.*, 2002) induced protection of mice from intravaginal challenge with HSV-2 or rVV expressing HIV-1 *gag*, respectively. Thus we concluded that, among those tested, CpG ODN and PP together is the optimal adjuvant formulation for intranasal immunization against BRSV in mice (Mapletoft *et al.*, 2008). This conclusion agreed with our above stated hypothesis.

Our goals were to test whether or not mucosal immunization with BRSV vaccines formulated with CpG ODN was feasible, in terms of inducing a protective immune response against BRSV, and superior to parenteral immunization. Having demonstrated not only the

feasibility of mucosal immunization against BRSV in mice, but also that the combination of CpG ODN and PP appears to be the optimal adjuvant formulation, we next sought to compare mucosal and parenteral immunization against BRSV. Thus we completed two studies to further examine the potential of BRSV vaccines co-formulated with CpG ODN and PP. Our hypothesis for these studies was that intranasal delivery of BRSV vaccines co-formulated with CpG ODN and PP would be superior to subcutaneous delivery, in terms of inducing immune responses and protection against BRSV in mice. In the first study, we compared intranasal and subcutaneous delivery of FI-BRSV formulated with CpG ODN and/or PP. In the second study, we introduced intranasal prime/subcutaneous boost (intranasal/subcutaneous) and subcutaneous prime/intranasal boost (subcutaneous/intranasal) immunization protocols for FI-BRSV co-formulated with CpG ODN and PP, as well as a comparison of intranasal and subcutaneous delivery of recombinant BRSV F protein co-formulated with CpG ODN and PP. Mice were assayed for BRSV-specific IgG, IgA and virus neutralizing antibodies in the serum and lungs, IFN-γ and IL-5 secretion by *in vitro* restimulated splenocytes, as well as eotaxin, IL-5, eosinophilia and viral replication in the lungs.

Ultimately, we demonstrated that intranasal delivery of FI-BRSV co-formulated with CpG ODN and PP is superior to subcutaneous delivery in virtually all respects, and generally superior, to intranasal/subcutaneous and subcutaneous/intranasal delivery protocols, as well. We also showed that intranasal delivery of recombinant BRSV F protein co-formulated with CpG ODN and PP is generally superior to SC delivery. Intranasally-delivered FI-BRSV co-formulated with CpG ODN and PP was observed to perform better than intranasally-delivered recombinant BRSV F protein co-formulated with CpG ODN and PP, in terms of increasing IFN-γ secretion by splenocytes *in vitro* restimulated with inactivated BRSV, and reducing viral replication in the

lungs. These two vaccines, however, performed virtually identically in every other assay that was performed (BRSV-specific IgG and IgA production, in both the serum and in lung fragment cultures, and IL-5 secretion by *in vitro* restimulated splenocytes, as well as lung eotaxin and IL-5 production, and lung eosinophilia). Thus, we concluded that intranasal delivery of FI-BRSV and recombinant BRSV F protein vaccines co-formulated CpG ODN and PP is superior to subcutaneous delivery in terms of inducing greater humoral, cell-mediated, and mucosal immunity, as well as protection against BRSV in mice. This conclusion agreed with our above stated hypothesis. Furthermore, because of the similarities between BRSV and HRSV, and the similarities between their interactions with their respective hosts, we believe that co-formulation of intranasally delivered RSV vaccines with CpG ODN and PP could prove important in the development of a vaccine that induces safe and protective immunity against HRSV in humans.

Our most important findings were essentially threefold: 1) the value of CpG ODN as an adjuvant in vaccines against RSV, for parenteral delivery in calves, as well as mucosal delivery in mice; 2) the value of co-formulation of CpG ODN and PP in mucosally delivered vaccines against RSV; and 3) the superiority of mucosal delivery, compared to parenteral delivery, with regards to immunization with CpG ODN- and/or PP-adjuvanted vaccines against RSV. The value of CpG ODN as an adjuvant in vaccines against RSV, particularly FI vaccines, did not come as a surprise. FI-RSV vaccines have a long history of inducing disease enhancement, which has been attributed to the induction of Th2-biased immune responses in mice. An adjuvant that promotes a Th1-type immune response, such as CpG ODN, is therefore an obvious choice for incluision in inactivated or subunit RSV vaccines. While Oumouna *et al.* demonstrated the value of CpG ODN in parenterally delivered BRSV vaccines in mice, we demonstrated their

value in parenterally delivered FI-BRSV in calves, as well as their value in mucosally delivered FI-BRSV and F protein vaccines in mice.

PP, on the other hand, are a relatively new type of adjuvant that had not been used in any RSV vaccine formulations prior to our work. Furthermore, the combination of CpG ODN and PP has only been used by us, as well as collaborators of ours (Mutwiri *et al.*, 2008). Their results paralleled ours in the sense that co-formulation of hepatitis B virus surface antigen (HBVsAg) with CpG ODN and PP was better than formulation with either adjuvant individually, in terms of inducing immune responses against HBVsAg, but it must also be kept in mind that they immunized their mice subcutaneously.

It was not surprising when we observed that mucosal delivery of BRSV vaccines co-formulated with CpG ODN and PP resulted in the induction higher levels of mucosal immunity, as well as higher levels of protection against BRSV than those resulting from parenteral delivery. A finding that could possibly be considered surprising, however, was that mucosal delivery was as good as, if not better than, parenteral delivery in terms of inducing humoral immune responses against BRSV. For a mucosal pathogen such as RSV, however, the induction of immunity and protection at the site of infection is paramount, and mucosal delivery of BRSV vaccines co-formulated with CpG ODN and PP was definitely better than parenteral delivery in these respects.

There are essentially two problems that need to be solved with regards to immunization against RSV: 1) induce immunity in neonates, in the face of maternal antibodies that may neutralize vaccine antigen prior to the initiation of the immune response; and 2) avoid vaccine-enhanced disease upon subsequent infection. The first problem is difficult to address. Although we were able to increase cellular immunity against BRSV in calves with low levels of maternal

antibodies (median serum IgG1 titers in the hundreds at the time of vaccination), it is difficult to determine why some vaccination protocols are successful while others are not. There are many documented instances in which preexisting antibodies (a model for maternal antibodies) against RSV has been associated with the suppression of subsequent immune responses against RSV in infants and children (Murphy *et al.*, 1986a), calves (Kimman *et al.*, 1989), neonatal mice (Bangham, 1986), and cotton rats (Murphy *et al.*, 1989; Murphy *et al.*, 1988; Murphy *et al.*, 1991; Prince *et al.*, 1982). In infants and children it was observed that preexisting antibody affected the antibody response to the HRSV G protein during primary HRSV infection (Murphy *et al.*, 1986a). In neonatal mice, it has been observed that monoclonal antibodies against HRSV, whether injected directly or transferred via the mother's milk, are associated with a reduction in the generation of HRSV-specific T-cell precursors (Bangham, 1986). It has also been demonstrated that passive transfer of hyperimmune HRSV antiserum to cotton rats prior to intradermal immunization with rVV expressing HRSV glycoproteins results in the suppression of antibody responses against the glycoproteins, and that these cotton rats were more susceptible to infection with live HRSV than control animals that received serum with no antibodies against HRSV (Murphy *et al.*, 1988). Interestingly, however, the suppressive effects of the passively transferred HRSV antiserum can be partially overcome by delivery of the rVV recombinants by the intranasal route (Murphy *et al.*, 1989). The same is true for intramuscular immunization with live HRSV, which is blocked in seropositive cotton rats, in contrast to intranasal immunization with live HRSV, which is not blocked in seropositive cotton rats (Prince *et al.*, 1982). The results of these studies indicate a possible advantage for mucosally delivered RSV vaccines with respect to avoiding the suppressive effects of maternal antibodies.

There are also a few studies in which researchers claim to have achieved vaccine efficacy in the presence of maternal antibodies against RSV in calves (Hagglund *et al.*, 2004; Mawhinney & Burrows, 2005; Patel & Didlick, 2004) and neonatal mice (Siegrist *et al.*, 1999). In one of the studies they examined the efficacy of two commercially available vaccines: Rispoval RS (Pfizer Animal Health), a modified-live vaccine containing the RB94 strain of BRSV; and Bovipast RSP (Intervet UK), a trivalent vaccine against BRSV, parainfluenza virus type 3 and *Mannheimia haemolytica* with alhydrogel and Quil A as adjuvants (Mawhinney & Burrows, 2005). Although vaccine induced seroconversion and protection against live challenge with BRSV were observed following a single parenteral immunization with either vaccine, the calves in this study, like those in our experiment, possessed low levels of maternal antibodies (mean serum total IgG titers of approximately 400 and 500) at the time of vaccination.

The other two studies, however, documented vaccine induced protection against BRSV in calves with high levels of maternal antibodies. In the first study, with calves with mean serum virus neutralizing antibody titers between 3.0×10^5 and 1.7×10^7 at the time of vaccination, protection was induced by parenteral immunization with β-propiolactone-inactivated BRSV mixed with aluminum hydroxide and aluminum phosphate (Patel & Didlick, 2004). In the second study, with calves with a median serum IgG1 titer of approximately 3.0×10^5 at the time of vaccination, protection was induced following parenteral immunization with BRSV immunostimulating complexes (ISCOMs) (Hagglund *et al.*, 2004). ISCOMs are mixtures of Quil-A, cholesterol, phosphatidylcholine and amphipathic antigens, such as viral membrane proteins. The Quil-A, cholesterol and phosphatidylcholine combine to form organized hollow cage-like structures that allow hydrophobic interactions with amphipathic molecules. It is difficult to explain why these two immunization protocols succeeded in the presence of high

levels of maternal antibodies when so many others have failed, but in the second instance, it may be partly or wholly attributed to the use of ISCOMs.

In a study in neonatal mice with a mean serum total IgG titer of approximately 3 x 10^5 at the time of vaccination, induction of protective immunity against HRSV was observed following intraperitoneal, intramuscular or subcutaneous immunization with the recombinant fusion protein BBG2Na adjuvanted with AdjuPhos (aluminum phosphate) or TiterMax (a water-in-oil emulsion made up of non-ionic block copolymer surfactant CRL-8941 and squalene) (Siegrist *et al.*, 1999). BBG2Na is a recombinant fusion protein consisting of amino acids 130 to 230 of the HRSV Long strain G protein (G2Na) fused to the albumin-binding region (BB) of streptococcal protein G. G2Na contains a conserved HRSV subgroup A-specific protective epitope (Akerlind-Stopner *et al.*, 1990; Trudel *et al.*, 1991), as well as a stretch of amino acids (aa 164 to 176) that are conserved among all known HRSV A and B isolates (Garcia *et al.*, 1994; Sullender *et al.*, 1991), and BB significantly enhances the *in vivo* half-life and immunogenicity of its fusion partner (Libon *et al.*, 1999; Makrides *et al.*, 1996; Sjolander *et al.*, 1997). BBG2Na is produced by prokaryotic expression in *Escherichia coli* as a nonglycosylated protein. BBG2Na has also been evaluated in phase I clinical trials (Siegrist *et al.*, 1999). Again, it is difficult to explain why BBG2Na is able to successfully induce protection against challenge with BRSV in the presence of high levels of maternal antibodies, but the authors of this study speculate that BBG2Na avoids inhibition by maternal antibodies because the immunogenicity of the nonglycosylated G2Na fragment differs from that of the native heavily glycosylated HRSV G protein (Siegrist *et al.*, 1999).

With regards to vaccine-enhanced disease upon subsequent infection, we believe, as indicated in the previous chapters, that the technology exists to overcome this problem. By

delivering RSV antigen (not likely in the form of FI-RSV) mucosally, with one adjuvant that promotes Th1-type immune responses (e.g. CpG ODN), and another adjuvant that protects the other vaccine components (e.g. PP), we believe that protection against RSV can be induced without inducing vaccine-enhanced disease upon subsequent infection. We do not necessarily believe that CpG ODN and PP are the only or even the best adjuvants to accomplish this, but simply that they represent the types of adjuvants that will be necessary parts of a safe effective mucosally delivered RSV vaccine. The next step, therefore, would be to begin testing and optimizing mucosally delivered RSV vaccines co-formulated with CpG ODN and PP in large outbred animals, such as calves. Efficacy will have to be demonstrated across a wide range of preexisting antibody levels, and not even the slightest evidence of disease enhancement should be tolerated. Because of their history, FI whole virus vaccines will never be perceived as safe in humans, regardless of their actual safety, thus alternate RSV antigen strategies (e.g. recombinant viral proteins, defined deletion mutants, or viral vectored vaccines) will have to be used. In conclusion, however, we believe that the combination of CpG ODN and PP is an excellent candidate adjuvant system for mucosally delivered RSV vaccines in calves and humans.

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