Development and Evaluation of New Generation Vaccines Against Bovine Respiratory Syncytial Virus

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Cover: Schematic representation of the effects of BRSV infection in calves (top), and the protective effect that may be afforded by prior vaccination (bottom).

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Abstract

Bovine respiratory syncytial virus (BRSV) is a major cause of respiratory disease in cattle worldwide. Improved BRSV vaccines are needed, with better efficacy and longer duration of protection, in particular when used in calves with specific maternally derived antibodies (MDA). New generation BRSV vaccines should also be DIVA compliant, and thus allow continued seromonitoring in vaccinated herds, including continuous evaluation of vaccine safety and efficacy.

In this thesis work, classic BRSV immunostimulating complexes (BRSV-ISCOMs) were shown to overcome inhibition by specific MDA, and induce a high level of protection, probably mediated by strong humoral and Th1 type T cell responses.

Characterization of proteins in BRSV-ISCOMs was performed to facilitate future standardization or improvement of BRSV-ISCOMs, and to serve as a basis to design efficient subunit vaccines (SU). SU consisted of recombinant human RSV (HRSV) proteins P, M2-1 and N nanorings with epitopes from BRSV proteins F and G.

Three DIVA-compatible vaccines, all omitting the SH protein, were evaluated: two vaccines formulated using SU, adjuvanted by either Montanide ISA71^{VG} (SUMont) or AbISCO-300 (SUAbis); and ΔSHrBRSV, a live recombinant BRSV with deleted SH gene. The safety, immunogenicity and protective efficacy of SUMont, SUAbis and ΔSHrBRSV were compared in calves with specific MDA in a BRSV infection model with high clinical expression, which was also developed in this thesis work.

Both ΔSHrBRSV and SUMont induced protection against BRSV infection, seemingly by activating different immunological pathways. ΔSHrBRSV induced almost complete clinical and virological protection, which appeared to rely mainly on mucosal IgA and systemic neutralizing antibodies directed against viral surface proteins, and T cell priming in the airways. SUMont induced a good level of protection, which appeared to be mediated by HRSV/BRSV cross-reactive specific T cells. SUAbis induced limited protection from BRSV challenge.

The three vaccines BRSV-ISCOMs, ΔSHrBRSV and SUMont, individually identified as promising vaccine candidates, are described and discussed in this thesis, including possibilities to improve their immunogenicity and protective efficacy, and to further investigate induced immunity and DIVA compliancy.

Keywords: BRSV, vaccine, ISCOM, subunit, modified live, DIVA, infection model

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Dedication

To my wife Rejoice and my little boys Tsodilo and Savuti.

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Hägglund, Sara; Hu, Kefei; Vargmar, Karin; Poré, Lesly; Olofson, Ann-Sophie; Blodörn, Krister; Anderson, Jenna; Ahooghalandari, Parvin; Pringle, John; Taylor, Geraldine; and Valarcher, Jean-François (2011). Bovine respiratory syncytial virus ISCOMs-Immunity, protection and safety in young conventional calves. *Vaccine* 29(47), 8719–8730.

II Hägglund, Sara; Hu, Kefei; Blodörn, Krister; Makabi-Panzu, Boby; Gaillard, Anne-Laure; Ellencrona, Karin; Chevret, Didier; Hellman, Lars; Lövgren Bengtsson, Karin; Riffault, Sabine; Taylor, Geraldine; Valarcher, Jean-François; and Eléouët, Jean-François (2014). Characterization of an experimental vaccine for bovine respiratory syncytial virus. *Clinical and vaccine immunology: CVI* 21(7), 997–1004.

III Blodörn, Krister; Hägglund, Sara; Gavier-Widen, Dolores; Eléouët, Jean-François; Riffault, Sabine; Pringle, John; Taylor, Geraldine; and Valarcher, Jean-François. A bovine respiratory syncytial virus model with high clinical expression in calves with specific passive immunity (submitted manuscript).

IV Blodörn, Krister; Hägglund, Sara; Fix, Jenna; Dubuquoy, Catherine; Makabi-Panzu, Boby; Thom, Michelle; Karlsson, Per; Roque, Jean-Louis; Karlstam, Erika; Pringle, John; Eléouët, Jean-François; Riffault, Sabine; Taylor, Geraldine; and Valarcher, Jean-François (2014). Vaccine Safety and Efficacy Evaluation of a Recombinant Bovine Respiratory Syncytial Virus (BRSV) with Deletion of the SH Gene and Subunit Vaccines Based On Recombinant Human RSV Proteins: N-nanorings, P and M2-1, in Calves with Maternal Antibodies. *PLoS One* 9(6), e100392.

Papers I, II and IV are reproduced with the permission of the publishers.

The contribution of KB to the papers included in this thesis was as follows:

I Contributed to animal experiments, laboratory work, analysis of results and authoring of manuscript.

II Contributed to laboratory work, analysis of results and authoring of manuscript.

III Main author, primary contributor to laboratory work and analysis of results, and substantial contribution to animal experiments.

IV Main author, substantial contribution to laboratory work, analysis of results, and animal experiments.

Abbreviations

ACS	Accumulated clinical score
APC	Antigen presenting cell
AVS	Accumulated virus shed
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BRSV-Dk	BRSV, isolate no. 9402022, Denmark
BRSV-Snk	BRSV, Snook strain
BSA	Bovine serum albumin
BT	Bovine turbinate (cells)
BVDV	Bovine viral diarrhea virus
CK	Calf kidney (cells)
COD	Corrected optical density
CTL	Cytolytic $CD8^+$ T lymphocyte
DALY	Disability Adjusted Life Year
DC	Dendritic cell
DIVA	Differentiation of infected from vaccinated animals
DMEM	Dulbecco's modified Eagle medium
DMEM-FCS	DMEM supplemented with the stated concentration of FCS
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FOT	Forced oscillation technique
HRP	Horseradish peroxidase
HRSV	Human respiratory syncytial virus
i.m.	Intramuscular
i.n.	Intranasal
i.t.	Intratracheal
IFN	Interferon
IL	Interleukin
ISCOM	Immunostimulating complex (adjuvant)

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LN	Lymph node
mAb	Monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MDA	Maternally derived antibodies
MHC	Major histocompatibility complex
MLV	Modified live vaccine
NK	Natural killer (cell)
OD	Optical density
ORSV	Ovine respiratory syncytial virus
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pfu	Plaque-forming unit
PID	Post-infection day
PM	Post-mortem
PRR	Pattern recognition receptors
PVD	Post-vaccination day
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (medium)
RSV	Respiratory syncytial virus (BRSV and HRSV)
RT-qPCR	Reverse transcription quantitative real-time PCR
s.c.	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
$TCID_{50}$	50% Tissue Culture Infective Dose
TCR	T cell receptor
Th	T helper (cell)
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	Tumor necrosis factor alpha

BRSV and HRSV proteins are abbreviated in Table 1 on page 16.
Recombinant proteins used in paper IV are abbreviated in Table 2 on page 40.

1 Introduction

1.1 Background

Bovine respiratory syncytial virus (BRSV) is a major cause of respiratory disease in cattle, and an important cause of enzootic pneumonia in calves worldwide (Valarcher & Taylor 2007).

The antigenically and genetically closely related human respiratory syncytial virus (HRSV), which causes similar disease in humans, was first isolated from chimpanzees in 1956 and named chimpanzee coryza agent (Morris et al. 1956). In 1957 the same virus was isolated from human infants, and was later renamed HRSV, based on the formation of multinucleated giant cells or syncytia in inoculated human cell culture (Chanock et al. 1957; Chanock et al. 1962).

Already in 1963, it was noted that certain calf sera inhibited *in vitro* cytopathic effects of HRSV (Taylor-Robinson & Doggett 1963), and in 1968 Doggett et al. suggested this inhibition was mediated by bovine antibodies (Doggett et al. 1968). Subsequently, in 1970 a virus identified as BRSV was isolated from cattle with respiratory disease in Switzerland (Paccaud & Jacquier 1970) and Japan (Inaba et al. 1970), and later in the United States (Smith et al. 1975).

Since then, both BRSV and HRSV have been shown to be highly prevalent, with high morbidity in calves and children, respectively (Meyer et al. 2008). Great efforts have been made to understand both viruses, and to develop preventive and therapeutic strategies.

1.2 Epidemiology

BRSV is host specific to cattle (International Committee on Taxonomy of Viruses 2011), although occasional isolations and serological evidence indicate that sheep, goat and wild ruminant species may be infected (Evermann et al. 1985; Lehmkuhl et al. 1980; Dunbar et al. 1985; Van der Poel et al. 1995; Gaffuri et al. 2006). While it appears that lambs are not very permissive to experimental BRSV infection alone, BRSV may play a role in predisposing or aggravating *Mannheimia haemolytica* infections in lambs (F. J. Trigo et al. 1984; Sharma & Woldehiwet 1990a). Serological and *in vitro* studies with BRSV and HRSV have also suggested that swine may be infected with one or both of these viruses, or possibly a porcine pneumovirus, but such a virus has yet to be isolated (Fishaut et al. 1978; Allan et al. 1998).

In cattle, BRSV is highly prevalent, with seroprevalence rates ranging from 50-100%, with variation over time, geography and mean age of animal groups, and is considered endemic in large parts of the world (Van Vuuren 1990; Paton et al. 1998; Hägglund et al. 2006; Beaudeau et al. 2010; Saa et al. 2012). In a recent study involving 120 calves in 45 dairy and beef herds in eastern Poland, BRSV antigens were detected in nasal secretion from 64% of calves with signs of respiratory disease, and from 56% of calves with no respiratory signs (Urban-Chmiel et al. 2014).

In regions with temperate climate, outbreaks of BRSV in cattle herds occur predominantly in the cold season, with clinical signs primarily in immunologically naïve animals (Stott et al. 1980).

As BRSV do not appear to spread over great distances by aerosol, recurring outbreaks in herds are likely due to either reintroduction by direct or indirect transmission, or by reactivation of latent infection (Van der Poel et al. 1993). Although resumed virus shedding in persistent latent infection with BRSV in cattle has never been confirmed, low levels of viral protein expression has been demonstrated in B cells from pulmonary lymph nodes collected up to 71 days after infection (Valarcher et al. 2001).

1.3 Impact of respiratory syncytial virus

1.3.1 Impact of BRSV and its role in the bovine respiratory disease complex

Bovine respiratory disease (BRD) is most often multifactorial, involving various viral, bacterial and environmental factors (Callan & Garry 2002). Therefore, the specific impact of BRSV on economics and animal welfare is impossible to calculate, although BRSV alone can cause severe lower respiratory disease in calves (Callan & Garry 2002), and is often the only

identified viral etiology during outbreaks of respiratory disease (Elvander 1996; Uttenthal et al. 1996; Thea B. Klem et al. 2014).

BRD constitutes a large proportion of the total morbidity of cattle, especially in young stock, causing extensive financial loss for producers in way of decreased production and increased veterinary expenses (Fulton 2009; Van der Poel et al. 1994). In the UK alone, an estimated 1.9 million calves were affected, and 157 000 calves died from BRD in 1995 (Reeve-Johnson 1999). This corresponds to economical losses to the cattle industry of £150 million per year in the UK, and by extrapolation, could amount to as much as €576 million per year in Europe as a whole (Reeve-Johnson 1999). According to another study which modeled BRD-associated costs, including associated increased morbidity, mortality, treatment and premature culling, and reduced production, the average added cost by BRD per heifer and year in a typical Dutch dairy herd, could be as high as €43 (van der Fels-Klerx et al. 2001). In the United States, 29% of calf losses in 2010 were caused by respiratory problems, excluding free-ranging calves lost to predators (USDA, National Agricultural Statistics Service 2011).

1.3.2 Impact of HRSV

HRSV is the foremost cause of lower respiratory disease worldwide (Nair et al. 2010), and the leading viral cause of pneumonia in children under the age of 5 years (Rudan et al. 2011). In turn, pneumonia is the leading cause of mortality from infectious disease in this age group, with 900 000 deaths worldwide in 2013 (Liu et al. 2014). Infants are particularly vulnerable, and hospitalization rates peak in 1 month old infants, especially those born preterm (Hall et al. 2013). When the disease burden of respiratory viruses in hospitalized humans was scored using the Disability Adjusted Life Year (DALY) model, HRSV received the highest score in children (<5 years old; DALY score 67.7) and immunocompromised patients (DALY score 6.8), but it also received a high score in elderly patients (>65 years old; DALY score 41.0), for whom only influenza constituted a greater disease burden (Gaunt et al. 2011). Therefore, developing effective control measures for HRSV is considered a priority by the WHO (Rudan et al. 2011).

1.4 Virology

1.4.1 Taxonomy

BRSV is an enveloped negative sense single strand RNA (-ssRNA) virus in the *Paramyxoviridae* family, *Pneumovirinae* subfamily, and *Pneumovirus* genus (International Committee on Taxonomy of Viruses 2011). Analysis of a

limited number of RSV isolates from sheep and goats suggest that, whereas isolates from goats may be considered strains of BRSV, ovine RSV (ORSV) is more divergent (Evermann et al. 1985; M Trudel et al. 1989; Duncan & Potgieter 1993). HRSV is in the same taxonomic genus, but is more distantly related (International Committee on Taxonomy of Viruses 2011).

1.4.2 Morphology

The typical mature Paramyxoviridae virus particle is a spheroid virion, approximately 150 nm in diameter, but filamentous forms are often seen (International Committee on Taxonomy of Viruses 2011). The glycoprotein-studded lipid envelope, together with the enclosed nucleocapsid and traces of non-structural viral proteins, form the BRSV virion. At the core of the spiral-shaped nucleocapsid is the 15k nucleotide viral genome, with 10 genes, coding for 11 proteins (Table 1) (Valarcher & Taylor 2007).

Table 1. *BRSV proteins and their main characteristics*

Abbr.	Protein name	Size[a]	RSV identity[b]	Major functions[c]
F	Fusion protein	574	81	Membrane fusion
G	Attachment glycoprotein	257/263	30	Membrane attachment
SH	Small hydrophobic protein	73	38	Promote attachment and fusion, suppression of apoptosis
M	Matrix protein	256	89	Virion packaging
M2-1	Matrix protein 2-1	186	80	Promote read-through at gene junctions during transcription
M2-2	Matrix protein 2-2	95	43	Mediate switch from transcription to replication
NS1	Non-structural protein 1	136	69	Type I IFN inhibition and resistance
NS2	Non- structural protein 2	124	84	Type I IFN inhibition and resistance
N	Nucleoprotein	391	93	Protect genome from degradation
P	Phosphoprotein	241	81	Unclear; interact with N, L and RNA
L	Large protein	2161	84	Form the polymerase complex with N and P

[a] Number of amino acids (AA) in protein.
[b] AA identity (%) between BRSV and HRSV, as determined in other studies (Lerch et al. 1991; Lerch et al. 1990; Anderson et al. 1992; Samal & Zamora 1991; Zamora & Samal 1992; Pastey & Samal 1995; Samal et al. 1991; Mallipeddi & Samal 1992; Yunus et al. 1998).
[c] Major protein function/role in virulence and pathogenesis, as described further in the text.

1.4.3 Replication

As the virion of a respiratory syncytial virus (RSV) buds off an infected host cell, the nucleocapsid is enclosed by an envelope of cellular membrane. The genome is stabilized and protected by the nucleoprotein (N) and the phosphoprotein (P); both essential for virion packaging (Teng & Collins 1998). Also associated with the nucleocapsid is the large protein (L) which, together with the N and P proteins, is proposed to form the polymerase complex (Tawar et al. 2009).

Species-specific attachment and fusion of BRSV and HRSV with bovine and human cells respectively, is mediated by subunit F2 of the fusion protein (F), whereas contributions of the attachment glycoprotein (G) and the small hydrophobic (SH) protein to host cell attachment are less specific (Heminway et al. 1994; Schlender et al. 2003).

Following fusion of the viral and cell membranes, the viral genome is released into the host cell cytoplasm, along with viral proteins carried in the virion. Using the viral genome as a template, the viral polymerase produces mRNA for protein synthesis, and positive sense RNA as templates for genome replication. The balance between protein synthesis and replication is modulated by transcription interruption at gene junctions, mediated by several viral proteins: the non-structural proteins 1 and 2 (NS1 and NS2), the two matrix-2 proteins (M2-1 and M2-2), and the N and P proteins. The NS1 and NS2 proteins promote protein synthesis, whereas the N and M2-2 proteins mediate a switch to viral replication, which is then sustained by the M2-1 protein. The role of the P protein in this process is not clear, but it is associated with the N, as well as the M2-1 protein (García et al. 1993). Protein synthesis from viral mRNA templates, as well as post-translational modifications of viral proteins, is achieved by utilizing the host cell machinery.

During infection, the viral glycoproteins F, G and SH localize to the cellular membrane, where the F and SH proteins, and to a lesser degree the G protein, contribute to the formation of syncytia of infected cells (Heminway et al. 1994). Oligomers of SH proteins may also increase membrane permeability by forming small molecule channels (Carter et al. 2010) and suppress apoptosis by inhibiting TNFα signaling (Fuentes et al. 2007; Taylor et al. 2014a).

Finally, during exit from the cell, the initial bud is created by assemblies of F protein in the cell membrane, the tails of which associate with the matrix (M) protein (Liljeroos et al. 2013). The M protein, together with the M2-1 protein, extends the bud by forming tubules, and attracts the nucleocapsid. The G and SH proteins are assembled onto the bud, through association with the M and F

proteins. Tubules formed by the M protein elongate the bud, which eventually separates from the infected cell (Liljeroos et al. 2013).

Thus, a new virion is created: with F, G and SH proteins on the outer surface of the lipid envelope; M, M2-1, and M2-2 proteins on the inner surface; enclosing the genome, N, P and L proteins in the nucleocapsid; along with small amounts of NS1 and NS2 proteins; and residual cellular proteins. Interactions between M proteins, on the inner surface of the initially elongated virions, tend to transform virions into spheroids (Liljeroos et al. 2013).

1.4.4 Antigenic and genetic variability

All BRSV isolates are of the same serotype, with less than 11% nucleotide divergence in the gene coding the G protein, which is the most variable gene (Larsen et al. 2000). Neutralization assays with monoclonal antibodies against the G protein indicate four different antigenic subgroups (A, AB, B, and untyped) (Schrijver et al. 1996). Shown in Figure 1, as modified from Valarcher et al. 2000 (Valarcher et al. 2000), gene sequencing and phylogenic analysis revealed six genetic subgroups (I-VI) based on variation in the gene coding for the G protein, and five subgroups, with less variation for the genes coding for the F and N proteins.

The genetic distance between BRSV and HRSV is heterogeneous among the viral genes, as shown in Table 1 and Figure 1. For example, the amino acid identity between BRSV and HRSV genes coding for the G protein is only 30% (Lerch et al. 1990), whereas for the N protein it is 93% (Samal et al. 1991). However, as shown in Figure 1, the genetic variations within BRSV isolates are consistently much smaller for the genes coding for the F, G and N proteins, compared to the nucleotide distance to corresponding HRSV genes.

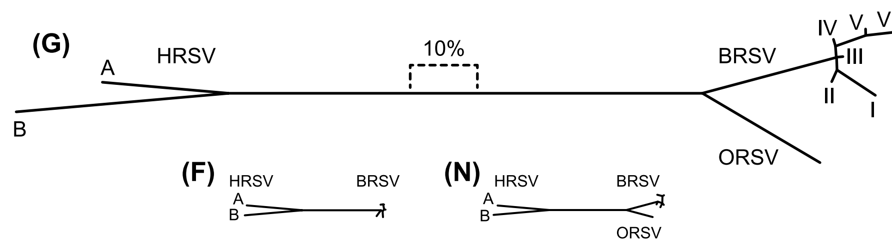

Figure 1. Phylogenetic trees showing the relative nucleotide distance between isolates of HRSV (subtypes A and B), ORSV and the six suggested genetic subgroups of BRSV (I-VI), in genes coding for the attachment glycoprotein (G), the fusion protein (F) and the nucleoprotein (N), as reported by Valarcher et al. (Valarcher et al. 2000). The F gene was not sequenced for the ORSV isolate in that study. The dashed line indicates a nucleotide distance of 10%, in all trees.

1.5 Clinical signs and pathology

Manifest primary BRSV infection is mostly seen in immunologically naïve animals; usually young calves where transfer of BRSV-specific maternally derived antibodies (MDA) have been inadequate, and older calves, with declining levels of specific MDA (Stott et al. 1980; Kimman et al. 1988). Commonly observed clinical signs are those of an upper airway infection, which may progress to bronchiolitis and interstitial pneumonia. Infection usually begins in the nasal mucosa or conjunctiva, with nasal and ocular discharge, and progresses to the lower airways, with coughing, abdominal dyspnea, tachypnea and adventitious lung sounds, which may include both wheezes and crackles (Bryson et al. 1983). In addition, severely affected animals may show non-specific signs, such as fever, depression, and reluctance to stand up or to eat.

Typically, the severity of respiratory clinical signs observed corresponds with the severity and extent of lung pathology (Leruste et al. 2012). Macroscopically, lesions appear as patchy or coalescent areas of consolidated lung tissue, with a swollen and dark red appearance, predominantly in the cranioventral lobes (Bryson 1993). In severe cases, bullous emphysema may be observed, typically in the caudal lobes (Kimman, Straver, et al. 1989).

Histologically, purulent bronchiolitis and alveolitis is typically seen in acute cases, with focal bronchiolar epithelial hyperplasia and necrosis, thickening of alveolar septa, and alveolar collapse (Bryson et al. 1978). Mucus, purulent debris and increased numbers of neutrophils are observed in bronchoalveolar lavage from BRSV-infected calves (Kimman et al. 1986).

In addition to clinical signs attributed to BRSV replication and inflammation, co-infections (e.g. species of pasteurella and mycoplasma) are common, and the etiology of individual clinical signs and lesions cannot always be assigned to a specific agent (Bryson et al. 1978; Callan & Garry 2002).

In humans, severe HRSV disease in infancy has been linked to chronic HRSV sequelae such as asthma and wheezing later in life, and has been linked to permanent remodeling of the airways following severe HRSV infection in early infancy (Szabo et al. 2013; Sigurs et al. 2010). In contrast, similar long-term complications following BRSV infection have not been reported in cattle, and moderate BRSV-induced lung pathology may be reversible (Castleman et al. 1985).

1.6 RSV experimental infection models

1.6.1 BRSV infection models

The study of BRSV and the evaluation of vaccines and therapies hinges on a reliable calf infection model. To be applicable to field conditions, and useful in e.g. vaccine evaluation studies, such a model needs to induce clinical signs in calves both with and without passive immunity. Ever since BRSV was first isolated four decades ago, many experimental infections have been published, often with virus shedding following infection, but limited specific respiratory clinical signs (Belknap et al. 1995; Taylor 2013). In 1972, Inaba and colleagues experimentally infected a seronegative calf by intranasal (i.n.) and intratracheal (i.t.) routes, with little resulting respiratory clinical signs despite virus shedding being detected in nasal secretions, and seroconversion occurring two weeks after infection (Inaba et al. 1972). A similar lack of clinical signs has been reported using i.n. and/or i.t. inoculation of both seronegative and seropositive calves (Jacobs & Edington 1975; Thomas et al. 1984; van der Poel et al. 1996). By employing repeated i.n. and i.t. inoculations of calves with or without passive immunity, some studies have reported more severe clinical signs (Bryson et al. 1983; Ciszewski et al. 1991; Belknap et al. 1991). Other studies however, still report only mild signs of BRSV infection, despite the repeated inoculations (Kimman, Westenbrink, Schreuder, et al. 1987; Otto et al. 1996). Additionally, some studies have reported severe clinical signs following experimental infection, but could not determine the importance of co-infections (Ciszewski et al. 1991), or preclude comparison with other studies by omitting key clinical parameters, such as changes in rectal temperature following challenge (Xue et al. 2010).

Inhalation of aerosolized BRSV has been the most successful calf model in inducing clinical signs in seropositive calves: either alone (Hägglund et al. 2004; Ellis et al. 2013), or in combination with i.t. inoculation (Larsen et al. 1999; Tjørnehøj 2000; Tjørnehøj et al. 2003). Small aerosol droplets (<5µm diameter) have the potential to reach and simultaneously infect the entire lung (Hatch 1961), but whether such droplets can convey infectious BRSV has not been determined.

To demonstrate clinical significance of the treatment effect of evaluated vaccines or antivirals, the infection model needs to induce clear clinical signs and marked pathology, besides virus shedding (Nakagawa & Cuthill 2007).

Apart from the generally low level of clinical expression in published experimental BRSV infection studies in calves, the diversity of methodology in sampling and reporting clinical signs and pathology, makes comparison difficult and meta-analysis problematic.

Lambs, which are less costly than calves and highly available, have also been experimentally infected with BRSV but appear to be less permissive than calves. Although some infected lambs shed virus and seroconvert following challenge, they demonstrate very limited clinical signs and minimal airway pathology (Cutlip & Lehmkuhl 1979; Lehmkuhl & Cutlip 1979a; Lehmkuhl & Cutlip 1979b; F J Trigo et al. 1984; Sharma & Woldehiwet 1990b).

1.6.2 HRSV infection models

Although experimental HRSV infection has been studied in healthy adult volunteers (Bagga et al. 2013), studies in the populations at risk for severe lower respiratory infection (i.e. infants, elderly and immunosuppressed) are restricted to studying cases of natural infection. Thus, a multitude of animal models have been explored (Bem et al. 2011). Chimpanzees are highly permissive to HRSV infection, but the high cost and unavailability of seronegative individuals have resulted in only a limited number of studies in this species (Byrd & Prince 1997). In contrast, mice and cotton rats are widely used in HRSV challenge studies, and are highly available, as are immunological reagents for these species (Bem et al. 2011). Other species, like macaques and lambs, constitute a compromise, in being less permissive to HRSV infection than chimpanzees and less immunologically defined than mice, but also being readily available and more similar in size and physiology compared to human infants (Bem et al. 2011).

Because of the similarities in epidemiology and clinical manifestation between HRSV and BRSV, experimental BRSV infection in calves is highly suited to study RSV pathogenesis in a natural host, and to evaluate vaccine candidates based on conserved proteins or identical attenuation approaches.

1.7 Immune responses and immunity

1.7.1 Passive immunity

Due to high seroprevalence, BRSV-specific MDA, primarily of isotype IgG_1, will be detectable in sera from most young calves in endemic regions, when fed sufficient quality and quantity colostrum in the first 24h post-partum (Uttenthal et al. 2000). These MDA have a serum half-life of approximately 36 days and therefore most unexposed calves will be seronegative by 3 to 7 months of age (Baker et al. 1986; Fulton et al. 2004). However, exposure during this period is highly likely in many herds, but might not be detected serologically due to suppression of humoral responses by specific MDA in calves (Kimman, Westenbrink, Schreuder, et al. 1987).

Maternal leukocytes present in colostrum are also taken up in the intestine and transported to lymphoid tissue (Liebler-Tenorio et al. 2002), where they react, up to 1 week after ingestion of colostrum, when stimulated with specific pathogens (Donovan et al. 2007). While the duration of colostrum-derived pathogen-specific T cell responses appear to be short lived, these may stimulate the neonatal immune system and augment its development in a non-specific manner (Reber et al. 2005). It might therefore be important to feed calves with fresh rather than exclusively frozen-thawed colostrum, in which cells are destroyed.

Both protection from clinical disease, and the immunological memory induced, are related to serum titer of specific MDA at the time of primary infection, because specific MDAs tend to suppress the initial humoral response (Kimman et al. 1988; Belknap et al. 1991). Thus, calves with high titers of specific MDA may be partially protected from clinical disease, but vulnerable to reinfections, due to limited immune memory. Consequently, in colostrum-fed calves disease is usually seen in calves older than 2 weeks old, with the most severe clinical signs observed in calves 1 to 3 months old (Kimman et al. 1988).

1.7.2 Innate immune responses

In an early stage during BRSV infection, the innate immune system is activated by several pattern recognition receptors (PRRs) in host cells, which recognize conserved pathogen-associated molecular patterns (PAMPs) in BRSV proteins and RNA (Zeng et al. 2012). This triggers several signaling pathways, resulting in a cascade of pro-inflammatory and antiviral cytokines, including IFNγ, TNFα, IL-1, IL-6, IL-8 and type I IFN (IFNα/β) (Knott et al. 1998; Grell, Tjørnehøj, et al. 2005; Røntved et al. 2000; Gershwin 2012; Guzman & Taylor 2014). IL-1, IL-6, and TNFα have local effects such as increasing diapedesis, and systemic effects, such as induction of fever and increased pain sensitivity. IL-8 is secreted by infected endothelial cells and activated macrophages, and induces neutrophil chemotaxis and phagocytosis (Baggiolini & Clark-Lewis 1992). IFNγ is produced and secreted in response to primary BRSV infection, mainly by NK cells, dendritic cells, macrophages, and γδT cells, and acts by promoting specific and non-specific cellular immune responses to intracellular pathogens (Knott et al. 1998). Once the virus is replicating and viral proteins are expressed, the production of type I IFN is down-regulated by BRSV (Valarcher et al. 2003).

In contrast to most other animals, cattle, and particularly young calves, have a high percentage of γδT cells (up to 60%-70% of T cells in calves) (Jutila et al. 2008). γδT cells are important in bovine neonatal innate immunity, and may

augment the acquired immune response to BRSV, following PRR-activation (TLR3 and TLR7) of γδT cells by BRSV (McGill et al. 2013). However, the numbers of γδT cells, and their systemic and local distribution, appear to be unaffected by primary BRSV infection in young calves (Mcinnes et al. 1999). γδT cells are also important immune regulators in cattle, partly by secreting the anti-inflammatory cytokine IL-10 (Guzman et al. 2014).

1.7.3 Acquired immune responses

Acquired immune responses to primary BRSV infection or vaccination are more or less suppressed by BRSV-specific serum MDA in calves, although immune memory may still be induced (Kimman, Westenbrink, Schreuder, et al. 1987). For many other pathogens, passively acquired serum antibodies suppress humoral, but not cellular, immune response (Niewiesk 2014); this also appears to be the case for BRSV (van der Sluijs et al. 2010).

Protective acquired immunity to RSV depends on both humoral and cellular components. Whereas high titers of virus neutralizing serum IgG and mucosal IgA antibodies can potentially stop initiation of infection (Kimman, Westenbrink, Schreuder, et al. 1987; Lee et al. 2004), a cytotoxic T cell response is essential for effective clearance of established infection in the lower airways (Taylor et al. 1995; Rossey et al. 2014). Calves intranasally inoculated with BRSV developed specific cell mediated immune responses, which were detectable after 5 days, peaked after 21 days, and persisted for at least 42 days (Field & Smith 1984). Neutralizing antibodies, both systemic and local, are mainly directed at the F and G proteins (Walsh et al. 1987; Westenbrink et al. 1989; Taylor et al. 1997; Taylor et al. 2005), whereas cytotoxic T cell activity is induced by the F, N, M2-1 (Openshaw et al. 1990; Gaddum et al. 2003) and P proteins (G. Taylor, 2014, pers. comm.).

In seronegative (colostrum-deprived) calves, primary infection induces serum antibodies of isotype IgM and IgA, IgG_1 and IgG_2, which are detectable by ELISA starting 8-10, 10-14 days, 13-17 days and 1-3 months after infection, respectively (Kimman, Westenbrink, Schreuder, et al. 1987). Similarly, neutralizing antibodies can be detected in serum 2 weeks after infection (Inaba et al. 1972). The duration of detectable serum antibodies, following primary infection of seronegative calves, varies from 2-4 weeks for IgM and IgA, and at least 4 months for IgG_1 (Kimman, Westenbrink, Schreuder, et al. 1987; Uttenthal et al. 2000). The duration of IgG_2 antibodies is unclear, but is likely longer than 4 months (Larsen 2000).

In adult cattle, which may have a history of repeated BRSV exposure, IgG_1 antibodies can persist for at least 3 years (T. B. Klem et al. 2014).

In contrast to seronegative animals, primary infection of calves with moderate levels of specific MDA (colostrum-fed) induces no change in serum or local respiratory antibody titer, except for occasional weak IgM and IgA responses (Kimman, Westenbrink, Schreuder, et al. 1987).

On the other hand, calves without serum antibodies, but with circulating BRSV-specific T cells (indicating immune memory from previous exposure), demonstrate more rapid and stronger humoral and cellular responses when challenged with BRSV, compared to unprimed calves (Sandbulte & Roth 2002).

Although no single immunological parameters has been shown to perfectly correlate with protection against BRSV following primary infection or vaccination, protection has been associated with humoral responses, in particular mucosal IgA secretion (Kimman, Westenbrink, Schreuder, et al. 1987), as well as with cellular responses (Riffault et al. 2010).

1.8 Pathogenesis

1.8.1 Course of infection

Based on experimental studies, natural BRSV infection is postulated to initiate as an infection of the upper airways, that can progress to pneumonia (Viuff et al. 1996). Upper respiratory and mild systemic clinical signs may occur as early as 2 days post-infection, whereas severe lower respiratory clinical signs usually culminate 7 days post-infection, and most clinical signs subside within 10 to 15 days after onset (Inaba et al. 1972; Hägglund et al. 2004). During this progression, BRSV replicates initially in epithelial cells in the nasal cavity and over approximately 2 days extends into the trachea, bronchi, bronchioles and alveoli (Viuff et al. 1996). In other studies where calves were experimentally infected, BRSV was demonstrated in nasal secretion up to 12 days following infection (titers peaking at 4 to 6 days) (Elazhary et al. 1980), and in lungs between 3 and 13 days (Jacobs & Edington 1975; Castleman et al. 1985; Viuff et al. 2002; Valarcher et al. 1999).

1.8.2 Viral mechanisms of pathogenicity

The initial clinical signs and pathology observed during primary RSV infection are a consequence of viral replication. Ventilation is impeded in the lower airways when replication in epithelial cells causes impaired ciliary function and an excess of luminal cell debris and mucus, along with structural collapse of alveoli (Kimman, Straver, et al. 1989; Bryson et al. 1991; Viuff et al. 1996). Bronchoconstriction further impedes ventilation, and in primary BRSV infection might be partially mediated by the viral peptide virokinin (produced

by post-translational proteolytic cleavage of the BRSV F protein) (Zimmer et al. 2003; Valarcher et al. 2006). This, along with the intrinsically poor collateral alveolar ventilation in the bovine lung, can facilitate development of emphysema due to air trapping in alveoli (Kimman, Straver, et al. 1989). In addition, tissue damage, poor ventilation, and the impaired mucociliary escalator, predisposes the host to secondary bacterial infections.

1.8.3 Effects of the primary immune response and viral countermeasures

Apart from the direct effects of viral replication, inflammatory responses in the host account for a considerable part of clinical signs and pathology, both during primary and secondary infection (Tregoning & Schwarze 2010). Whereas BRSV inoculation of tissue cultures causes obvious cytopathology, very little cytopathology is seen when monocultured bovine respiratory epithelial cells are infected (Valarcher & Taylor 2007). Such differences can be explained by the fact that certain viral mechanisms may influence the immune response *in vivo.* For example:

- HRSV replication induces transcription of IL-8, an important mediator of mucosal inflammation, through cytoplasmic-nuclear translocation of NF-κB p65 subunit (RELA) in human alveolar epithelial cells (Garofalo et al. 1996).
- The non-structural RSV proteins NS1 and NS2 interfere with type I IFN (IFNα/β) production and signaling (Valarcher et al. 2003), and render infected cells resistant to the antiviral effects of IFNα/β (Schlender et al. 2000; Bossert & Conzelmann 2002). Furthermore, wild-type HRSV demonstrate increased activation of IL-4 producing $CD4^+$ T cells and decreased activation and proliferation of Th17 cells, compared to NS1-gene-deleted HRSV, suggesting a Th2 type immune response, independent of IFNα/β signaling (Munir et al. 2011).
- The SH protein of BRSV and HRSV inhibits tumor necrosis factor alpha (TNFα) signaling (Fuentes et al. 2007; Taylor et al. 2014a), and thereby suppresses apoptosis in infected cells. Apoptosis is an important means of defense against intracellular pathogens such as BRSV (Viuff et al. 2002).
- A truncated and soluble form of the attachment protein G is secreted from HRSV infected cells, starting before the budding off of virions (Hendricks et al. 1988). This secreted form of the G protein is proposed to thwart immune responses, by tying up antibodies in the humoral response, and through its mimicry of the leukocyte chemoattractant CX3CL1 (fractalkine in humans) (Tripp et al. 2001) of the innate and adaptive cellular immune response.

- Purified HRSV F protein up-regulates the toll-like receptor 4 (TLR4), making airway epithelial cells more sensitive to present bacteria (Monick et al. 2003), but potentially also aiding the clearance of virus (Haynes et al. 2001). On the other hand, low expression of TLR4 in cord blood from infants, has been associated with increased risk of developing severe HRSV disease (Inchley et al. 2013).

The individual impact of these various mechanisms on RSV pathogenesis, and the extent to which extrapolation between BRSV and HRSV is valid, remains to be fully elucidated. Furthermore, there may be strain-dependent differences in virus/host interactions, that may effect the course and manifestation of infection and host responses (Valarcher & Taylor 2007).

1.8.4 Immunopathogenesis

Apparently immune-mediated aggravated disease caused by natural HRSV infection, after prior vaccination with a formalin inactivated (FI) HRSV, was first reported in 1969 by Kim et al., following a vaccine trial in infants and children (Kim et al. 1969).

It was concluded that this exacerbated disease was caused by factors relating to the virus in the vaccine, as both unvaccinated children and children immunized with a parainfluenza type 1 vaccine, produced similarly by the same manufacturer, did not suffer exacerbated disease in the same HRSV outbreak (Chanock et al. 1970).

A later study, which analyzed sera from that vaccine trial, showed that FI-HRSV induced antibodies that bind to the F and G HRSV proteins, but failed to inhibit cell fusion and neutralize virus (Murphy et al. 1986; Murphy & Walsh 1988), possibly caused by distortion of viral epitopes by formalin inactivation.

FI-RSV vaccine-induced immunopathology has been reproduced using FI-HRSV in small animal models (Graham et al. 1993; Connors et al. 1994), macaques (De Swart et al. 2002), and using FI-BRSV in calves (Antonis et al. 2003).

Results from these animal models corroborated the induction of non-neutralizing antibodies by FI-HRSV (Graham et al. 1993; Connors et al. 1994), and showed that FI-HRSV induced a Th2 biased immune response (Connors et al. 1992; Hussell et al. 1998), characterized by an influx of eosinophils in the lungs (Openshaw et al. 2001). It has also been suggested that these non-neutralizing antibodies may form complexes with antigen in the lungs (Graham et al. 1993; Connors et al. 1994), which may in turn induce a detrimental inflammatory response (Kimman, Daha, et al. 1989; Nadal & Ogra 1990).

However, despite the use of several commercially available inactivated BRSV vaccines for the past several decades, there is only one report of aggravated disease in calves immunized with inactivated BRSV and subsequently naturally exposed to BRSV (Schreiber et al. 2000), suggesting that this phenomenon is a rarity in cattle.

Thus, after 45 years of extensive studies in animal models to explain the FI-HRSV vaccine-induced immunopathology observed in children in the 1960's, uncertainties remain.

1.9 Laboratory diagnostics

1.9.1 Indirect diagnostics

One commonly used diagnostic tool to attribute an outbreak of respiratory disease in a herd to BRSV infection is analysis of paired serum to detect seroconversions or significant increases of antibodies in an individual or a herd. Titers of antibodies may be determined either using a virus neutralization assay by quantifying the ability of sera to reduce infection in BRSV inoculated cells, or by ELISA where antigen-specific antibodies are quantified. Total BRSV antigen produced by inoculation of cells is commonly used, either to coat wells in an indirect ELISA, or as the antigen in a capture ELISA. However, specific antibodies can also be detected using ELISAs coated with individual proteins, such as G (Taylor et al. 1995) and N (Samal et al. 1993). For routine detection of seropositivity or seroconversion, BRSV-specific IgG antibodies are often measured (Hägglund et al. 2006). Assays selectively detecting IgA, IgM, IgG_1 and IgG_2 may be employed to establish timing of an outbreak (Kimman, Westenbrink, Straver, et al. 1987).

1.9.2 Direct diagnostics

Pre-existing high titers of antibodies, passively or actively acquired, may prevent or mask humoral responses. Thus direct detection of live virus, viral antigen, or viral RNA may be required to attribute an outbreak of respiratory disease to BRSV (Kimman et al. 1986).

The presence of live BRSV in nasal secretion or bronchoalveolar lavage (BAL) may be ascertained by virus isolation, and virus quantified by titration and characterized by virus neutralization assay. This delicate process involves inoculation of the sample onto bovine kidney (Thomas et al. 1982), turbinate (Elvander 1996), or lung cells (Larsen et al. 1999), followed by either observation of virus specific cytopathic effect by light microscopy, staining of virus antigen by immunohistochemistry, or detection of virus RNA by molecular beacons (Santangelo et al. 2006).

Although demonstration of live virus is most convincing, inoculation of cell culture is not always practical, and there may be uncertainties about the viability of virus in samples collected in the field. In this context, demonstration of virus antigen(s) or RNA is an alternative.

Immunofluorescent staining can be used to illustrate BRSV antigen in histological sections (Kimman et al. 1986; Viuff et al. 1996), whereas antigen ELISAs can be used in the field to detect BRSV antigens in body fluids (Valarcher et al. 1999; Tjørnehøj et al. 2003). Recently, a rapid HRSV antigen detection test, containing monoclonal antibodies against HRSV proteins F and N, was shown to yield results with a high level of agreement with reverse transcription quantitative PCR results (RT-qPCR), when nasal secretions from calves were analyzed (Urban-Chmiel et al. 2013; Urban-Chmiel et al. 2014).

Whereas isolation can prove etiology, and antigen immunofluorescent staining can indicate the extent of viral antigen present, conventional or RT-qPCR is more sensitive (Larsen et al. 1999; Hakhverdyan et al. 2005; Boxus et al. 2005), and can detect viral RNA in BAL fluid up 13 days following experimental infection in calves, compared to 5 days for virus isolation and immunofluorescent staining (Valarcher et al. 1999).

Recently, a method termed droplet-real-time PCR reportedly shortened the assay time to 10 min (Uehara et al. 2014), when detecting BRSV using previously published RT-qPCR primers and probe (Willoughby et al. 2008)

1.10 Vaccines

Since BRSV is highly prevalent worldwide, eradication on a herd or regional level would leave naïve animals susceptible to reintroduction, with occurrence of potentially severe clinical disease in cattle of all ages.

On the other hand, effective vaccination of unexposed animals and herds can protect individual animals from clinical disease, and in addition protect herds by stopping viral replication, thereby reducing the risk of transmission into and within that herd.

Calves need to be vaccinated at an early age, to protect them when they are co-mingled. However, the development of an effective BRSV vaccine in young calves faces four basic obstacles: (i) immunological immaturity in young calves tend to attenuate vaccine responses (Chase et al. 2008); (ii) immune responses are often suppressed by BRSV-specific maternally derived antibodies (MDA) in sera of young calves; (iii) vaccination needs to induce a balanced and long lasting immune response, exceeding that induced by natural infection in young calves with specific MDA; and (iv) vaccination must be

safe, and avoid vaccine-induced immunopathology, as reported for some RSV vaccines.

In addition, to facilitate serological analysis to monitor virus transmission, as well as vaccine efficacy and safety, vaccination should induce an immune response that can be differentiated from that induced by infection.

1.10.1 Commercially available BRSV vaccines

Live BRSV vaccines, attenuated by serial cell culture passages, have been commercially available for 40 and 30 years, in Europe and the USA respectively (Meyer et al. 2008; Fulton 2009). Mostly due to safety concerns, these modified live vaccines (MLV) were all licensed for parenteral use, with limited ability to overcome the inhibitory effect of specific MDA in young calves (van der Poel et al. 1999).

As with many other pathogens, mucosal immunity to RSV is more effectively induced through mucosal immunization, compared to parenteral immunization, especially in the presence of specific MDA (Belyakov & Ahlers 2009; Kimman, Westenbrink, Schreuder, et al. 1987; Kimman, Westenbrink, et al. 1989).

Consequently, after demonstrating increased efficacy in calves with passive immunity using the mucosal route, compared to the parenteral route of vaccination, one of these MLVs was licensed for intranasal use (Vangeel et al. 2007). Nonetheless, both clinical and virological protection following challenge were only partial, and of short duration (Ellis et al. 2013). Moreover, when conventional colostrum-fed calves, with varying levels of serum specific MDA, were vaccinated intranasally with that vaccine, they shed virus in nasal secretions for up to 20 days after vaccination (Timsit et al. 2009).

Thus, safety concerns with MLVs remain: specifically the risks of reversion to virulence, of exacerbating disease when administered during a natural BRSV outbreak (Kimman, Sol, et al. 1989), and of vaccine contamination, e.g. with bovine viral diarrhea virus (BVDV) (Palomares et al. 2013). Furthermore, although MLVs induce immune responses similar to those following natural infection in calves, these might not be sufficient (Gershwin 2012).

Inactivated vaccines circumvent the risk of genetic reversion, and by utilizing different adjuvants the induced immune response can be directed and extended. The immunomodulating effects of adjuvants are achieved through various mechanisms, such as increased non-specific inflammation at the site of immunization (e.g. aluminum hydroxide), slowed release and prolonged exposure to the antigen (e.g. emulsions), enhanced uptake of antigen by

antigen presenting cells (APCs) (e.g. immunostimulating complexes, ISCOMs), and by activating additional arms of the immune system (e.g. TLR agonists, cytokines and antigen specific antibodies) (Aucouturier et al. 2001; Getahun & Heyman 2006; Tritto et al. 2009; Heegaard et al. 2011; Gregory et al. 2013; Garg et al. 2014). Commercially available inactivated BRSV vaccines use either aluminum hydroxide, Quil A, or a combination of the two (van der Sluijs et al. 2010; Ellis et al. 2007; Vangeel et al. 2007).

1.10.2 HRSV vaccine development

In contrast to the many commercially available BRSV vaccines, there is currently no available vaccine against HRSV (Rudraraju et al. 2013). Historical HRSV vaccine candidates have failed either due to safety concerns (Kim et al. 1969), or have proven ineffective in children with specific MDA (Belshe et al. 1982). However, several candidate vaccines are in clinical stages of evaluation, including recombinant live HRSV vaccines (i.e. ClinicalTrials.gov id NCT01459198 and NCT01968083) and recombinant vector vaccines expressing HRSV protein (ClinicalTrials.gov id NCT01805921).

Based on the successful use of passive immunoprophylaxis in at-risk infants, using humanized monoclonal anti-HRSV antibodies (Wright & Piedimonte 2011), the focus in HRSV vaccine research has recently shifted somewhat from infant immunization, to maternal immunization during pregnancy (Kaaijk et al. 2013). Maternal immunization has proven effective for many other pathogens, and may prove effective for HRSV, especially for preterm neonates (Chu & Englund 2014).

1.10.3 Differentiation of infected from vaccinated animals

Vaccination programs using any of the commercially available BRSV vaccines require repeated boosts to fully protect calves during the first year of life, and have an additional drawback: infection in a vaccinated animal or herd cannot be detected serologically, because animals vaccinated with a complete virus vaccine cannot be distinguished serologically from infected animals. On the other hand, exclusion from the vaccine formulation of at least one viral protein, which is immunogenic yet non-essential for protection, will enable continued serological differentiation of infected from vaccinated animals (DIVA) (van Oirschot et al. 1986), as illustrated in Figure 2. Ideally, a companion DIVA assay enables identification of animals or herds that were infected and were contagious, after prior vaccination. This allows continued serosurveillance in regions where vaccination programs are instituted, and furthermore provides the means to monitor changes in vaccine efficacy and safety.

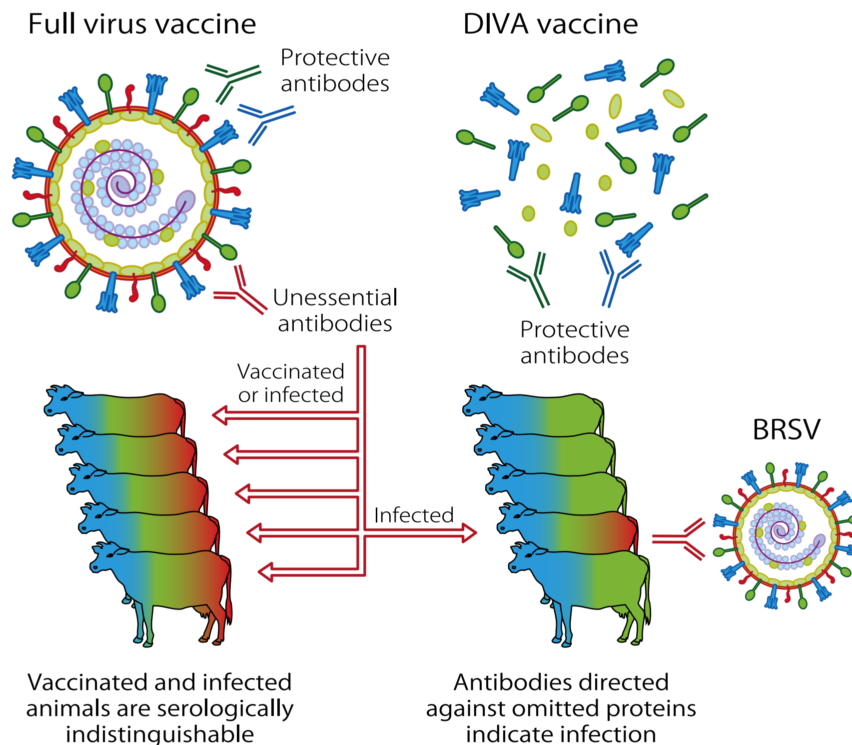

Figure 2. Exclusion of at least one viral protein from the vaccine formulation allows serological differentiation between infected and vaccinated animals (DIVA), in contrast to full virus vaccines.

1.10.4 New generation RSV vaccine strategies

In summary, vaccination of young colostrum-fed calves with currently available commercial BRSV vaccines does not offer full clinical and virological protection, or requires repeated boosts during the first year of life. Furthermore, their use precludes seromonitoring for circulating virus, or to monitor vaccine efficacy in the field. The key to effective immunity against BRSV in young calves seems to be induction of a balanced Th1/Th2 immune response, with neutralizing antibodies, as well as $CD4^+$ and $CD8^+$ T cell activity (Meyer et al. 2008; Gershwin 2012). Also, in contrast to those acting on a single arm of the immune system, multimodal immune responses are more likely to induce long lasting memory (Bevan 2011).

New generation RSV vaccines use novel adjuvants and rationally selected antigens to overcome the shortcomings of currently available commercial vaccines, with the aim to induce balanced and long lasting immune responses, and are DIVA-compatible by omitting at least one viral protein. Viral proteins

can be selected by specific purification of infected cell lysate, with varying levels of purity. However, scientific and technological advances in genetic engineering allow definitive exclusion of specific viral proteins, as well as the introduction of attenuating point mutations in selected genes.

The NS1, NS2, SH, G, F and M2-1 genes have been targeted for attenuation in live vaccines (Valarcher et al. 2003; Malkin et al. 2013). Point mutations may impart sufficient attenuation (Wright et al. 2000), but carry the risk of reversion to virulence (Lin et al. 2006). This risk can be drastically reduced by recombinant deletion of entire viral genes.

Deletion of the SH gene is one of the most promising attenuation strategies for live recombinant BRSV (ΔSHrBRSV) (Taylor et al. 2014a) and HRSV (MEDI-559) (Malkin et al. 2013). ΔSHrBRSV induced protective immunity in calves with low levels of specific MDA, after prior intranasal immunization (Taylor et al. 2014a), but requires additional investigations in calves with levels of specific MDA mimicking field condition, to further evaluate safety, immunogenicity and protective efficacy.

Genetic engineering has also produced DNA vaccines (Taylor et al. 2005) and virus vectored vaccines (Taylor et al. 1997; Bian et al. 2014; Kim et al. 2014; ClinicalTrials.gov id NCT01805921), often including the immunogenic proteins F, G, N and M2-1 proteins. Recombinant RSV proteins can be produced in cell culture, such as human embryonic kidney cells (McLellan et al. 2010), insect cells (Smith et al. 2012) and *E.* coli (Tran et al. 2007), or in plants (Lau & Korban 2010). Several subunit vaccines involving the HRSV F protein have demonstrated immunogenicity in cotton rats (Raghunandan et al. 2014), mice (Glenn et al. 2013) and macaques (McLellan et al. 2013), with induction of predominantly neutralizing antibodies.

Proteins conserved between BRSV and HRSV (e.g. F, N, P and M2-1) may provide cross-protection. The HRSV N protein, presented as N nanorings (Tran et al. 2007), have been shown to induce strong N-specific memory T cell responses and partial protection against experimental BRSV infection in seronegative calves (Riffault et al. 2010). Furthermore, when the ectodomain of the matrix protein 2 from influenza H1N1 was recombinantly attached to HRSV N nanorings, immunized mice demonstrated protection from both HRSV and influenza challenge (Hervé et al. 2014), suggesting that attaching epitopes from BRSV to N nanorings may similarly induce protective responses.

The protein content in RSV vaccines can also be modulated by different methods of specific or non-specific purification of infected cell lysate, and by antigen/adjuvant formulation. Classic BRSV-ISCOMs, i.e. purified BRSV-infected cell lysate formulated with immunostimulating complexes, were

shown to contain proteins F and N (M. Trudel et al. 1989; Trudel et al. 1992) and induced both neutralizing antibodies and cytotoxic T cell responses in mice (M. Trudel et al. 1989; Trudel et al. 1992). Moreover, BRSV-ISCOMs induced neutralizing serum and mucosal antibody responses in calves with BRSV-specific MDA and a high level of clinical and virological protection, which exceeded that induced by an inactivated commercial vaccine (Hägglund et al. 2004).

2 Aims of the thesis

The overall objective of this thesis work was to develop and evaluate new generation vaccines against BRSV, specifically:

- To evaluate, in calves with specific maternal immunity, the safety, immunogenicity and protective efficacy of a BRSV vaccine based on whole inactivated virus antigens in classic ISCOMs, and to characterize the antigen content in this formulation.
- To formulate two subunit DIVA-compatible vaccines against BRSV.
- To establish and characterize a BRSV-challenge model in calves with passive immunity, by combining protocols and methods from previous studies.
- To evaluate the safety, specific immune responses and protection induced by two subunit formulations and one recombinant live BRSV vaccine in calves with maternal antibodies.

3 Materials and methods

The intention of the text in this section is to clarify methods used in each experiment involved in this thesis, and to indicate similarities and differences between experiments. In some sub-sections, for further technical details the reader is directed to publications that are included in this thesis. In other sub-sections, details are given where they were omitted in publications.

3.1 Cells and viruses

3.1.1 Cells

Calf kidney (CK) cells, bovine turbinate (BT) cells and Vero cells at low passages were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM, Lonza, Belgium), with 20 ml 1 M Hepes buffer (Lonza, Belgium), 10 ml 200 mM l-glutamine (Lonza, Belgium), 10 ml 0.9% NaCl solution, 60 mg benzyl penicillin sodium (Recip AB, Sweden) and 100 mg streptomycin sulfate salt (Sigma Aldrich, Sweden), added per liter DMEM. Fetal calf serum (FCS, PAA Laboratories GmbH, Austria) was added to the supplemented DMEM, at concentration of 5%, 10% or 20% (DMEM-FCS 5%, 10% and 20%, respectively).

3.1.2 Viruses

Two isolates of BRSV were used for experimental challenges described herein and are referred to as: BRSV-Dk (no. 9402022, Denmark (Viuff et al. 1996), and BRSV-Snk (Snook strain, (Thomas et al. 1982)). BRSV-Dk was isolated and passaged five times in fetal bovine lung cells (Viuff et al. 1996), followed by two (study I) or three (study III) passages in BT cells. After initial isolation in CK cells (Thomas et al. 1982), BRSV-Snk was passaged three consecutive times in gnotobiotic calves, where each inoculum was prepared from BAL and administered by respiratory route, as described in detail elsewhere (Valarcher

et al. 2003). Both inocula were verified to be free of contaminating viruses, including BVDV, and bacteria, including mycoplasma, as described in paper I and III. Inocula and other virus sample titers were determined by plaque assay on CK cells, with the unit pfu/ml, as previously described (Stott et al. 1980), or by endpoint dilution assay on BT cells, with the unit $TCID_{50}$/ml, as previously described (Hägglund et al. 2004).

3.2 Candidate vaccine production and formulation

3.2.1 Production of BRSV antigen and BRSV-ISCOM vaccine

BRSV antigen and BRSV-ISCOMs were produced before the start of this thesis project. Briefly, BRSV antigens used for vaccine formulation in study 1 and 2, as well as for RT-qPCR positive control and ELISA antigen, were produced using BRSV-Dk, as described for the BRSV-Dk inoculum, except propagation and titration which were done in Vero cells. These BRSV antigens were purified by ultracentrifugation over sucrose, solubilized using octyl glucoside and separated through a sucrose gradient, as described in detail in paper II. ISCOMs with incorporated BRSV proteins (BRSV-ISCOMs) were produced by incubating a fraction of the solubilized antigen with cholesterol, phosphatidylcholine and Quillaja saponin, followed by purification by dialysis, ultracentrifugation and sterile filtering, as described in detail in paper I and II. BRSV protein used as control antigen in paper I and II, was processed as described for BRSV-ISCOMs, without adding lipids and Quillaja saponin. As adjuvant control for the BRSV-ISCOMs in paper I, a commercial ISCOM matrix adjuvant, containing cholesterol, phosphatidylcholine and Quillaja saponin from the same batch, was used (AbISCO-300, Novavax, previously Isconova, Uppsala, Sweden). In addition, ISCOMs formulated with uninfected Vero cell lysate (Vero-ISCOMs), otherwise identical to BRSV-ISCOM formulation, was used as control when characterizing BRSV-ISCOMs in paper II. Presence of infectious BRSV in the BRSV-ISCOM vaccine was ruled out by negative virus isolation.

3.2.2 Characterization of BRSV-ISCOMs

Aliquots of the undiluted BRSV-ISCOM vaccine formulation were analyzed to characterize the protein content. Aliquots of undiluted BRSV proteins and Vero-ISCOMs were used as controls. The protein concentrations in BRSV-ISCOMs, BRSV proteins and Vero-ISCOMs were 1.46 mg/ml, 1.43 mg/ml and 0.73 mg/ml, respectively; as determined by the Bradford assay (Bradford 1976) and amino acid analysis (Amino Acid Analysis Center, Uppsala University, Sweden) (Moore et al. 1958).

The morphology and size of BRSV-ISCOMs were investigated by negative-stain transmission electron microscopy (TEM; FEI Tecnai 10 G2 microscope; 2kx2k Morada CCD camera; iTEM control software, Olympus Soft Imaging Solutions GmbH; Vironova AB, Sweden).

BRSV proteins were identified by size and antigenicity, using SDS-PAGE, Western blot (Towbin et al. 1979), and dot-blot, with protein specific monoclonal antibodies, and hyperimmune sera from calves experimentally infected with BRSV (BRSV-Dk or strain 391-2), as described in detail in paper II. In addition, visually detectable bands on SDS-PAGE gels from three separate batches of BRSV-ISCOMs were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF; Uppsala University, Sweden; and INRA, France) (Tran et al. 2009), and entire lanes were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS; PAPPSO platform, INRA, France) (Arfi et al. 2013). The size and intensity of bands in digital images of SDS-PAGE gels was used to estimate the relative quantity between individual proteins, using ImageJ analysis software) (Schneider et al. 2012). Data from LC-MS/MS were used to find matches for proteins in the following protein databases: *Macaca mulatta* (UniprotKB, 06/24/2013), BRSV (strain A519086, UniprotKB 06/21/2013, and BRSV-Dk), and an in-house contaminant database (INRA, France).

3.2.3 Subunit vaccines containing HRSV P, M2-1 and N-nanorings with BRSV epitopes

Construction, expression and purification of recombinant RSV proteins

Recombinant HRSV protein with epitopes from BRSV included in the subunit vaccine formulations in paper IV, were constructed, produced and purified by a project partner in this thesis work, and this process is described in detail in paper IV. In brief, *E*. coli were transformed with plasmids to express full-length HRSV M2-1 or -P proteins, or HRSV N protein nanorings decorated with epitopes from BRSV (Table 2). N nanorings, with 10 or 11 protomers, were constructed and formed by co-expression of full-length HRSV N protein and amino acid residues 161-241 of the HRSV P protein, as described elsewhere (Tran et al. 2007; Tawar et al. 2009). The BRSV epitope-decorated N-nanorings (eN) used in the subunit vaccines were constructed by recombinantly attaching selected residues to the N or C terminus of the N protein, and expressed and purified as described for N nanorings (Table 2). The attached residues represent key epitopes on BRSV proteins F (AA 255-278 and 422-438) and G (AA 174-187), and one combinatorial peptide mimicking antigenic site II on the BRSV F protein (mimotope) (Chargelegue et al. 1998) (Table 2). All HRSV recombinant protein were constructed using the HRSV

Long strain sequence (GenBank accession no. AY911262), and BRSV epitopes using BRSV-Dk sequences (Larsen et al. 1998), and constructs verified by sequencing. Expressed protein concentration was measured by absorption at 280 nm (M2-1 and P) or the Bradford method (eN).

Table 2. *Recombinant HRSV proteins and BRSV epitopes used in subunit vaccine formulation. Recombinant HRSV proteins P and M2-1 were included as full-length proteins. N nanorings are formed by co-expression of the HRSV N protein (Long strain) and a fragment of HRSV P protein (Long strain, AA residues 161-241; Tran et al. 2007). N nanorings with recombinant epitopes attached to each of the 10 or 11 protomers (eN) were constructed by grafting indicated residues to the N or C terminus of the N protein.*

Protein product	Description	Sequence origin
P	Full length P protein	HRSV[a]
M2-1	Full length M2-1 protein	HRSV[a]
eN-$F_{255-278}$	Residues 255-278 of F at N terminus of N in eN (AA: SELLSLINDMPITNDQKKLMSSNV)	BRSV[b]
eN-$F_{422-438}$	F residues 422-438 at C terminus of N in eN (AA: CTASNKNRGIIKTFSNG)	BRSV[b]
eN-F_{mimo}	Residues mimicking epitope on F[c] at C terminus of N in eN (AA: HWSISKPQ)	Combinatorial peptide
eN-$G_{174-187}$	G residues 174-187 at N terminus of N in eN (AA: STCEGNLACLSLCQ)	BRSV[b]

a HRSV Long strain (GenBank accession no. AY911262).
b BRSV strain 9402022 (Larsen et al. 1998).
c Antigenic site II (AA residues 422-438) on protein F, as described by Chargelegue et al. (1998).

Formulation of subunit vaccines

In paper IV, subunit vaccines SUMont and SUAbis were formulated, both with 25 µg/dose each of recombinant proteins HRSV M2-1, HRSV P, eN-$F_{255-278}$, eN-$F_{422-438}$, eN-F_{mimo} and eN-$G_{174-187}$, diluted in PBS (Table 2). For each dose of SUMont, 0.6 ml of protein suspension (150 µg of total protein) was mixed and emulsified with 1.4 ml of Montanide ISA71^{VG} (SEPPIC, France), according to the manufacturer's instructions. For each dose of SUAbis, 144 µl (390 µg) of AbISCO-300 (Novavax, Sweden) and 150 µg of total protein were diluted in PBS to a final volume of 2 ml. A placebo vaccine, formulated as SUAbis without protein, was used as control in paper IV.

3.2.4 Recombinant SH-gene-deleted live BRSV vaccine

The recombinant BRSV with deleted SH gene (ΔSHrBRSV) used as live vaccine in paper IV was constructed by a project partner in this thesis, using full-length cDNA from BRSV strain A51908 (Mohanty et al. 1975), variant Atue51908 (GenBank accession no. AF092942), as previously described (Karger et al. 2001). ΔSHrBRSV was propagated in Vero cells and verified to be free of BVDV, as previously described (Valarcher et al. 2003). The use of ΔSHrBRSV, a genetically modified microorganism, for experimental intranasal vaccination of calves in paper IV, was approved by the Swedish Work Environment Authority (registration number 202100-1868 v8a1).

3.3 Calves

Calves of Swedish Holstein or Swedish red and white breed were obtained from two conventional dairy herds (herd 1 and herd 2) for experiments reported in papers I, II, III and IV (Table 3).

Both herd 1 and 2 were certified to be free of BVDV and bovine leucosis. Seromonitoring in both herds (as described in detail in each paper) for detection of BRSV infections indicated that included calves were BRSV naïve. However, as discussed below for paper III, this appeared not to be valid for one animal.

Group allocation for each experiment aimed to generate homogenous groups, with regard to age and titer of BRSV-specific MDA. The assigned treatment for each group, within each experiment, was randomized.

Groups of calves in paper I and II were housed in separate pens, whereas groups of calves in paper III and IV were housed in separate rooms with anteroom and negative pressure ventilation, and separate protective clothing for staff. All calves had free access to water and hay, and rations of calf pellets twice daily. In addition, calves were fed milk replacer twice daily, except in paper III.

All animal experiments described herein were carried out in accordance with the EU Directive 86/609, and were approved by the Ethical Committee at the district court of Uppsala, Sweden (Ref. no. C68/10 and C330/11).

Table 3. *Experimental groups and calf ages. Calves were obtained for experimental vaccination and BRSV challenge (reported in paper I, II and IV), or for experimental BRSV challenge alone (reported in paper III), or as sentinels for transmission of live vaccine (paper IV), or as uninfected controls (paper III), as described in material and methods. Calf identities used to refer to calves in this thesis are the same as those used in the paper indicated.*

Paper	Group	n	Calf identities	Age at immunization (weeks)	Age at challenge (weeks)
I & II	BRSV-ISCOM	5	a-e	6 (3-8)	11 (8-13)
	BRSV protein	5	f-j	6 (5-8)	11 (10-13)
	Adjuvant	5	k-o	6 (5-7)	11 (10-12)
	PBS	5	p-t	6 (4-8)	11 (9-13)
III	BRSV-Snk	3	A1-A3		10 (8-13)
	BRSV-Dk	3	B1-B3		10 (8-11)
	Uninfected	3	D1-D3		13 (8-15)[a]
IV	ΔSHrBRSV	5	a1-a5	7 (4-9)	12 (9-14)
	SUMont	5	b1-b5	6 (3-10)	11 (8-15)
	SUAbis	5	c1-c5	7 (2-9)	12 (7-14)
	Controls	5	d1-d5[b]	6 (2-11)	11 (7-16)
	Sentinels	3	s1-s3	6 (4-7)	

a Refers to age at commencement of experiments, equivalent to the day of challenge in infected calves.
b Referred to as calves C1-C5 in paper III.

3.4 Experimental vaccination

3.4.1 Paper I

In paper I, four groups of five calves each (Table 3) were immunized twice with an interval of two weeks, subcutaneously with: (i) BRSV-ISCOMs containing 188 µg protein in 2 ml PBS; (ii) 188 µg BRSV protein in 2 ml PBS; (iii) 390 µg AbISCO-300 in 2 ml PBS; or (iv) 2 ml PBS.

3.4.2 Paper IV

In paper IV, four groups of five calves each (Table 3) were immunized: group (a) i.n. with ΔSHrBRSV ($5x10^6$ pfu in 6 ml of DMEM); group (b) intramuscularly (i.m.) with 2 ml of SUMont; group (c) subcutaneously (s.c.) with 2 ml of SUAbis; and group (d) s.c. with 2 ml of placebo. Group (a) was immunized once, whereas groups (b), (c) and (d) were immunized twice, with an interval of three weeks.

To avoid spread of the live vaccine to the other groups, group (a) was housed in a separate room until three weeks after vaccination, in a separate unit of the animal facility that had separate ventilation. In addition to the measures

described to isolate each room in a unit, each unit had separate staff, and showers were required to exit each unit.

To detect possible transmission of the live vaccine (ΔSHrBRSV), three seronegative sentinel calves were co-housed with group (a), starting the day after vaccination, for six consecutive days. During this time, and for two additional weeks after being isolated from group (a), sentinels were clinically, immunologically and virologically monitored for indications of transmission of vaccine virus. Replication of the live vaccine (ΔSHrBRSV) in the upper airways was also monitored in group (a) following vaccination by collecting and analyzing nasal secretion for the presence of BRSV RNA by RT-qPCR.

Three weeks after first vaccination, one calf (c5) in group (c) was euthanized due to a traumatic leg injury.

3.4.3 Post-vaccination monitoring of calves (papers I and IV)

Calves were monitored clinically following vaccination, including daily recording of rectal temperature, and any local or systemic adverse effect of immunization. If any, local swelling at the site of injection was classified as mild (<5x5 cm), moderate (<10x10 cm), marked (<15x15 cm) or severe (>15x15 cm) (paper IV).

3.5 Experimental infection of calves by aerosol inhalation

On post-infection day (PID) 0 all calves, except sentinels and uninfected controls, were challenged by inhalation of aerosolized virulent BRSV, in three different experiments (papers I, III and IV). In paper I calves were challenged with BRSV-Dk ($10^{5.0}$ $TCID_{50}$ in 3 ml DMEM), in paper III with either BRSV-Dk ($10^{4.4}$ pfu in 5 ml DMEM; calves B1-3) or BRSV-Snk ($10^{4.0}$ pfu in 5 ml DMEM; calves A1-3), and in paper IV with BRSV-Snk ($10^{4.0}$ pfu in 4 ml DMEM). In both vaccine evaluation experiments (papers I and IV), calves were challenged five weeks after first vaccination (two weeks after boost, where applicable). Inocula were aerosolized by a compressor/nebulizer system (Super Dandy Inhaler, PARI, Germany), and the resulting aerosol inhaled via a tightly fitting face mask (Swevet Piab AB, Sweden) (Figure 3), which was well tolerated by calves.

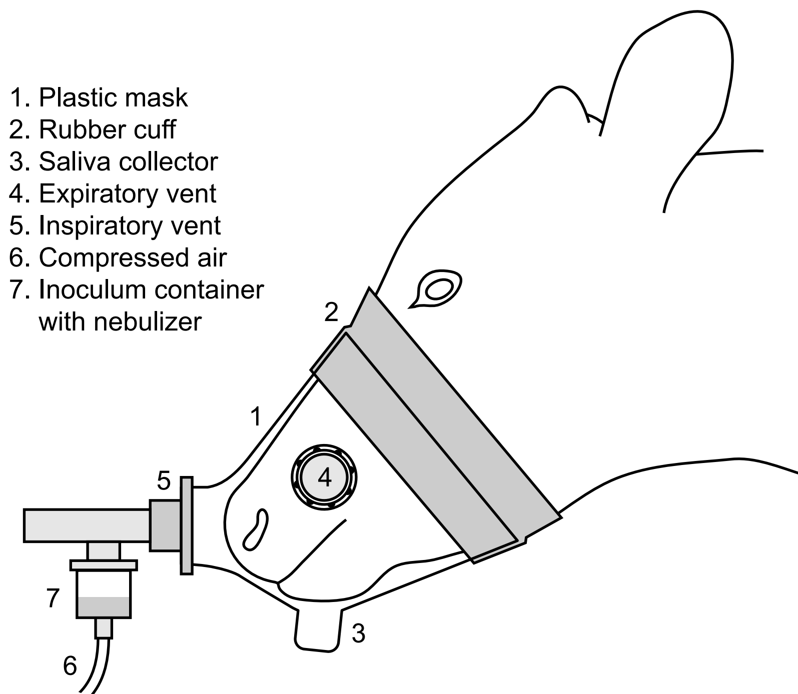

Figure 3. Face mask and nebulizer used for aerosol BRSV challenge in calves.

3.6 Clinical monitoring after challenge

Following challenge on PID 0, daily clinical examinations of all calves were carried out, and clinical signs were recorded.

Clinical examinations included: general state and appetite; recording rectal temperature and respiratory rate; presence of ocular or nasal discharge, or of spontaneous coughing, breathing pattern and reaction to cough provocation; auscultation of lungs for increased or adventitious respiratory sounds; as well as direct or indirect signs of diarrhea.

Clinical scores were calculated from recorded clinical signs, using two different scoring systems (detailed in paper I for that experiment, and paper III for the other experiments). Accumulated clinical scores (ACS) were calculated using the Trapezoid method. Note that, even though ACS represents the area under curves of clinical scores from infection to termination of each experiment, the experiment reported in paper I was one day shorter in duration, and used a different clinical scoring system, compared to experiments reported in paper III and IV.

3.7 Passive measurement of lung function

The effect of BRSV infection on the lung function of calves was determined by the forced oscillation technique (EquineOsc Calf measurement head, EEMS, Harts, UK), on PID -1 (paper I) or 0 (paper III) and again on PID 6. Airway resistance and reactance (kPa/L/s) were measured at 3, 5, 7 and 10 Hz, as described elsewhere (Reinhold et al. 1996), using the same tightly fitting face mask used for aerosol inhalation (Figure 3). For daily calibrations, a 2.26 m long tube with a 21 mm internal diameter was used. On each occasion, every calf was tested in two series, and series were repeated if coughing or breath-holding was observed. The series with optimal coherence was selected (coherence>0.9).

3.8 Live sampling before and after challenge

Heparinized and whole blood samples, for extraction of peripheral blood mononuclear cells (PBMCs) and serum respectively, and nasal secretion for detection of BRSV by RT-qPCR and virus isolation, were collected before and after challenge, as indicated in Figure 4. Additionally, in paper IV, nasal secretions were collected following vaccination; from animals vaccinated with ΔSHrBRSV on post-vaccination day (PVD) 2, 4-7, 11 and 14, and from three seronegative calves acting as sentinels for transmission of live vaccine on PVD 0-8, 11 and 14. Serum was also collected from sentinel calves on PVD 20. In paper III, BAL was performed in sedated calves (calves A1-3 and B1-3), on PID -1, as described in detail in paper III.

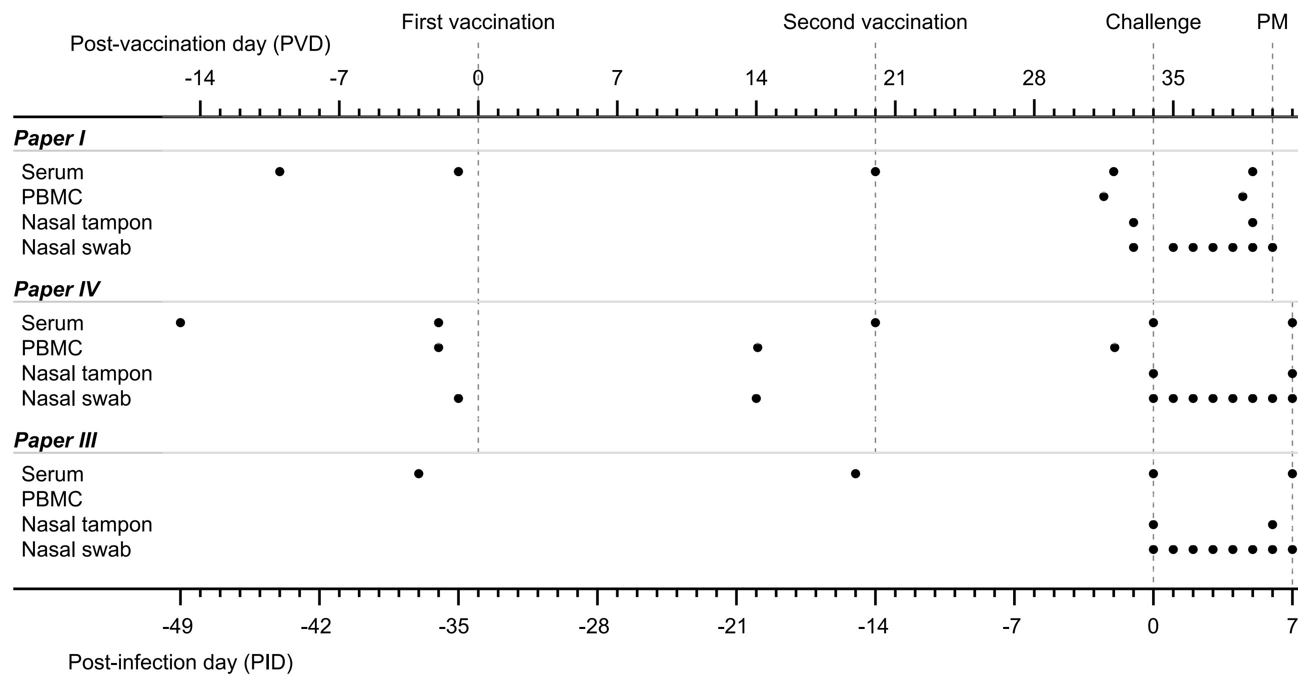

Figure 4. Samples collected from calves challenged with BRSV in three separate experiments. Additional samples of serum and nasal secretion were collected following vaccination in paper IV; from calves immunized with live vaccine, and from calves acting as sentinels

3.9 Post-mortem examination and sampling

All infected calves were euthanized on PID 6 (paper I) or PID 7 (papers III and IV) by an intravenous overdose of ketamine (5 mg/kg) and pentobarbital sodium (15 mg/kg), followed by exsanguination.

During post-mortem examination lungs were photographed, palpated and lesions recorded on lung charts. The extent of lung lesions (%) was calculated by digital image analysis of lung charts (Adobe Illustrator CS5, version 15.1 for Macintosh).

In all infected calves, as well as uninfected control calves in paper III (Table 3), BAL was performed on excised lungs; 500 ml of PBS was poured into the trachea, with lobes in the right lung clamped off, and then BAL fluid was collected by gravity into a sterile glass bottle and stored on ice until a centrifugation step (200 x g, 10 min) to separate BAL cells and BAL supernatant. BAL cell pellets were resuspended in DMEM-FCS 20% for virus isolation or RLT buffer (Qiagen, Sweden) for RNA extraction, and stored at -70°C. BAL supernatant was also stored at -70°C until subsequent antibody and cytokine analysis.

Lung tissue samples were collected post-mortem for histological examination; one tissue sample from the accessory lobe, and three (paper I) or one (paper III and IV) tissue sample(s) from each of the remaining three lobes in the right lung, as well as a sample from the trachea, were fixed in 10% buffered formalin, and paraffin sections prepared. In each lung lobe, grossly abnormal areas were sampled, where available. In paper I, an additional pneumonic lung tissue sample was homogenized in DMEM-FCS 20% for virus isolation.

3.10 Detection of BRSV

3.10.1 Quantification of BRSV RNA

Total RNA was extracted from nasal secretions and BAL cells, and BRSV RNA quantified by BRSV F gene RT-qPCR, as described in papers I, II, IV and elsewhere (Hakhverdyan et al. 2005). Equivalent titers of RNA ($TCID_{50}$ equivalent) were derived by correlation with a standard curve, constructed by

the inclusion in each run of dilutions of a virus sample with known titer ($10^{5.0}$ $TCID_{50}$ in paper I and $10^{5.8}$ $TCID_{50}$ in papers III and IV).

Accumulated virus shed (AVS), was calculated using the Trapezoid method, based on BRSV RNA detected in nasal secretion collected daily, from infection to euthanization (PID 6 for paper I; PID 7 for papers III and IV).

3.10.2 Detection of BRSV antigen

The process of immunohistochemical staining of BRSV antigen in sections of lung tissue is described in detail in paper III. In brief, microwave heat-induced epitope retrieval was used to unmask antigens. This was followed by deactivation of endogenous peroxidases using 3% H_20_2, blocking unspecific binding using 2% bovine serum albumin (BSA), incubation with RSV-specific monoclonal antibodies, binding to primary antibodies by secondary antibodies attached to a dextran polymer backbone with peroxidase enzymes, staining with diaminobenzidine, and counterstaining with haematoxylin.

3.10.3 Isolation of live BRSV

Isolation of live BRSV was attempted by inoculation of samples suspended in DMEM onto 95% confluent BT cells in 25 cm^2 culture flasks. After incubation for 1h at 37°C, inocula were poured off and replaced with DMEM and incubated at 37°C for six (paper I) or seven (papers III and IV) days. During this incubation, cultures were examined daily using light microscopy and any cytopathic effects recorded. Samples inducing cytopathic effects were considered positive for live BRSV. Consecutive passage of negative samples, as indicated in respective results, was done by transfer of cell lysate into new 25 cm^2 culture flasks with fresh BT cells, and monitored as described for the first passage.

3.11 Measuring immunological parameters

3.11.1 Quantification of antibodies

BRSV neutralizing antibodies were measured in heat-inactivated samples by a plaque reduction assay using fetal calf kidney cells, as described previously (Kennedy et al. 1988).

BRSV-specific IgG_1 antibodies were measured by a commercial indirect ELISA (BRSV-Ab ELISA, Boehringer Ingelheim Svanova, Sweden), according to instructions. Corresponding kits from the same manufacturer were also used to detect IgG antibodies against BVDV, bovine coronavirus and bovine parainfluenza virus type 3 in paper I.

BRSV-specific IgG_2 antibodies were measured by exchanging the conjugated antibody in the BRSV-specific IgG_1 ELISA, for mouse-anti-bovine-IgG_2 followed by rat-anti-mouse-IgG_1 conjugated with horseradish peroxidase (HRP).

BRSV-specific IgA was measured by capture ELISA (as modified from Uttenthal et al. 2000), as described in paper I. Briefly, plates were coated with mouse-anti-bovine-IgA antibodies and blocked with BSA, followed by sequential incubation with: sample, BRSV or uninfected cell lysate, mouse-anti-BRSV N protein antibody, rat-anti-mouse-IgG_1:HRP, tetramethylbenzidine (TMB) substrate; and H_20_2.

IgG antibodies specific for BRSV G, F, M, P and SH were analyzed by ELISA, as described previously for the G protein (Taylor et al. 1995). Antigen and control antigen for these ELISAs were produced in chick embryo fibroblasts infected with recombinant fowlpox or wild-type fowlpox viruses, as described previously (Gaddum et al. 2003).

IgG antibodies against HRSV F, N, P and M2-1 were measured by ELISA as described previously for N (Riffault et al. 2010). Antigens were produced and purified as previously described for N (N, P and M2-1) (Roux et al. 2008) and F (McLellan et al. 2011). IgA antibodies against HRSV N were measured by coating microtiter plates with N protein (as for IgG), followed by blocking with BSA, and sequential incubation with: sample, mouse anti-bovine IgA; rat anti-mouse IgG_{2a}:HRP; TMB substrate; and H_20_2.

Antibodies competing with monoclonal antibody 19 (mAb 19), known to neutralize both HRSV and BRSV (Arbiza et al. 1992), were analyzed by competitive ELISA, wherein plates were coated with BRSV protein (control antigen in paper I and II) and blocked with 2% horse milk, followed by sequential incubation with serially diluted samples, mAb 19, rat-anti-mouse-IgG_{2a}: HRP, TMB substrate and H_2O_2.

For all antibody ELISAs, optical density (OD) was measured at 450 nm. After the corrected optical density (COD) was calculated by subtraction of relevant background OD, levels of antibodies were presented either as (i) titer, derived from serial dilution of each sample, followed by calculation of end-point titer by linear regression using a negative sample as cut-off reference; or (ii) as percent COD (%COD) of a positive reference sample at dilution 1:25.

3.11.2 BRSV-specific lymphocyte proliferation assay

PBMCs were isolated from heparinized blood, diluted 1:1 in PBS, by centrifugation at RT (1100 x *g*, 30 min) over Ficoll Paque Plus medium (GE Healthcare, Sweden). After this PBMCs were recovered in the interface layer and washed three times by centrifugation in PBS (500 x *g* 10 min, then twice

200 x *g*, 10 min), then resuspended in RPMI medium (Lonza, Belgium, supplemented as described for DMEM, and betamercaptoethanol at 5 $x10^{-5}$ M) with 5% FCS. After mechanical disruption of tracheobronchial lymph nodes (LN), mononuclear cells were isolated as described for PBMCs, but centrifuged for 8 minutes at 800 x *g* over Ficoll Paque Plus medium. Isolated PBMCs and LN mononuclear cells were seeded into wells on 96-well cell culture plates, and restimulated in triplicate using BRSV antigen (heat-inactivated BRSV-Dk infected BT cell lysate) and control antigen (heat-inactivated uninfected BT cell lysate), and then incubated at 37°C with 5% CO_2. To detect cell viability in each stimulation condition, and relative cell proliferation, Alamar Blue® (Invitrogen, Sweden) was added to each well after six (paper I) or seven (paper IV) days of incubation. After an additional six (PBMCs paper I), eight (PBMCs paper IV) or twenty-four (LN cells) hours of incubation, optical densities were measured in each well at 570 nm (OD_{570nm}) and 595 nm (OD_{595nm}) (Multiskan EX 355, Thermo Fisher Scientific, USA). Mean COD was calculated by subtracting the adjusted OD (OD_{570nm}-OD_{595nm}) of control antigen-stimulated wells, from the adjusted OD of BRSV antigen-stimulated wells, and calculating a triplicate mean. Supernatants of stimulated wells were recovered after centrifugation (200 x g, 5 min, 20°C), and stored at -80°C for cytokine analysis.

3.11.3 Flow cytometric analysis of BRSV-specific IFNγ-producing lymphocytes

For flow cytometric analysis, tracheobronchial LN mononuclear cells were isolated and restimulated as described for the proliferation assay, except they were restimulated in duplicate wells, and incubated for 18h, with brefeldin A (10 µg/ ml; Sigma, Sweden) for the last 15h. Glycoproteins CD4 and CD8 were stained in stimulated cells (MCA1653F:FITC (CD4), MCA837A647: AlexaFlour 647 (CD8), AbD Serotec, Sweden). Dead cells, with compromised membranes, were stained by a dye reacting with free amines in the cell interior (LIVE/DEAD Fixable Near-IR Dead Cell Stain, Life Technologies, Sweden). After fixation (4% paraformaldehyde, 10 min) and membrane permeabilization (FACS permeabilization solution 2, BD Biosciences, Sweden), cells were stained for intracellular IFNγ (MCA1783PE: RPE (IFNγ), AbD Serotec, Sweden). The fluorescence of stained cells was analyzed by flow cytometry (FACSVerse, FACSuite software, BD Biosciences, Sweden). Gates for live, non-aggregating cells were based on fluorescence at 783/56 nm and light-scattering properties. Gates for $CD8^+$, $CD4^+$ and IFNγ producing cells were set based on Fluorescence Minus One controls.

3.11.4 Detection of cytokines

Detection of cytokines by immunosorbent assays

In paper IV, supernatant from PBMCs restimulated with BRSV (described for the proliferation assay) was analyzed using commercially available kits detecting IFNγ and IL-4 (Bovine IFNγ ELISA, MCA5638KZZ and Bovine IL-4 ELISA, MCA5892KZZ, Bio-Rad, Sweden). Both assays were performed according to instructions from the manufacturer for respective kits, and concentrations of cytokines calculated based on an included standard sample, and expressed as ng/ml.

After 20X concentration of BAL supernatant (BAL20X), by filtered centrifugation (UFC900324, Amicon Ultra-15, 3kDa, Merck Millipore, Sweden; swinging bucket rotor, 4000 x g, 25-30 min), IFNγ and IL-4 were analyzed in BAL20X as described for PBMC supernatant. Similarly, by following instructions of commercial ELISA kits and deriving sample concentrations from a relevant standard sample, IL-6 (ESS0029 Bovine IL-6 ELISA, Pierce, USA), IL-8 (ABIN414016 Bovine IL-8 ELISA, Antibodies Online, Germany) and TNFα (VS0285B-002 Bovine TNFα ELISA, Divbio Science Europe, The Netherlands) were analyzed in BAL20X.

Detection of cytokines by RT-qPCR

In paper I, PBMCs were isolated and restimulated with BRSV, as described for the proliferation assay, with the following modifications for analysis by RT-qPCR: after 24h of incubation, cells were pelleted by centrifugation (200 x *g*, 5 min, 20°C) and total RNA extracted (RNeasy Mini Kit, Quiagen, Sweden). Samples were analyzed using bovine-specific IFNγ-, IL-4- and 28S-RT-qPCR (iScript One-Step RT-PCR kit for probes and an IQ5 realtime PCR machine, Bio-Rad, Sweden), as previously described (Rosbottom et al. 2007). IFNγ and IL-4 were analyzed in triplicates, and 28S in duplicates. The house-keeping gene 28S was used to standardize detected cytokine levels, and results are expressed as: copies of cytokine mRNA / 1000 copies of 28S rRNA.

3.12 Histological assessment and scoring of severity of inflammation in lesioned lung sections

Slides with sections of trachea and lung tissue with gross lesions, were stained with: (i) hematoxylin and eosin (HE) for morphological description, or (ii) Luna's eosinophil stain (paper I) or carbol chromotrope stain (paper III and IV) to indicate eosinophils. A pathologist described and scored the severity of histopathology and inflammation in each slide as either normal (0), mild (1),

moderate (2) or severe (3). A mean score of histopathological severity was calculated for each calf, based on all lung tissue slides for that animal. In paper III, additional corresponding slides from each animal were stained by BRSV-specific immunohistochemistry (described in detail in the section 3.10.2 Detection of BRSV antigen).

3.13 Data analysis

3.13.1 Ranking of animals

Within each calf experiment presented in this thesis, calves were ranked with regard to three measurable outcomes of experimental BRSV infection: accumulated clinical score or ACS (clinical rank), extent of lung lesions (lung lesion rank), and accumulated virus shed in nasal secretion or AVS (viral-shed rank). In each experiment, rank 1 was assigned to the calf with the lowest ACS, least lung lesions and lowest AVS, in each of the three ranks respectively. More affected calves were assigned consecutively higher ranks. Statistics were calculated based on the individual sum of these three ranks. For each group of calves, three rank sums were calculated, as well as a total rank sum.

3.13.2 Statistical analysis

Results are presented as group mean ± one standard deviation (SD), where not otherwise stated. Standard deviations of percentages are presented as percentage point (pp). Data were analyzed using one-way ANOVA followed by Dunnett's test (paper I and II; Minitab 16.1.1, Minitab Inc.) or Student's t-test (papers III and IV; JMP 10 for Mac, SAS Institute Inc.), or Kruskal–Wallis analysis followed by Wilcoxon test (papers III and IV; JMP 10 for Mac, SAS Institute Inc.). Significance was assumed when $p \leq 0.05$.

4 Results

4.1 Evaluation of immunogenicity and protective efficacy of BRSV-ISCOMs in calves with BRSV-specific maternal antibodies (Paper I)

To confirm and extend previously reported immunogenicity and high level of protective efficacy of BRSV antigen in classic ISCOMs (BRSV-ISCOMs) in older calves, young calves with specific MDA were immunized with BRSV-ISCOMs, and challenged with BRSV. Furthermore, to elucidate the influence of the adjuvant in BRSV-ISCOMs, additional calves were immunized with BRSV protein alone, or adjuvant alone. Finally, calves injected with PBS acted as double negative controls.

4.1.1 Immunogenicity of BRSV-ISCOMs

Immune responses were investigated by analyzing isotypes of antibodies in serum, nasal secretion and BAL supernatant; and by *ex-vivo* BRSV restimulation of isolated PBMCs, and subsequent analysis of cell proliferation and estimation of IFNγ and IL-4 gene expression through mRNA detection.

Vaccinated calves had varying titers of colostrum-derived BRSV-specific serum IgG antibodies before first vaccination (mean±SD $\log_{10}$ titer 1.9±0.3; Table 2 in paper I), which were declining until first vaccination. Corresponding titers in calves immunized with adjuvant or PBS continued to decrease throughout the experiment. Adventitious BRSV infection was furthermore ruled out by repeated seromonitoring of seronegative sentinel animals.

In contrast to all control calves, BRSV-ISCOM vaccinated calves demonstrated increasing levels of BRSV-specific serum IgG after a single immunization (4/5 calves), with an augmented increase after boost (5/5 calves).

Serum concentrations of IgG_1 and IgG_2 antibodies in BRSV-ISCOM-immunized calves were significantly elevated on PID -2 and PID 5, compared

to all controls ($p \leq 0.0001$). Similarly, levels of mucosal IgA in nasal secretion and BAL were significantly elevated on PID 5 and 6, respectively, compared to all controls ($p \leq 0.005$ and $p \leq 0.01$).

In addition, after challenge (on PID 4), 4/5 BRSV-ISCOM-immunized calves demonstrated BRSV-specific proliferative responses in isolated circulating lymphocytes, compared to none of the calves in all other groups. Furthermore, in this group significantly increased quantities of IL-4 and IFNγ mRNA were detected in restimulated cells, compared to calves immunized with adjuvant or PBS for IL-4 ($p \leq 0.05$) and for IFNγ ($p \leq 0.05$) when compared to cells from all other calves. The mean ratio of IL-4 to IFNγ mRNA was 1:46 in cells from BRSV-ISCOM-immunized calves, suggesting a Th1 type immune response.

No adverse reactions to immunization were observed following the first vaccination, or following the second vaccination with BRSV protein or PBS. However, mild to moderate local swellings and elevated rectal temperature (40.0-40.3°C) was observed 12h (persisting up to 48h) after second immunization with either BRSV-ISCOMs or adjuvant alone.

4.1.2 Protection induced by BRSV-ISCOMs

Following challenge in paper I, calves immunized with BRSV-ISCOMs showed no or very minor clinical signs, indicating a high level of clinical protection, whereas mild to severe respiratory disease was observed in all other calves. Calves immunized with BRSV proteins alone showed predominantly mild to moderate clinical signs of respiratory disease, indicating they were afforded at least partial clinical protection. On the other hand, calves immunized with adjuvant alone or PBS developed moderate to severe clinical signs of disease, including purulent nasal discharge, coughing, wheezing lung sounds, increased respiratory rate (peak 76 breaths/min), elevated rectal temperature (peak 41°C), and subjectively, various degrees of depression. When the accumulated clinical score (ACS) was calculated, BRSV-ISCOM-immunized calves received the lowest ACS (mean±SD 1.3±1.9), followed by BRSV-protein (mean±SD 2.9±1.0), PBS (mean±SD 6.7±6.7), and adjuvant immunized calves (mean±SD 9.0±5.9 ACS). The difference in ACS between BRSV-ISCOM and adjuvant immunized calves was significant ($p \leq 0.05$).

The accumulated clinical signs observed in each group corresponded to the extent of consolidated lung lesions on post-mortem examination at PID 6. The least amount of lung lesions were observed in calves immunized with BRSV-ISCOMs (mean±SD 3.8±2.9%), followed by calves immunized with BRSV protein (mean±SD 7.5±6.0%), adjuvant (mean±SD 15.7±14.5%), and PBS (mean±SD 16.1±8.7). Similarly, when a pathologist scored ten areas per calf,

the extent of gross lung pathology corresponded to the severity of histopathology: BRSV-ISCOM being lowest (mean rank 5.6), followed by BRSV protein (mean rank 9.2), adjuvant (mean rank 11.8), and highest being PBS (mean rank 15.4).

The accumulated virus shed (AVS), representing BRSV RNA detected in nasal secretion from PID 0 to PID 6, was also significantly reduced in BRSV-ISCOM-immunized calves (mean±SD 0.3±0.5 log_{10} $TCID_{50}$ eq.), compared to all control calves ($p \leq 0.0001$), which shed more than seven logs more virus following challenge (mean±SD for BRSV protein alone, adjuvant alone and PBS, 7.5±2.5, 7.4±2.4 and 7.9±2.8 log_{10} $TCID_{50}$ eq., respectively).

Thus, BRSV-ISCOM-immunized calves were both clinically and virologically protected from virulent BRSV infection. Taken together, BRSV-ISCOM vaccination afforded immunized calves significant protection, compared to all other groups of calves, when total rank sums were calculated ($p \leq 0.05$; Figure 5). In contrast, calves immunized with BRSV protein alone were not protected.

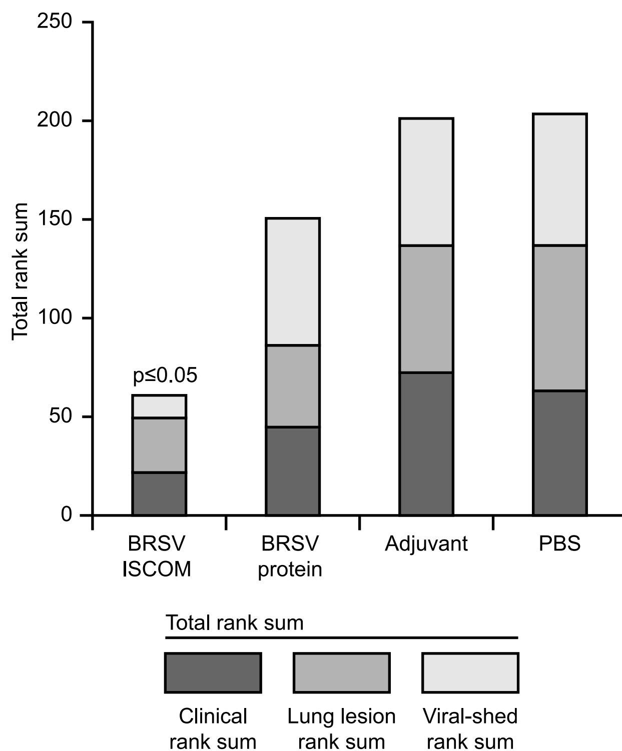

Figure 5. Ranking of clinical signs, extent of lung lesions, and virus shed in calves following experimental BRSV infection after prior immunization. Four groups of five colostrum-fed calves were immunized twice at an interval of three weeks with BRSV-ISCOMs, BRSV protein, adjuvant alone, or PBS; and challenged with BRSV by aerosol inhalation two weeks later. Following challenge, clinical signs and virus shed in nasal secretion was recorded for six days, when lung lesions were recorded at post-mortem. Low ranks were assigned to those with low accumulated clinical signs (low clinical rank), sparse lung lesions (low lung lesion rank), and low accumulated virus shed (low viral-shed rank). The figure shows each rank sum per groups, and each stack represent the group total rank sum. The significantly lower total rank sum of the BRSV-ISCOM-immunized calves, compared to all other groups ($p \leq 0.05$), indicates they were protected from BRSV infection.

4.2 Characterization of BRSV-ISCOMs (Paper II)

The promising results in paper I prompted further investigation of the BRSV-ISCOM vaccine, to enable further improvement and standardization of the BRSV-ISCOM formulation, or to develop a recombinant subunit vaccine. Therefore, the morphology and antigenicity of BRSV-ISCOMs was

investigated in paper II, by electron microscopy and both qualitative and semi-quantitative characterization of the protein content, using gel electrophoresis, Western blot, dot blot and mass spectrometry (MALDI-TOF and LC-MS/MS).

Electron microscopy imaging of the BRSV-ISCOM vaccine revealed pleomorphic cage-like ISCOM particles with diameters of 40-90 nm (mean±SD 56.4±11.1 nm, n=1644), as well as smaller and less defined particles (10-20 nm diameter).

BRSV proteins F and N were clearly identified in BRSV-ISCOMs by size separation in Coomassie blue stained SDS-PAGE gels. Using digital image analysis of these gels, and by measuring the total protein concentration in the BRSV-ISCOM vaccine used for immunizations, each dose of vaccine (188 µg total protein) was estimated to contain 77 µg of F and 17 µg of N protein.

Analysis by MALDI-TOF verified that these bands, cut out from SDS-PAGE gels, contained the F and N proteins, respectively. In addition to these viral proteins, Vero cell derived integrins, CD9 and histone H4, were identified by MALDI-TOF, in separate cut out bands from the same SDS-PAGE gels. Visually identical results were obtained with three different batches of BRSV-ISCOMs and selected bands were confirmed by MALDI-TOF.

Dot blot and Western blot analysis, using a panel of monoclonal antibodies and hyperimmune calf sera, confirmed the presence of the F and N proteins, but also demonstrated the presence of G, M, M2-1, and P proteins in both the BRSV-ISCOM and the BRSV protein vaccine formulations.

Using the highly sensitive method LC-MS/MS to identify peptide sequences in BRSV-ISCOMs, the F, N, M, M2-1, and P proteins were demonstrated, but also the SH protein and Vero cell derived proteins (e.g. integrins). However, the G protein was not detected when lanes from SDS-PAGE gels were analyzed by LC-MS/MS, which might be due to limited migration of the glycosylated G protein.

To investigate the immunogenicity of identified proteins, sera from calves in paper I were further analyzed in paper II.

Analysis of sera from BRSV-ISCOM-immunized calves, collected 2 weeks after second vaccination, but before challenge, demonstrated significantly elevated titers of antibodies directed against proteins F, N and G (N $p \leq 0.001$; F and G $p \leq 0.05$), compared to calves immunized with ISCOM matrices alone (AbISCO-300; Figure 6). Furthermore, low titers of antibodies against proteins M and P, and high titers of antibodies against the SH protein were detected in BRSV-ISCOM-immunized calves, although this was not statistically significant compared to calves immunized with adjuvant alone (Figure 6).

In addition, mAb 19 competing antibody titers were measured to approximate the neutralizing capacity of sera from BRSV-ISCOM-immunized calves, and these were significantly higher ($p \leq 0.001$) than those of all controls (mean (range) $\log_{10}$ titer 2.4 (1.7-2.6), 1.5 (0-2.2), 0.3 (0-1.0) and 1.2 (0-1.5) for calves immunized with BRSV-ISCOM, BRSV proteins, AbISCO-300 or PBS, respectively).

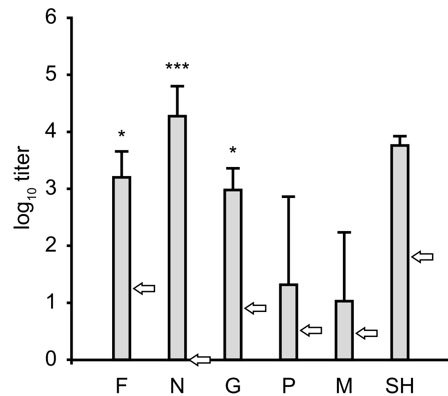

Figure 6. BRSV protein specific antibodies in sera from calves immunized with BRSV-ISCOMs. Five calves were immunized with BRSV-ISCOMs, as described in section 3.4.1. In addition, five calves were immunized identically, but with ISCOM matrices alone (adjuvant). The figure shows mean titer of antibodies directed against viral proteins F, N, G, P, M and SH, in sera collected from BRSV-ISCOM-immunized calves before challenge, two weeks after second vaccination. Vertical lines indicate standard deviation. Arrows indicate corresponding mean titer of calves immunized with adjuvant alone, and asterisks indicate statistical difference compared to these calves ($p \leq 0.05$ (*); $p \leq 0.001$ (***)).

4.3 Refinement and characterization of a BRSV infection model in calves (Paper III and IV)

In paper III, to enable the joint vaccine evaluation described in paper IV, which involved three project partners, and to facilitate comparability with follow-up studies, protocols and methods previously used by project partners were consolidated into a common BRSV calf infection model. This process involved the evaluation of two previously used inocula, BRSV-Snk passaged in gnotobiotic calves and BRSV-Dk passaged in cell culture, to determine which induced the most field-like BRSV disease. Two groups of three calves with low levels of BRSV-specific serum IgG_1 antibodies (mean±SD 4.1±4.8 %COD on PID 0) were challenged by aerosol inhalation of either inoculum.

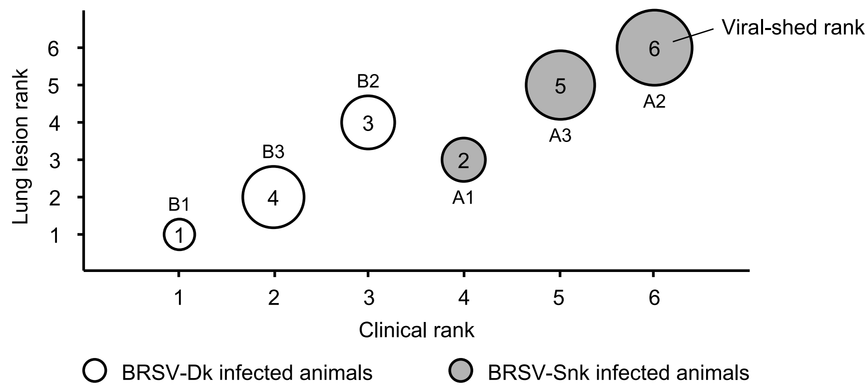

Figure 7. Ranking of clinical, pathological and virological outcomes in calves experimentally infected by aerosol inhalation of either BRSV-Dk or BRSV-Snk. Following challenge, nasal secretions were collected daily, and calves were clinically monitored for seven days, when calves were euthanized. Lung lesions were recorded post-mortem. Based on this data, ranks were assigned as described in Figure 5. Clinical rank is indicated on the x-axis, lung lesion rank on the y-axis, and viral-shed rank by the number inside and the size of each bubble. Calf identities are indicated next to each bubble.

4.3.1 Characterization of clinical, pathological and virological expression of two inocula (Paper III)

All infected calves were markedly affected by experimental infection, although BRSV-Snk infected calves were consistently more affected, as illustrated by clinical, pathological and virological ranks (Figure 7). Individual rank sums were significantly higher in BRSV-Snk infected calves ($p \leq 0.01$), and their total group rank sum was twice as high, compared to BRSV-Dk infected calves (42 and 21, respectively). This was despite the fact that one BRSV-Snk infected calf (A1) rapidly seroconverted after challenge (BRSV-specific IgG_1 in sera diluted 1:25: 5 %COD on PID 0 and 91 %COD on PID 7), suggesting this calf had been previously exposed to BRSV, despite seromonitoring in the herd of origin from 17 days after the birth of this calf.

The difference in rank sums reflects that, following challenge, BRSV-Snk infected calves were significantly more clinically affected (ACS: 46.2±4.5, compared to 20.0±13.5; $p \leq 0.05$), had more extensive lung lesions (38.5±26.3%, compared to 12.8±14.6%), and shed more virus in nasal secretion (AVS: 12.6±8.1 $TCID_{50}$ eq., compared to 8.0±4.0 $TCID_{50}$ eq.), compared to calves infected with BRSV-Dk.

The difference in clinical score, in turn, reflects the marked difference between the two groups of calves, in clinical disease observed following

challenge. On PID 7, all BRSV-Snk infected calves were depressed and had reduced or absent appetite, whereas only one BRSV-Dk infected calf (B2) had an affected general state, and none demonstrated reduced appetite.

A full account of clinical signs of respiratory disease observed in these calves is given in paper III. In brief, clinical signs observed in BRSV-Snk infected calves following challenge were characterized by coughing, tachypnea, enhanced and wheezing lung sounds, abdominal dyspnea, pyrexia, with general depression and reduced appetite.

Lung lesions observed in BRSV-Snk infected calves on PID 7 were predominantly located in the cranioventral lobes. Macroscopically, lesions were dark red and consolidated, with excessive mucus and pus in bronchi. Histologically, lesions were characterized by moderate to severe bronchointerstitial pneumonia, purulent bronchitis and bronchiolitis with prominent peribronchiolar lymphoid-cell hyperplasia, and fibrinosuppurative alveolitis.

BRSV-Dk infected calves demonstrated similar lung lesions, but less extensive and less severe, both macroscopically and histologically (mild to moderate bronchointerstitial pneumonia).

Both groups of calves had similar histological lesions in the trachea on PID 7: with degeneration, desquamation and focal hyperplasia of trachea epithelium, and intraepithelial infiltrate of polymorphonuclear leukocytes, predominantly neutrophils. Interestingly, whereas immunohistochemically stained BRSV antigen was abundant in the tracheae, but almost undetectable in the lungs of BRSV-Dk infected calves, the opposite was true for 2/3 BRSV-Snk infected calves (A2 and A3). No BRSV antigen was detected by immunohistochemistry in the trachea or lungs of the BRSV-Snk infected calf A1, which seroconverted after challenge.

This calf (A1) also shed substantially less virus in nasal secretion collected from PID 0 to PID 7, and in BAL on PID 7 (AVS/BAL: 3.6/1.7 $\log_{10}$ $TCID_{50}$ eq.), compared to the other two BRSV-Snk infected calves (AVS/BAL: (A2) 19.3/3.3 $\log_{10}$ $TCID_{50}$ eq.; (A3) 14.9/3.5 $\log_{10}$ $TCID_{50}$ eq.). Virus shed varied in BRSV-Dk infected calves (AVS/BAL: (B1) 3.5/2.8 $\log_{10}$ $TCID_{50}$ eq.; (B2) 9.4/2.3 $\log_{10}$ $TCID_{50}$ eq.; (B3) 11.1/2.7 $\log_{10}$ $TCID_{50}$ eq.), but was less than that of calves A2 and A3.

However, live BRSV was isolated in the first passage, in both nasal secretions from PID 6 and BAL fluid from PID 7 from all infected calves.

When BAL cells collected before and after challenge (PID -1 and PID 7) were analyzed, a significant increase in total number of cells following challenge was evident in calves infected with BRSV-Snk, both compared to before challenge ((PID -1) $1.0 \pm 0.2 \times 10^6$ cells/ml; (PID 7) $6.9 \pm 2.0 \times 10^6$

cells/ml; $p \leq 0.01$), and compared to BRSV-Dk infected calves before ((PID -1) 1.1±0.2 x10^6 cells/ml; $p \leq 0.01$) and after challenge ((PID 7) 0.8±0.7 x10^6 cells/ml; $p \leq 0.01$).

Both groups of calves demonstrated a shift in BAL cell type composition following challenge, from predominantly macrophages before infection ((BRSV-Snk) 63.0±26.0%; (BRSV-Dk) 69.0±10.1%), to predominantly neutrophils after infection ((BRSV-Snk) 79.0±4.4%; (BRSV-Dk) 80.3±6.0%).

4.3.2 Further characterization of the BRSV-Snk infection model in calves with BRSV-specific maternal antibodies (Papers III and IV)

The infection model and the BRSV-Snk inoculum were verified to also be effective in calves with specific MDA and were further characterized, using data and samples collected from unvaccinated calves challenged in the experiment described in paper IV (calves d1-d5, Table 3). These calves had moderate titers of serum specific MDA (moderate MDA; mean±SD 2.0±0.2 $\log_{10}$ titer) at the time of experimental infection. Corresponding data and samples from three uninfected calves were used as control (calves D1-D3, Table 3).

Calves with moderate MDA were severely affected after aerosol inhalation of virulent BRSV (BRSV-Snk, $10^{4.0}$ pfu), comparable to that observed in BRSV-Snk infected calves with very low levels of specific MDA (low MDA) and without prior priming (calves A2 and A3, paper III).

Clinically, mild to moderate signs were first noticed on PID 3 and 4 in calves with moderate MDA, including cough and nasal discharge. By PID 7, these mild signs had progressed to severe signs of upper and lower respiratory disease, including: severely depressed general state with reduced or absent appetite; pyrexia (mean max 40.8°C; range 40.2-41.2°C); tachypnea (mean max±SD 81.6 SD±4.6 breaths/min) with moderate to severe abdominal dyspnea and wheezing lung sounds.

Virus shedding in nasal secretion in infected calves with moderate MDA coincided with onset of clinical signs (PID 3), but peaked on PID 5 (mean±SD 2.2±0.36 $\log_{10}$ $TCID_{50}$ eq.), and was declining on PID 7 (mean±SD 1.1±0.74 $\log_{10}$ $TCID_{50}$ eq.) In the lower airways, high amounts of viral RNA was detected in BAL cells collected from control calves on PID 7 (mean±SD 5.0±0.62 $\log_{10}$ $TCID_{50}$ eq.), and live BRSV was isolated from these BAL cells (in passage one or two).

At gross post-mortem examination, infected calves with moderate MDA had massive consolidated lung lesions, involving 36-64% of the lung tissue, and in addition, two calves (d3 and d4) had moderate lung pleural

emphysemas. Consolidated lung lesions were dark red and dense, mainly located in the cranioventral lobes.

Lung tissue samples from consolidated areas were collected for histopathological examination, in each of the lobes in the right lung, from infected calves with moderate MDA. These exhibited severe inflammation microscopically (score 2.8-3.0, mean 2.9, scale 0-3), with severely affected lung parenchyma, including severe thickening of alveolar walls, lined with scattered type II-cells, and massive infiltration of inflammatory cells, mainly mononuclear and neutrophils. A few syncytial cells, as well as very few eosinophils, were observed.

To further characterize inflammatory and immunological responses to experimental infection, post-mortem BAL samples collected from infected and uninfected calves were analyzed. Compared to identically collected BAL samples from uninfected controls, calves with moderate MDA demonstrated a significant elevation of total number of BAL cells after infection ($1.1 \pm 0.2 \times 10^6$ cells/ml and $11.0 \pm 3.7 \times 10^6$ cells/ml, respectively; $p \leq 0.005$). As with the post-infection influx of BAL cells observed in calves with low MDA infected with BRSV-Snk, BAL cells observed in calves with moderate MDA after challenge consisted predominantly of neutrophils (68.6±14.4%), followed by macrophages (27.8±13.0%) and lymphocytes (3.6±2.0%). Regardless of specific MDA status, very few eosinophils were seen when BAL cells were enumerated (<0.5%).

Coinciding with this influx of neutrophils, supernatant from post-mortem BAL from infected calves contained significantly elevated concentrations of IFNγ when measured by ELISA, compared to that of uninfected calves (mean±SD 0.53±0.19 and 0.06±0.04 ng/ml, respectively; $p \leq 0.005$). In contrast, no significantly elevated concentrations of IL-4, IL-6, IL-8 and TNFα were detected in BAL supernatant, although IL-6 concentrations tended to be higher in infected calves, compared to those of uninfected controls (mean±SD 9.47±2.07 and 5.68±2.32 ng/ml, respectively; $p=0.08$). Interestingly, the IFNγ concentration in BAL supernatant (collected in the left lung) from BRSV-Snk infected calves was inversely correlated with the extent of lung lesions in the left lung ($r^2=0.82$, $p \leq 0.05$; Figure 8).

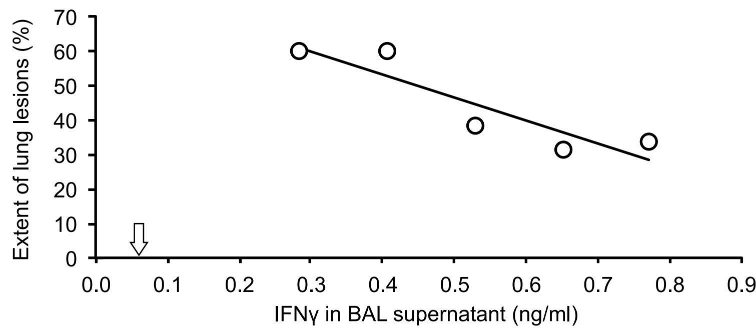

Figure 8. Correlation between IFNγ in BAL supernatant and extent of lung lesions, seven days after experimental BRSV infection of calves with moderate levels of maternally derived BRSV-specific serum antibodies. Calves were challenged by aerosol inhalation with the Snook strain BRSV (BRSV-Snk), and seven days later lung lesions were recorded post-mortem, and bronchoalveolar lavage (BAL) was performed on the left lung. When concentrations of IFNγ (ng/ml) in BAL supernatant were measured by ELISA (x-axis), and lesions in the left lung were quantified by digital image analysis (% of whole left lung; y-axis), an inverse correlation was identified (r^2=0.82, p≤0.05; solid line). The arrow represents the mean IFNγ concentration in BAL supernatant of three uninfected calves, sampled identically.

The impact of infection on lung function in calves with moderate MDA infected with BRSV-Snk was evaluated by passive measurement of airway resistance and reactance using the forced oscillation technique, before and after challenge (on PID 0 and 6). Following challenge, infected calves demonstrated significantly decreased airway reactance (PID 0 and 6: 0.03±0.03 and -0.02±0.04 kPa/L/s at 10Hz; p≤0.05) and a tendency of increased airway resistance (PID 0 and 6: 0.17±0.03 and 0.20±0.06 kPa/L/s at 10Hz; p=0.2).

4.4 Evaluation of immunogenicity and protective efficacy of three DIVA-compatible vaccines - two formulations of a subunit vaccine and one gene-deleted live BRSV vaccine - in calves with BRSV-specific maternal antibodies (Paper IV)

In paper IV, we used the BRSV infection model presented in paper III to evaluate three DIVA-compatible vaccine candidates, derived from previous studies by the three project partners in this thesis work.

A composition of recombinant subunits containing HRSV proteins P, M2-1 and self-assembling nanorings of the HRSV protein N with recombinantly grafted epitopes from BRSV proteins F and G, was formulated with either the oil emulsion Montanide ISA71^{VG} (SUMont) or ISCOM matrices, AbISCO-300 (SUAbis). Both subunit vaccines were administered twice parenterally with an interval of three weeks, SUMont intramuscularly and SUAbis subcutaneously,

to be consistent with routes used in preceding experiments (Hägglund et al. 2004; Riffault et al. 2010). In addition, one recombinant live BRSV with the SH gene deleted (ΔSHrBRSV) was administered once intranasally. Four groups of five young calves with moderate titers of serum specific MDA (log_{10} BRSV-specific IgG_1 antibody titers: 1.9-3.3, mean 2.3) were each vaccinated with one of these three vaccines, or adjuvant alone (controls), and challenged by aerosol inhalation five weeks after first immunization.

4.4.1 Clinical signs, virus shedding and lung pathology following challenge

Unvaccinated control calves were severely affected after BRSV challenge

All unvaccinated control calves were severely affected following challenge, as described in detail in section 4.3.2 and in paper IV; with severe respiratory clinical signs (accumulated clinical score, ACS; mean±SD 73.2±29.1) and high titers of virus in nasal secretion following challenge (accumulated virus shed, AVS; mean±SD 11.0±2.2 log_{10} $TCID_{50}$ eq.), and extensive lung lesions at post-mortem (mean±SD 48.3±12.0%).

Calves immunized once intranasally with ΔSHrBRSV were highly protected from BRSV infection

In contrast to control calves, calves immunized with ΔSHrBRSV and challenged with BRSV-Snk five weeks later, developed only very mild signs of respiratory disease, with a mean accumulated clinical score of 3.7 (SD±3.6). One calf (a1) had no clinical signs, one calf (a5) had mild dyspnea and slight wheezing lung sounds on PID 7, whereas clinical signs in the remaining calves (calves a2-a4), included coughing on provocation, slight serous nasal discharge, and slightly enhanced lung sounds, and were restricted to PID 6 and PID 7. Throughout the challenge, none of the calves immunized with ΔSHrBRSV exhibited depression, loss of appetite, or rectal temperatures exceeding 39.6°C, and their mean maximum respiratory rate was 49.6 breaths/min (SD±3.6 breaths/min).

Only 2/5 calves immunized with ΔSHrBRSV shed virus in nasal secretion following challenge, and only in low quantities detected by RT-qPCR (max mean±SD 0.1±1.1 log_{10} $TCID_{50}$ eq.), and for only 2 and 3 days in calves a3 and a5, respectively. The accumulated virus shed in ΔSHrBRSV-immunized calves (mean±SD 1.4±2.2 log_{10} $TCID_{50}$ eq.) was nine logs lower than that of controls. Live virus could not be isolated (after three passages) and only low amounts of viral RNA were detected in BAL collected on PID 7 (mean±SD 2.1±0.6 log_{10} $TCID_{50}$ eq.), which was significantly less than that of controls ($p \leq 0.001$).

ΔSHrBRSV-immunized calves had the least gross lung lesions (3.9±3.6%), and the least severe histopathological inflammation (score 0.8-2.3, mean 1.3) in sampled lesions, compared to all other groups of calves. Furthermore, ΔSHrBRSV-immunized calves had significantly reduced concentrations of cells in BAL (mean±SD 5.2±3.5 cells x 10^6/ml) collected on PID 7, compared to controls (mean±SD 11.0±3.7 x10^6 cells/ml; $p \leq 0.01$).

Calves immunized twice intramuscularly with SUMont were well protected against BRSV infection

The clinical signs observed in SUMont-immunized calves following challenge were intermediate to the severe signs described for control calves and the very mild signs described for ΔSHrBRSV-immunized calves. The mean accumulated clinical score of SUMont-immunized calves was 18.0 (SD±14.0). Mild clinical signs were first observed in SUMont vaccinated calves on PID 3, and peaked with moderate signs on PID 6, including spontaneous coughing, serous nasal discharge, slight wheezing lung sounds, slight to moderate dyspnea and elevated respiratory rate (mean max±SD 54.4±8.3 breaths/min). Throughout the challenge, only 2/5 SUMont-immunized calves had rectal temperatures ≥39.6°C, and only on one day each (calf b2: 39.9°C on PID 4; calf b4: 39.7°C on PID 2).

Compared to controls, SUMont-immunized calves shed seven logs less virus in nasal secretion following challenge (accumulated, mean±SD 3.6 ± 2.6 $\log_{10}$ $TCID_{50}$ eq.), but two logs more than ΔSHrBRSV-immunized calves. In the lower airways, virus could only be isolated from BAL (PID 7) from one SUMont-immunized calf (b3, 3rd passage), and amounts of viral RNA (mean±SD 3.2±1.3 $\log_{10}$ $TCID_{50}$ eq.) were significantly reduced compared to control calves ($p \leq 0.001$), but one log higher than those of ΔSHrBRSV-immunized calves.

Similarly, the extent of lung lesions recorded at post-mortem in SUMont-immunized calves (8.8±10.2%), was significantly reduced compared to control calves ($p \leq 0.05$), but more extensive than those of ΔSHrBRSV-immunized calves. Histologically, SUMont-immunized calves demonstrated similar lesions, with similar severity (score 0.6-2.5, mean 1.6), compared to that described for ΔSHrBRSV-immunized calves. Compared to controls, calves immunized with SUMont had less cells in BAL on PID 7 (mean±SD 3.1±1.8 cells x 10^6/ml; $p \leq 0.01$).

Calves immunized twice subcutaneously with SUAbis were afforded limited protection against BRSV infection

Clinical signs observed in SUAbis-immunized calves following challenge were less severe than those observed in control calves, but more severe than those observed in calves immunized with SUMont, which was reflected in the intermediate accumulated clinical score (mean±SD 31.0±15.0). The nature of clinical signs observed in SUAbis-immunized calves were similar to those described for calves immunized with SUMont, but with higher mean peak respiratory rate (61.0±6.8 breaths/min) and rectal temperatures (3/4 calves ≥39.7°C).

SUAbis-immunized calves shed three logs less virus in nasal secretion (mean AVS±SD 7.9±3.4 log_{10} $TCID_{50}$ eq.), compared to controls, but four logs more than SUMont ($p \leq 0.05$), and six logs more than ΔSHrBRSV-immunized calves ($p \leq 0.005$). Viral RNA was detectable in nasal secretion from calves immunized with SUAbis from PID 3 to PID 7 (mean 4.5 days). Live virus was isolated from BAL cells from 3/4 SUAbis-immunized calves in the third passage, compared to 5/5 in the first or second passage for control calves. Similarly, less viral RNA was detected in BAL from SUAbis-immunized calves (mean±SD 4.2±1.2 log_{10} $TCID_{50}$ eq.) compared to controls, although this was not significant ($p=0.07$).

On PID 7, calves immunized with SUAbis had extensive consolidated lesions in the lungs (mean 20.5±8.8%), although significantly less extensive than those of controls ($p \leq 0.05$). Histological lesions were moderate to severe (score 2.0-3.0, mean 2.5), but morphologically similar to the severe lesions described for controls. Compared to controls, calves immunized with SUAbis had significantly lower concentrations of cells in BAL on PID 7 (mean±SD 3.2±1.9 cells x 10^6/ml; $p \leq 0.01$).

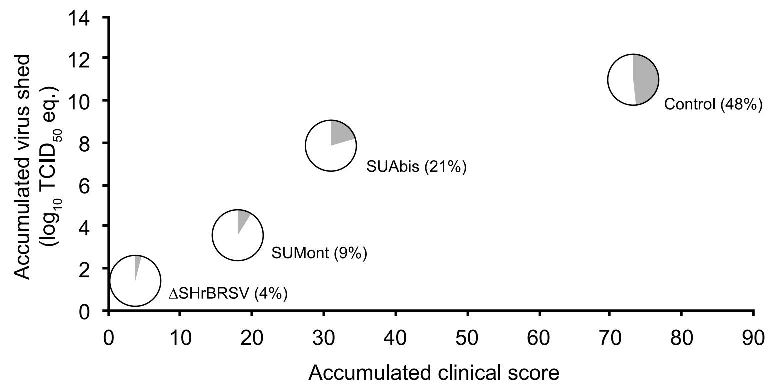

Figure 9. Agreement between clinical signs, lung pathology and virus shed in nasal secretion, when calves were experimentally infected after prior immunization. Four groups of five calves were immunized with one of three formulations (as described in section 3.4.2): ΔSHrBRSV, SUMont, SUAbis, or adjuvant alone (Control). One SUAbis vaccinated calf was euthanized, due to traumatic injury. Five weeks after first immunization, all remaining calves were challenged by aerosol inhalation with BRSV-Snk. Clinical signs were scored and BRSV RNA in nasal secretion quantified for seven days following challenge, when accumulated clinical scores (x-axis) and virus shed (y-axis) was calculated (indicated by the center of each pie-chart). Extent of gross lung lesions (%) was quantified post-mortem, and is indicated by the gray portion of each pie-chart.

4.4.2 Order of degree of vaccine-induced protection

The different extent to which the three vaccinated groups of calves were affected by BRSV infection is illustrated in Figure 9, showing mean accumulated clinical scores and viral shed in nasal secretion, and mean extent of lung pathology. Figure 9 reveals a clear order of degree of vaccine-induced protection among groups, compared to controls:

1. ΔSHrBRSV (once i.n.) induced a high level of protection
2. SUMont (twice i.m.) afforded good protection
3. SUAbis (twice s.c.) offered limited protection

This order of degree of vaccine-induced protection from disease and virus replication, compared to controls, is supported in Figure 10, showing statistically significant differences in: (i) daily and accumulated clinical scores; (ii) macroscopic extent of lung lesions; (iii) histopathological severity of lung lesions; (iv) daily and accumulated virus titers detected by RT-qPCR in nasal secretion; and (v) virus titer in BAL on PID 7. The same relative order was seen when calves were ranked based on the three parameters in Figure 9, and group total rank sums were calculated (see Figure 5 in paper IV).

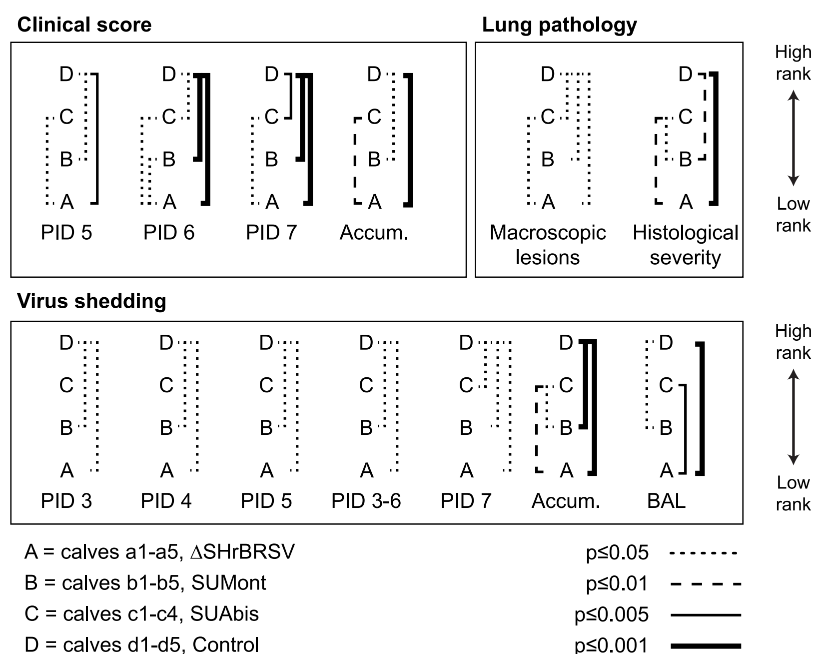

Figure 10. Summary of statistically significant differences between groups of calves experimentally infected with BRSV, after prior vaccination. Four groups of five calves were immunized as described in section 3.4.2, with ΔSHrBRSV (A), SUMont (B), SUAbis (C) or placebo (D, Control). Five weeks after first vaccination, calves were challenged with BRSV on post-infection day (PID) 0, and monitored for seven days, until PID 7. For each group, means and standard deviations were calculated for: daily and accumulated clinical scores, daily and accumulated virus titers in nasal secretion, as well as in bronchoalveolar lavage on PID 7, detected by RT-qPCR, and macroscopical extent and histological severity of lung lesions. For each parameter, the figure lists groups, ranked based on group mean for each parameter, from high to low. Note however, that calves immunized with ΔSHrBRSV had the lowest mean/rank across all presented parameters, consistently followed by SUMont, SUAbis and placebo immunized calves, in that order. Vertical lines indicate statistically significant differences between groups, where p-values for dashed and solid lines are indicated in the figure.

4.4.3 Immune responses to vaccination and experimental challenge

This section present immunological results from paper IV, including: serum BRSV-specific IgG_1, and antibodies directed against proteins F, G, N, P and M2-1, as well as virus neutralizing antibodies (Figure 6 in paper IV); local antibodies directed against total BRSV antigen (IgA and IgG_1) and the N protein (IgA), in nasal secretion and in BAL (Figure 7 in paper IV); and BRSV-specific proliferation in circulating lymphocytes (Figure 8 in paper IV).

In addition, BRSV-specific proliferation and cytokine production in cells extracted from tracheobronchial lymph nodes at PID 7 were analyzed in controls and calves immunized with ΔSHrBRSV (Table 3 in paper IV).

A single intranasal immunization with ΔSHrBRSV primed for strong anamnestic mucosal IgA and systemic neutralizing humoral responses and local IFNγ producing BRSV specific T cells

Following vaccination but before challenge, no humoral or systemic cellular immune responses were detected in calves immunized with ΔSHrBRSV ($p \leq 0.05$). Local BRSV-specific cellular immune responses were not investigated before challenge.

However, following challenge (PID 7) ΔSHrBRSV-immunized calves demonstrated rapid and significant increases in BRSV-neutralizing antibodies in serum ($p \leq 0.001$) and BRSV-specific IgA antibodies in nasal secretion ($p \leq 0.05$), compared to before challenge (PID 0). The post-infection increase in neutralizing antibodies in serum was also reflected in significant increases in protein specific IgG antibodies directed against F ($p \leq 0.01$), G ($p \leq 0.01$), N ($p \leq 0.01$) and M2-1 ($p \leq 0.05$), compared to controls.

In addition, a significant BRSV-specific proliferative response was detected in restimulated tracheobronchial lymph node lymphocytes from ΔSHrBRSV-immunized calves after challenge compared to controls ($p \leq 0.05$), with an increased proportion of BRSV-specific IFNγ-producing lymphocytes (FACS; $CD4^+$ $p \leq 0.05$; $CD8^+$ $p = 0.06$; compared to lymphocytes from ΔSHrBRSV-immunized calves stimulated with uninfected cell lysate), and significantly increased IFNγ concentrations detected in supernatant of BRSV-stimulated cells (ELISA; $p \leq 0.05$), compared to supernatant of BRSV-stimulated cells from controls.

Thus, the high level of protection from disease and virus replication observed in ΔSHrBRSV-immunized calves following challenge appears to have been mediated by rapid anamnestic BRSV-specific immune responses, including local cellular responses, as well as local and systemic virus neutralizing humoral responses.

Two intramuscular immunizations with SUMont induced IFNγ-producing T cell responses and non-neutralizing humoral responses directed against proteins N, P and M2-1 but not against F and G epitopes

Already after first vaccination, calves immunized with SUMont demonstrated both cellular and humoral immune responses. Significant proliferative BRSV-specific responses were detected in restimulated PBMCs from SUMont-immunized calves, compared to controls and all other vaccinated calves

($p \leq 0.001$), two weeks after first vaccination (one week before boost). Humoral responses following first vaccination consisted of modest increases in titers of serum IgG antibodies directed at proteins N, P and M2-1, but these increases were not statistically significant, and antibodies were not neutralizing virus. Furthermore, titers of BRSV-specific IgG_1 antibodies in serum continued to decline after first vaccination, which may be explained by concurrently declining titers of specific MDA.

The second SUMont vaccination boosted the responses described after first vaccination, with: (i) a significant BRSV-specific T cell proliferative response two weeks after second immunization ($p \leq 0.05$), now with significantly higher concentrations of IFNγ in the supernatant of BRSV-restimulated cells ($p \leq 0.05$), compared to BRSV-restimulated cells from all other calves; and (ii) significantly higher titers of serum IgG antibodies directed against proteins N ($p \leq 0.001$), P ($p \leq 0.05$) and M2-1 ($p \leq 0.001$) at PID 0, compared to controls. However, although the mean total BRSV-specific IgG_1 antibody titer of SUMont-immunized calves was slightly elevated before challenge, compared to controls, these antibodies were not directed against proteins F or G, and were not neutralizing virus. In addition to systemic humoral responses, SUMont-immunized calves demonstrated significantly higher levels of BRSV-specific IgA antibodies in nasal secretion after second vaccination, on PID 0, compared to all other calves ($p \leq 0.005$).

Following challenge, titers of protein specific antibodies tended to plateau, and serum from SUMont-immunized calves were still not neutralizing virus, despite a continued increase of mean BRSV-specific IgG_1 antibody in these calves. Mucosal BRSV-specific IgA antibodies were significantly elevated in BAL on PID 7, compared to controls ($p \leq 0.001$), as was IgA directed against the N protein, compared to controls and the other vaccinated calves ($p \leq 0.001$ and $p \leq 0.05$, respectively). Cellular responses were not investigated after challenge in SUMont-immunized calves.

In summary, the good level of protection observed in SUMont-immunized calves following challenge two weeks after second vaccination, was probably mediated by the strong systemic T cell responses characterized by IFNγ secretion, observed already before challenge. Although SUMont induced strong local and systemic BRSV-specific humoral responses, systemic humoral responses were non-neutralizing, and directed against the N, P and M2-1 proteins, and not the F and G epitopes.

Two subcutaneous immunizations with SUAbis induced non-neutralizing humoral responses directed against proteins N, P and M2-1, but no T cell response

Following first and second SUAbis vaccination, humoral immune responses detected in immunized calves were similar to those described for SUMont-immunized calves, but with less amplitude, and there was no indication of induction of a cellular response. Specifically, titers of serum IgG antibodies against N, P and M2-1 were all significantly elevated after challenge, compared to controls ($p \leq 0.001$, $p \leq 0.05$, $p \leq 0.001$, respectively). However, sera from SUAbis-immunized calves did not neutralize virus.

The limited protection observed in SUAbis-immunized calves was thus paralleled by equally limited protective immune responses: with an absence of neutralizing antibodies, which were prominent in ΔSHrBRSV-immunized calves, and an absence of T cell responses, which were detected in SUMont-immunized calves.

4.4.4 Vaccine safety

ΔSHrBRSV was appropriately attenuated and did not transmit to seronegative sentinel calves

Calves immunized once intranasally with ΔSHrBRSV and co-housed seronegative sentinel calves were monitored clinically, serologically and virologically following vaccination. Transmission of ΔSHrBRSV to sentinels was ruled out by: the absence of clinical signs of infection; the absence of BRSV RNA in nasal secretion; and the absence of BRSV-specific humoral responses three weeks after co-housing with ΔSHrBRSV vaccinated calves.

Furthermore, calves vaccinated with ΔSHrBRSV showed only very slight upper respiratory signs following vaccination and shed no (4/5 calves), or marginal amounts (calf a2; ≤0.36 $TCID_{50}$ eq. unit; 5-7 days post-vaccination), of virus in nasal secretion.

Subunit vaccines were safe and did not induce any severe adverse reactions to vaccination

Calves immunized twice parenterally with SUMont, SUAbis or placebo vaccine, were clinically monitored following first and second vaccination, with special attention to possible swellings at injection sites.

Following vaccination with SUMont, calves demonstrated elevated rectal temperature (mean calf max±SD 40.8±0.29°C and 40.0±0.11°C, 1st and 2nd vaccination, respectively) and slight depression, with no or mild local

swellings after first vaccination, and moderate to marked local swellings after second vaccination. These adverse reactions waned within 2-3 days.

Calves immunized with either SUAbis or adjuvant alone demonstrated elevated rectal temperatures following first (mean max±SD 40.0±0.5°C), but except for one control calf (d5, max 40.1°C), no temperature elevations following second vaccination. Following both vaccinations, but more pronounced after first vaccination, these calves developed mild to moderate swellings at injection sites, which persisted for 2-3 days.

5 Discussion

The development of BRSV vaccines that can effectively induce long lasting protection in young calves with BRSV-specific maternally derived antibodies (MDA) remains a high priority to the cattle industry.

Disease is commonly seen in calves less than 1 year old, with a peak in morbidity between 1-3 months (Verhoeff et al. 1984). Young immunologically immature calves may be afforded some protection from BRSV infection by MDA (Chase et al. 2008; Kimman et al. 1988). However, as for many pathogens in veterinary and human medicine, specific MDAs often completely suppress antibody production and at least partially inhibit induction of humoral memory following vaccination or natural exposure, whereas cellular memory and responses are usually minimally affected (Kimman, Westenbrink, Schreuder, et al. 1987; Niewiesk 2014). For many commercial BRSV vaccines, MDA suppression of vaccine responses often results in insufficient immune responses when calves are experimentally or naturally exposed to virulent BRSV (Larsen et al. 2001; Hägglund et al. 2004).

Vaccination programs using commercially available BRSV vaccines interfere with epidemiological surveys, because the humoral responses induced by classic vaccines are indistinguishable from those induced by natural infection. New generation DIVA vaccines that omit at least one immunogenic viral protein in the rational vaccine design allow continued serological monitoring, and the ability to monitor vaccine safety and efficacy in the field.

Classic BRSV-ISCOMs have previously been shown to overcome the suppressive effect of specific MDA in older calves (7–15 weeks old) (Hägglund et al. 2004). In this thesis work, BRSV-ISCOMs were evaluated in younger calves (3–8 weeks old), and the vaccine formulation was characterized with regard to immunogenicity and protein content.

In addition, three DIVA-compatible new generation BRSV vaccines were evaluated in a severe BRSV calf infection model described in this thesis.

5.1 BRSV-ISCOMs overcame the suppressive effect of maternally derived antibodies and induced protective antibody and T cell responses in young calves (Paper I)

In agreement with previous results in older calves (Hägglund et al. 2004), the BRSV-ISCOMs evaluated in paper I overcame the suppressive effect of MDA in younger calves and induced a high level of clinical and virological protection following challenge. Protection observed in BRSV-ISCOM-immunized calves was associated with strong humoral responses and a Th1-oriented T cell response, which in turn was strongly linked to the ISCOM adjuvant, as calves immunized with BRSV proteins without adjuvant were significantly less well protected. These results suggest that BRSV-ISCOM vaccination can be effective in immunologically immature calves with specific MDA, which is relevant in field conditions. Additionally this suggests that BRSV-ISCOMs are safe in this target group, as no exacerbated disease following challenge was observed clinically or on investigation of pulmonary histopathology post-mortem.

BRSV-ISCOM-immunized calves demonstrated only limited respiratory signs and minimal lung pathology following challenge, in contrast to calves immunized with adjuvant alone or with PBS, which showed moderate to severe respiratory signs and moderate lung lesions.

The virological protection was even more effective, with no or only marginal virus RNA detected in nasal secretion from BRSV-ISCOM-immunized calves, and a mean area under curves of virus shed in nasal secretion from PID 0 to PID 6 (AVS) seven logs higher in all other calves.

Protection in BRSV-ISCOM-immunized calves was associated with strong anamnestic mucosal BRSV-specific IgA responses after challenge, despite nominal levels of specific IgA in serum and nasal secretion before challenge, indicating strong priming by BRSV-ISCOMs. In addition, elevated levels of BRSV-specific IgA antibodies were detected in lung lavage from BRSV-ISCOM-immunized calves six days after challenge, and correlated with protection within that group. The ability to mount a strong and rapid mucosal IgA response has previously been associated with protection (Kimman, Westenbrink, Schreuder, et al. 1987).

Elevated levels of BRSV-specific IgG (IgG_1 and IgG_2) in serum and nasal secretion were observed already before challenge in BRSV-ISCOM-immunized calves. Acquired or maternally derived pre-existing neutralizing antibodies in nasal secretion appeared to be protective against BRSV infection in one study (Mohanty et al. 1976). This suggests that nasal IgG in BRSV-

ISCOM-immunized calves may have contributed to protection, although the virus neutralizing capacity of nasal secretion was not investigated.

To approximate the neutralizing capacity of sera from immunized calves before challenge, serum mAb 19 competitive antibodies were quantified. Like with the commercially available humanized monoclonal antibody palivizumab for HRSV (Wu et al. 2005), mAb 19 binds to antigenic site IV on the F protein and neutralizes BRSV *in vitro* (Arbiza et al. 1992). Palivizumab competitive ELISAs are increasingly used in HRSV studies (Swanson et al. 2011; Smith et al. 2012), because these assays are less time-consuming and less costly, as well as being easier to standardize between different laboratories, compared to virus neutralization assays. One of these ELISAs has been shown to be indicative of neutralizing capacity of antibodies as the results correlate with a virus neutralization assay (Glenn et al. 2013). Further studies are needed to confirm such a correlation for BRSV and mAb 19. However, sera from BRSV-ISCOM-immunized calves did compete with mAb 19 (paper II), and were later confirmed to neutralize virus in a plaque reduction assay (data not shown), with titers similar to those previously reported for older BRSV-ISCOM-immunized calves (Hägglund et al. 2004).

Vaccine-induction of cytolytic CD8$^+$ T lymphocyte (CTL) activity and CD4$^+$ T helper cell cytokine production, which further stimulates both humoral and cellular responses and the generation of memory, are important in the combined immune response to BRSV infection (Taylor et al. 1995). In mice, modification of APCs, through CD40/CD40L binding by antigen specific CD4$^+$ T helper cells, induce necessary co-stimulation by APCs, to activate antigen specific naïve CD8$^+$ T cells, and to generate CTL immunity and memory (Clarke 2000), which could further explain why CD4$^+$ T helper cell responses are important in the initial response to BRSV in calves (Thomas et al. 1996).

Restimulated PBMCs from BRSV-ISCOM-immunized calves proliferated and produced both IL-4 and IFNγ, with a predominance of IFNγ. This is in line with previous studies using classic ISCOMs to immunize mice (Villacres-Eriksson et al. 1992; Maloy et al. pr1995; Sjölander et al. 1997), and suggests that a Th1 type immune response, dominated over a Th2 type immune response in mice (Zhu & Paul 2008), although cattle may have less Th1/Th2 polarized immune responses (Brown et al. 1998). These cellular responses likely contributed to the protection observed in BRSV-ISCOM-immunized calves, and extend findings from a previous study (Hägglund et al. 2004) in which cellular responses unfortunately were not investigated.

Inactivated virus used in the lymphocyte proliferation assay in paper I mainly stimulates CD4$^+$ T helper cells, and not CD8$^+$ T cells. However, CTLs

were likely primed *in vivo* by BRSV-ISCOM immunization, supported by the prominence of the IFNγ producing $CD4^+$ T helper cell population, as demonstrated *in vitro*. Nonetheless, better characterization of cellular responses is desirable, and could be achieved through isolation and specific assaying of PBMC subpopulations, such as measurement of the cytolytic activity of isolated $CD8^+$ T cells through flow cytometry of co-cultured autologous target cells (e.g. B cells) pulsed with relevant antigen (Mbitikon-Kobo et al. 2012).

The strong humoral and cellular immune responses observed in BRSV-ISCOM-immunized calves demonstrate that BRSV-ISCOMs overcame the commonly observed inhibitory effect of MDA on vaccine-induced immune responses.

Even low levels of serum specific MDA may completely suppress humoral responses, and may attenuate subsequent memory responses. This has been shown for BRSV and HRSV (Kimman, Westenbrink, Schreuder, et al. 1987; Crowe et al. 2001) and many other pathogens and vaccines, e.g. in infant vaccination against measles, a virus in the same family as RSV, *Paramyxoviridae* (Bertley et al. 2004; Gans et al. 2004).

The suppressive effect of specific MDA on B cell activation and antibody production are mainly mediated by two mechanisms: epitope masking and cross-linking of receptors on B cells (Niewiesk 2014).

Suppression by epitope masking is suggested to work by specific MDA binding to vaccine antigen, thereby physically blocking access by B cells (Karlsson et al. 1999). This problem might be partially overcome by increasing the amount of antigen(s) in the vaccine, as demonstrated by effectiveness of high titer measles vaccination in young children (Whittle et al. 1988). However, this approach was also shown to be harmful to individuals with low levels of specific MDA (Garenne et al. 1991), and may be detrimental to the induction of immune memory (Kalia et al. 2006). Additionally, high titer vaccines are costly to produce.

Complexes of multimeric antigen and pre-existing antibodies can interfere with B cell receptor (BCR) oligomerization and signaling, through cross-linking of BCRs and FcγIIB receptors (Liu et al. 2010). However, inhibition by cross-linking of receptors has been shown to be limited using small multimeric antigen carriers (Enriquez-Rincon & Klaus 1984), which might explain how BRSV-ISCOMs (56 nm mean diameter) avoided inhibition by MDA.

In addition, specific antibodies bound to a small multimeric antigen carrier (in the Å range) have been suggested to enhance uptake by APCs, and stimulation of APC maturation (Enriquez-Rincon & Klaus 1984; Getahun & Heyman 2006), a mechanism reported for monomeric antigen/antibody complexes (Schnurr et al. 2005). Internalization of classic ISCOMs by APCs is

partially mediated by the hydrophobicity of ISCOM particles, but may be enhanced by specific receptor interaction between ISCOM protein and APC surface protein (Villacres et al. 1998; Lövgren Bengtsson et al. 2011). Whether the presence of specific antibodies can enhance APC uptake of classic ISCOMs requires further studies.

Nonetheless, in the challenge infection of paper I, BRSV protein formulated with ISCOMs (BRSV-ISCOMs) overcame the suppressive effect of maternal antibodies and induced IgG antibody production, IgA memory, and Th1 type cellular responses, in contrast to calves immunized with BRSV proteins alone. This demonstrates that the adjuvant formulation played an essential role in the induction of protective immunity by BRSV-ISCOMs.

Thus, as BRSV-ISCOM vaccination successfully protected young calves with specific MDA against BRSV infection, and have previously been shown to induce protection exceeding that induced by a commercially available inactivated vaccine (Hägglund et al. 2004), this formulation is a very promising vaccine candidate. Characterization of BRSV-ISCOMs would facilitate batch-to-batch consistency of this vaccine, and provide knowledge that could be used in future rational vaccine design.

5.2 Characterization of BRSV-ISCOMs verified high concentrations of BRSV proteins F and N, and additionally identified BRSV proteins G, M, M2-1, P and SH, as well as further elucidated BRSV-ISCOM-induced immune responses (Paper II)

The protein makeup of BRSV-ISCOMs used in paper I was investigated in paper II, using gel electrophoresis, Western blot, dot blot and mass spectrometry (MALDI-TOF and LC-MS/MS). Each dose of BRSV-ISCOMs contained 188 µg of protein, consisting of approximately 77 µg F protein, 17 µg of N protein, and BRSV proteins G, M, M2-1, P and SH probably at lower concentrations, as well as several cell-derived proteins. BRSV proteins L, NS1, NS2 and M2-2 were not detected in the BRSV-ISCOMs used in paper I, even by the highly sensitive LC-MS/MS method (Smith et al. 2004).

A previous study in which HRSV-ISCOMs were prepared by dialysis, like BRSV-ISCOMs in paper I and II, identified the F and G proteins in HRSV-ISCOMs (Hu et al. 1998), whereas the F and N protein were identified in BRSV-ISCOMs prepared by ultracentrifugation (M. Trudel et al. 1989). The F protein was the most abundant protein in both BRSV- and HRSV-ISCOMs (M. Trudel et al. 1989; Hu et al. 1998). Thus, results in paper II confirmed previous findings regarding classic RSV-ISCOMs, and also enhance our understanding

of the makeup, of these vaccines by semi-quantification of these proteins and identification of BRSV proteins M, M2-1, P and SH, and cell-derived proteins in BRSV-ISCOMs.

Additionally, sera from BRSV-ISCOM-immunized calves in paper I were analyzed to determine to which BRSV proteins antibodies were directed. As expected, based on the high concentrations of proteins F and N in BRSV-ISCOMs, and the known immunogenicity of these proteins (Walsh et al. 1987; Riffault et al. 2010), significantly elevated titers of serum antibodies against proteins F and N were detected in BRSV-ISCOM-immunized calves. Similarly, the highly immunogenic glycoprotein G (Walsh et al. 1987; Taylor et al. 1997) induced G-specific serum antibodies in BRSV-ISCOM-immunized calves. Antibodies against the SH protein were also elevated in BRSV-ISCOM-immunized calves, compared to calves immunized with BRSV proteins alone or PBS, but not compared to calves immunized with adjuvant alone, which may be explained by individual variations in levels of specific MDA. Proteins P and M, detected at low concentration in BRSV-ISCOMs, did not appear to induce specific antibodies.

The individual contribution of each detected protein to BRSV-ISCOM-induced protection cannot be unequivocally identified based on available data. However, previous studies have shown that the glycoproteins F and G induce neutralizing serum antibodies and mucosal IgA antibodies (Westenbrink et al. 1989; Walsh et al. 1987; Taylor et al. 1997; Taylor et al. 2005), and that the F protein activate $CD8^+$ CTLs (Gaddum et al. 2003). Other studies have shown that while proteins N, M2-1 and P induce non-neutralizing antibodies (Riffault et al. 2010; paper IV), importantly these proteins have epitopes recognized by $CD8^+$ CTLs (Gaddum et al. 2003; Taylor G., personal communication). Antibodies directed against the SH protein do not neutralize virus *in vitro*, but have been shown to reduce virus replication in HRSV-infected mice, likely by connecting SH on the surface of infected cells to Fcγ receptors of alveolar macrophages (Schepens et al. 2014). In that study, protection from subsequent HRSV infection of SH immunized mice was concluded to be antibody mediated, since, although a mouse $CD4^+$ T cell epitope within the SH protein has been reported (Nicholas et al. 1989), no SH specific T cells were detected in vaccinated mice,. In this context, the BRSV-ISCOM-induced protective humoral and cellular responses observed in paper I may have been mediated by antibodies against proteins F (including antibodies competing with the neutralizing mAb 19), G and SH, and T cell responses against F, N, M2-1 and P.

The role of the cell-derived proteins detected in BRSV-ISCOMs is not known. These proteins may have adjuvant effects, but could also potentially

cause hypersensitivity and a Th2 type immune response in some individuals when administered repeatedly to calves, similar to that observed in mice inoculated with vaccine and HRSV inoculum containing residual bovine proteins (Ostler & Ehl 2002). However, there are likely residual cellular proteins in many commercial bovine vaccines, and the low frequency of reports of immunopathology in vaccinated cattle might be explained by differences in immune responses between cattle and mice (Brown et al. 1998). Nonetheless, although there was no clinical or histopathological indication of immunopathology in BRSV-ISCOM-immunized calves, the role of cellular proteins in this formulation deserves further investigation in extended studies, to minimize the risk of immunopathology in certain individuals.

The adjuvant formulation in BRSV-ISCOMs considerably enhanced protective immune responses, as demonstrated by the absence of similar responses and the poor virological protection induced by BRSV proteins alone. Classic ISCOMs have been shown to induce Th1 type cellular and humoral immune responses in mice (Villacres-Eriksson et al. 1992; Maloy et al. 1995; Sjölander et al. 1997), similar to those observed following BRSV-ISCOM vaccination of calves in paper I. ISCOMs recruit innate and adaptive effector cells to lymph nodes draining the site of subcutaneous vaccination, including IFNγ-producing NK cells, which activate dendritic cells (DCs) (Duewell et al. 2011). Following ISCOM-mediated uptake by DCs and B cells, antigen is processed and epitopes presented on major histocompatibility complex (MHC) class II molecules, which may activate specific $CD4^+$ T helper cells, and on MHC class I molecules through cross presentation, which may activate specific $CD8^+$ T cells (Robson et al. 2008; Schnurr et al. 2009). Although in paper I (when isolated PBMCs were restimulated with inactivated virus), only $CD4^+$ T helper cells were stimulated *in vitro*, specific $CD8^+$ T cells were also likely stimulated *in vivo* by BRSV-ISCOM immunization, as discussed earlier.

Membrane proteins readily associate with ISCOM particles (Morein et al. 1984), as do some internal viral proteins (M. Trudel et al. 1989; Polakos et al. 2001), depending on their relative hydrophobicity, and possibly through association with other proteins. Whether the proteins detected in the BRSV-ISCOM formulation in paper I were incorporated or associated with the ISCOM particles was not confirmed. However, this could be investigated by fractioning of the formulation by ultracentrifugation through a sucrose gradient, followed by analysis of lipid and protein content in each fraction.

In conclusion, the classic BRSV-ISCOM formulation evaluated in paper I overcame the suppressive effect of MDA, and induced a high level of clinical and virological protection. Strong humoral responses against proteins F and N, present at high concentrations in BRSV-ISCOMs, and in addition G and SH,

detected in low concentrations, were induced by BRSV-ISCOMs. Proteins P, M and M2-1, detected at low concentrations, did not induce significant humoral responses, but may have induced $CD4^+$ or $CD8^+$ T cell responses, although protein-specific T cell responses were not evaluated.

To be cost-effective and to ensure batch-to-batch consistency, large-scale propagation of BRSV and commercialization of classic BRSV-ISCOMs would require optimization of the described protocol. Alternatively, large-scale production of individual recombinant viral proteins is a viable option as it has become easier and less costly, and additionally facilitates rational design of the protein content and adjuvant used in the vaccine.

5.3 Merging methods from different laboratories produced a robust BRSV challenge model with high clinical expression in calves with passive immunity (Paper III & IV)

Information about the protein content in the BRSV-ISCOMs and preliminary results from separate vaccine evaluations performed by two other project partners in this thesis work, resulted in three vaccine candidates that warranted further evaluation.

The three involved research groups had previously carried out experimental BRSV infections to evaluate vaccines in calves of varying age and levels of specific MDA, using: (i) aerosol inhalation of BRSV-Dk, in genetic subgroup II (Valarcher et al. 2000), propagated in cell culture (paper I); (ii) i.n. nebulization and i.t. injection of the BRSV-3761 isolate, in genetic subgroup V (Meyer et al. 2008), passaged three times in neonatal calves, and recovered in each passage by BAL (Riffault et al. 2010); and (iii) similar to (ii), except the inoculum was BRSV-Snk, in genetic subgroup IV (Valarcher et al. 2000), passaged in gnotobiotic calves (Taylor et al. 2014a).

These experiments were in turn based on previously published studies, e.g. (Tjørnehøj et al. 2003; Hägglund et al. 2004; Taylor et al. 1995). In addition to the differences in inocula and mode of inoculation, the three laboratories had used different methods to monitor key outcomes of experimental infection.

Therefore, a common BRSV infection model in calves was developed, with the aim of producing consistently high clinical expression, along with consolidated methods for monitoring infection, in order to evaluate these three vaccines, and to enable comparability with follow-up studies performed in the different laboratories (e.g. studies on duration of immunity, or evaluation of heterologous prime/boost regimens).

Two groups of three calves were experimentally infected with BRSV; either BRSV-Snk (calves A1-A3) passaged in calves, or BRSV-Dk (calves B1-B3) with low number of cell culture passages. Both inocula were administered by aerosol inhalation using identical facemasks, nebulizers and air compressors.

Following challenge, regardless of inocula, all calves developed manifest BRSV disease, demonstrating that challenge by small droplet (≤5 µm) aerosolization of BRSV is effective in calves. How deep infectious BRSV reaches in the respiratory tract following aerosol inhalation by calves is unknown. One study using similar aerosol inhalation in combination with intratracheal injection to infect calves with BRSV reported that virus replication started in the upper airways, and progressed to the lower airways (Viuff et al. 2002). However, another study where cattle (9–12 month old) were infected with aerosolized foot-and-mouth disease virus, demonstrated simultaneous infection of the whole lung (Pacheco et al. 2010). Even if, as is the case in humans (Knight 1980), inhaled aerosol droplets reach the alveoli in calves, to the author's knowledge there are no published data showing to what extent infectious BRSV virions are carried in small aerosol droplets. Future studies on this topic, and studies to further investigate the pathogenesis of natural versus different methods of experimental BRSV infection in calves, might shed light on these issues, as well as add an additional perspective on the clinical relevance of different BRSV infection models, such as the one presented in paper III.

The BRSV-Snk inoculum was concluded to induce more severe disease, compared to the BRSV-Dk inoculum. BRSV-Snk-infected calves were consistently more severely affected by BRSV infection, across clinical, pathological and virological parameters. This difference in potency might simply be explained by differences in strain virulence, but may also have been influenced by passage and propagation in calves and in cell culture, for BRSV-Snk and BRSV-Dk, respectively.

Passage of BRSV-Dk in BT cells may have attenuated its virulence in calves, which would explain a higher level of clinical signs observed using the same inoculum with fewer passages (paper I), and when propagated in fetal lung cells (Hägglund et al. 2004). It is likely that changes in virulence following passage(s) in cell culture depend on the virus strain, cell type and number of passages. Some studies have demonstrated loss of virulence of BRSV or HRSV after passage *in vitro* (Deplanche et al. 2007; Kwilas et al. 2009), whereas other studies have suggested that passage in cell culture induces no significant changes in the gene coding for the attachment protein (Furze et al. 1997; Larsen et al. 1998).

In contrast to BRSV-Dk, the BRSV-Snk inoculum was passaged and propagated in gnotobiotic calves. Passage of both inocula in either gnotobiotic calves or cell culture prior to experimental infection would have facilitated better comparability of the virulence of the two strains, but that was not feasible, nor was it needed for the purpose of the study in paper III.

Following challenge, two out of three calves infected with BRSV-Snk (calves A2 and A3, Table 3) shed high amounts of virus in nasal secretion following challenge. The remaining calf (A1) shed considerably less virus in nasal secretion, and based on rapid seroconversion after BRSV-Snk inoculation, was subsequently determined to have been previously primed by natural BRSV infection.

All BRSV-Snk-infected calves (including calf A1) demonstrated severe clinical signs and moderate to severe lung pathology following challenge, similar to that described for natural BRSV infection in the field (Bryson 1993; Verhoeff et al. 1984). Contrary to previous reports (Baker et al. 1986; Van der Poel et al. 1994), the prior exposure of calf A1 did not appear to provide clinical protection against experimental BRSV reinfection.

The case of calf A1 illustrates that in the event of BRSV infection at a young age serum specific MDA can inhibit humoral responses despite establishment of immune memory, and that declining serum specific MDA titers can mask weak responses, which is in agreement with previous observations (Uttenthal et al. 2000; Kimman, Westenbrink, et al. 1989). Consequently, seromonitoring in herds delivering supposedly BRSV-naïve calves needs to be more stringent to conclusively rule out prior exposure.

BRSV-Snk, but not BRSV-Dk infection was shown to induce a significant increase of cells in post-mortem BAL (predominantly neutrophils), compared to cells in BAL before challenge, which have previously been reported in naturally BRSV-infected calves (Kimman et al. 1986). This suggests a difference in strain virulence or kinetics of infection.

A marked difference in virus localization between the two groups was revealed when BRSV antigens in sections of trachea and lung tissue were immunohistochemically stained post-mortem. This occurred despite virus isolation from BAL cells and high virus titers detected in BAL supernatant from all infected calves regardless of inocula (including calf A1). Abundant virus was observed in the lungs of BRSV-Snk-infected calves (except calf A1) and very little in the trachea, whereas the opposite was observed in BRSV-Dk-infected calves. This might be explained by a difference between the two inocula in progression of virus replication on PID 7, given that virus replication starts in the upper airways and progresses to the lower airways (Viuff et al. 1996).

The conclusion in paper III, that BRSV-Snk passaged in gnotobiotic calves was the most suitable inoculum for a BRSV infection model in calves, was confirmed when the model was repeated and further characterized in five unvaccinated calves (d1-d5) with moderate levels of specific MDA in the experiment described in paper IV. Following challenge with the BRSV-Snk inoculum in that experiment, calves d1-d5 demonstrated clinical signs and lung pathology similar, but even more severe, than that observed in calves A1-A3, and shed similar amounts of virus in the upper and lower airways.

When BAL cells collected from calves d1-d5 seven days after challenge were enumerated, an influx of neutrophils was observed, similar to that observed in calves A1-A3. In addition, elevated concentrations of IFNγ in BAL supernatant at PID 7 were detected, whereas IL-4 was present only at minimal concentrations, which is in agreement with previous findings in primary infected calves (Grell, Tjørnehøj, et al. 2005). IFNγ can be produced by cells both in the innate immune system, such as neutrophils, NK cells, alveolar macrophages and γδT cells, and by cells in the adaptive immune system, such as $CD8^+$ T cells (Mcinnes et al. 1999; Sohn et al. 2007; McGill et al. 2013). Thus, IFNγ detected in BAL supernatant might have been produced by $CD8^+$ T cells, which migrate to the lungs during acute BRSV infection (Antonis et al. 2006), or possibly by the increased numbers of neutrophils detected in BAL. Either way, this appeared to be a protective response, as there was an inverse correlation in calves d1-d5 between concentrations of IFNγ in BAL supernatant, and the macroscopic extent of lung pathology.

In contrast to IFNγ, concentrations of TNFα, IL-6 and IL-8 were not elevated in calves d1-d5 in BAL collected seven days after challenge, although increased expression of these cytokines has been associated with BRSV infection in calves (Røntved et al. 2000; Grell, Riber, et al. 2005; Grell, Tjørnehøj, et al. 2005) and lambs (Redondo et al. 2014). This discrepancy may be related to differences in timing of sampling in relation to the progression of infection.

Objective assessment of lung function to enable comparison between calves and between experiments is often limited to respiratory frequency. Auscultation of lung sounds, on the other hand, is subjective. Therefore the forced oscillation technique (FOT) was evaluated as a means to passively and objectively measure lung function during BRSV infection in calves. By using this technique the effect of BRSV infection on the respiratory function was demonstrated. FOT measurements in calves d1-d5, before and after challenge, indicated increased resistance and decreased reactance in the lower airways, which have been associated with bronchoconstriction and obstructive airway processes (Reinhold et al. 1996; Oostveen et al. 2003). Passive measurement of

airway resistance, using methods similar to FOT, have been used in mice (Davis et al. 2007) and infants (Drysdale et al. 2014) to assess the impact of HRSV on lung function, and could be an additional objective parameter when evaluating BRSV vaccines or therapeutics in calves.

In brief, protocols and methods from different laboratories were consolidated and refined, and validated as a robust BRSV infection model, with high clinical expression in calves with maternal antibodies. In addition, tools to monitor and evaluate key outcomes of BRSV infection were developed.

5.4 ΔSHrBRSV and SUMont induced almost complete and partial protection, respectively, through distinct immunological pathways (Paper IV)

The evaluation in calves of three separate vaccines described in paper IV was the culmination of this thesis work and the product of encouraging results from three different project partners in developing killed vaccines, such as BRSV-ISCOMs (Hägglund et al. 2004 and papers I & II) or N nanorings (Riffault et al. 2010), and live attenuated gene-deleted vaccines, such as ΔSHrBRSV (Taylor et al. 2014a).

Formulation of the subunit vaccines was based on knowledge of BRSV proteins involved in generating a protective immune response and results from studies on BRSV-ISCOMs (paper I and II). Different recombinant proteins were obtained or generated, and included in the subunit composition.

The nucleoprotein N was included as N nanorings, and because recombinant F and G proteins could not be obtained in sufficient quantity, known protective epitopes from BRSV F and G were attached to the N or C terminus of the HRSV N protein, producing epitope-decorated N nanorings (eN; Table 2).

Recombinant HRSV P and M2-1 were also added to the subunit composition, as these proteins contain epitopes recognized by CTLs, and were also identified in BRSV-ISCOMs, as discussed earlier.

The subunit composition was formulated into two vaccines: SUAbis, adjuvanted by ISCOM matrices (AbISCO-300), and SUMont, adjuvanted by an oil emulsion (Montanide ISA71^{VG}). Both types of adjuvants have been shown to enhance T helper cell, CTL and antibody responses (Lövgren Bengtsson et al. 2011; Aucouturier et al. 2001).

ΔSHrBRSV, a live recombinant BRSV vaccine, attenuated by deletion of the SH gene, had been shown to be effective and sufficiently clinically attenuated in seronegative calves (Taylor et al. 2014a). Similarly, a

recombinant live SH-gene-deleted HRSV vaccine is one of the most promising vaccine candidates in clinical trials in children (Malkin et al. 2013).

The SH protein was chosen as a common DIVA marker, and was omitted by rational design from all three vaccine candidates because it had been shown to be immunogenic (paper II), but non-essential to protection (Taylor et al. 2014a). A common DIVA marker enables development and use of a single DIVA assay, and combined vaccination with successful candidates.

The safety, immunogenicity and protective efficacy of these three vaccines were evaluated and compared in calves with specific MDA, using the regimen and route expected to be the most effective, and believed to comply with requirements in the field.

ΔSHrBRSV was administered once intranasally, five weeks before challenge, whereas the subunit vaccines were administered twice intramuscularly (SUMont) or subcutaneously (SUAbis), five and two weeks before challenge. Controls received ISCOM matrices alone, twice at three weeks of interval.

Immunization with these three vaccines offered different levels of protection against BRSV infection, clearly and consistently distinct from each other. Protection induced by ΔSHrBRSV and the subunit vaccines, respectively, was mediated by dissimilar immune responses.

Unvaccinated calves (d1-d5) all showed signs of general depression and severe respiratory signs of disease following challenge, shed high titers of virus in their upper and lower airways, and lesions of consolidation covered an average of 48% of their lungs at post-mortem.

In contrast, calves immunized with ΔSHrBRSV were almost completely protected following challenge, and showed only very mild signs of respiratory disease, shed only minimal or no virus in the upper and lower airways, and demonstrated minimal inflammation in the lungs at post-mortem. These results confirm findings in young (1-4 weeks old) seronegative calves, immunized with the same vaccine, and challenged with BRSV six months later (Taylor et al. 2014b), and extend findings to apply to young calves with specific MDA.

No immune responses were detected in ΔSHrBRSV-immunized calves before challenge, and the very high level of protection observed following challenge appears to have been mediated by strong anamnestic responses, both humoral and cellular, indicating that vaccination had primed immune memory sufficient to induce protection against BRSV five weeks later.

The rapid anamnestic responses of mucosal BRSV-specific IgA antibodies and neutralizing antibodies directed against the F and G proteins in serum, observed in ΔSHrBRSV-immunized calves, were in agreement with

observations in BRSV-ISCOM-immunized calves (paper I, paper II and Hägglund et al. 2004), and have previously been associated with protection (Kimman, Westenbrink, Schreuder, et al. 1987; Kimman, Westenbrink, et al. 1989; Ellis et al. 2007; Taylor et al. 1997).

Local ΔSHrBRSV-induced T cell memory in the airways was observed following isolation and BRSV restimulation of lymphocytes from tracheobronchial lymph nodes on PID 7, characterized by IFNγ rather than IL-4 production, suggesting a Th1-biased response. Corresponding analysis was not performed in subunit-immunized calves, due to logistical limitations. However, a previous study demonstrated N nanoring-specific proliferation and IFNγ production in lymphocytes isolated from tracheobronchial lymph nodes twenty days after challenge from calves immunized intranasally and intramuscularly with N nanorings, but not in calves immunized intramuscularly alone (Riffault et al. 2010).

SUMont-vaccinated calves were also protected following challenge, although significantly less protected than ΔSHrBRSV-immunized calves: with more severe clinical signs and virus shedding, and more inflammation in the lungs.

The good level of protection induced by SUMont vaccination appears to have been mainly mediated by cellular responses against the internal proteins included in the subunit composition: N, M2-1 and P.

Humoral responses were detectable already after first vaccination, but these were non-neutralizing and directed against N, M2-1 and P, and not against F and G. The BRSV F and G epitopes, recombinantly attached to N nanorings, and included in both subunit vaccines, were selected based on their ability to induce protective antibodies (Arbiza et al. 1992; Bastien et al. 1997; Thomas et al. 1998; Chargelegue et al. 1998; Wu et al. 2007), and have no known T cell epitopes in cattle (Fogg et al. 2001). Therefore, the protection observed in SUMont-immunized calves was likely mainly mediated by T cell responses against N, P and M2-1, whereas the F and G epitopes had no or a very limited impact.

HRSV proteins N, P or M2-1 in SUMont induced cross-reactive responses to restimulation with BRSV of circulating T cells, isolated after both first and second SUMont vaccination, and induced protection following challenge which exceeded the partial protection observed by Riffault et al., following immunization of calves with HRSV N nanorings alone (Riffault et al. 2010). Proteins N, P and M2-1 are all highly conserved between BRSV and HRSV (Samal et al. 1991; Mallipeddi & Samal 1992; Zamora & Samal 1992), and contain $CD8^+$ T cell epitopes in cattle (Gaddum et al. 2003). In contrast to SUMont, SUAbis failed to induce detectable T cell responses, and

consequently afforded immunized calves only limited protection following challenge.

The distinct degrees of protection afforded by the three vaccines can be explained by differences in the immune responses they induced. This in turn might be explained by differences in type of antigen (live/subunit), antigen quantity, adjuvant, as well as route and number of administrations.

Replication of ΔSHrBRSV in the airways of immunized calves and the subsequent expression of native viral proteins and replication of viral RNA in infected cells would have primed several humoral and cellular immunological pathways, as discussed below, although only very limited replication of vaccine virus was detected in ΔSHrBRSV-immunized calves.

SUMont contained relatively high amounts of recombinant HRSV N, P and M2-1 proteins, and induced cross-reactive immune responses, which would likely be difficult to achieve through inoculation with live HRSV, considering the poor replication of HRSV in calves (Thomas et al. 1984). Thus, SUMont overcame the BRSV/HRSV host species barrier, by administration of sufficient amount of appropriate HRSV antigen, in conjunction with a water-in-oil adjuvant.

Pre-existing BRSV-specific MDA did not suppress either humoral or cellular immune responses to SUMont vaccination. ΔSHrBRSV-immunized calves failed to seroconvert following vaccination, which is in contrast to that previously observed in calves with low levels of serum specific MDA (Taylor et al. 2014b), suggesting suppression of antibody production by serum MDA. On the other hand, the rapid anamnestic responses upon BRSV challenge observed in ΔSHrBRSV-immunized calves, indicate very good priming, despite moderate levels of serum specific MDA.

Antibody characteristics in these vaccine-induced humoral responses varied. Whereas humoral responses induced by ΔSHrBRSV were neutralizing, and likely induced by the F and G proteins on the surface of the vaccine virus, antibodies detected in calves vaccinated with SUMont or SUAbis were non-neutralizing, and were instead directed against the internal viral proteins N, P and M2-1. Serum and mucosal antibodies against proteins F and G have been shown to be protective through virus neutralization (Westenbrink et al. 1989; Walsh et al. 1987; Taylor et al. 1997; Taylor et al. 2005), whereas antibodies against N, P and M2-1 are less likely to contribute to protection, as these are internal viral proteins. However, the roles of these antibodies in the SUMont observed protection require further investigation.

Lack of immunogenicity of the BRSV F and G epitopes (attached to N nanorings) might be related to epitope conformation or accessibility in these constructs, even though the protein products of all constructs were verified by

epitope-specific monoclonal antibody ELISAs (data not shown). The lack of immunogenicity might also be explained by an insufficient exposure of these epitopes, which might be resolved by increasing the administered dose, or by increasing the number of F and G epitopes attached to N nanorings (Hervé et al. 2014).

Both ΔSHrBRSV and SUMont induced a local or systemic Th1-oriented $CD4^+$ T lymphocyte response. BRSV-specific T cells producing IFNγ were detected in PBMCs from SUMont-immunized calves after a single vaccination, and in tracheobronchial lymph node cells from ΔSHrBRSV-immunized calves after challenge. Due to logistical limitations, priming of T cell responses in circulating PBMCs was not analyzed after challenge, and only in tracheobronchial lymph node cells from ΔSHrBRSV-immunized calves and controls. Therefore, local priming of T cell responses by SUMont, and circulating T cells primed by ΔSHrBRSV, cannot be excluded. As discussed earlier, a strong and balanced $CD4^+$ T helper cell response is important to enhance antibody production, cytolytic activity, and immune memory (Taylor et al. 1995; Clarke 2000; Amanna & Slifka 2010).

The contribution of cytolytic $CD8^+$ T cells was not confirmed herein, but these can be expected to have been activated by ΔSHrBRSV and SUMont, since both contained antigens with epitopes recognized by $CD8^+$ CTLs and, based on studies in vitro, both were suggested to induce the proliferation of IFNγ producing CD4+ T helper cells, which would have stimulated activation of CTLs. Furthermore, replication of ΔSHrBRSV in host cells is likely to have activated antigen specific CTLs, following presentation of vaccine antigen on MHC class I molecules, and induction of relevant cytokine production (Masopust et al. 2007). SUMont may have activated BRSV cross-protective CTLs through cross presentation by APCs (Bevan 2006), or after adjuvant-mediated ingression into the cytoplasm of host cells (Rao & Alving 2000). Confirmation of induction of CTLs by ΔSHrBRSV and SUMont could be obtained by use of a specific $CD8^+$ T cell cytolytic activity assay, as discussed for BRSV-ISCOMs, and should be investigated in future studies.

Route of immunization likely also affected the degree of vaccine-induced protection. For live virus vaccines, the intranasal route has been shown to be more effective than parenteral routes in overcoming the suppressive effect of specific MDA, and inducing protective immunity (Kimman, Westenbrink, et al. 1989; Kimman, Westenbrink, Schreuder, et al. 1987; Vangeel et al. 2007), perhaps by activating antigen-specific memory lymphocytes in the mucosa with appropriate homing signals (Belyakov & Ahlers 2009). This is consistent with the strong local immune responses observed in ΔSHrBRSV-immunized

calves. However, immunity induced by mucosal live BRSV vaccination may have limited duration (Ellis et al. 2013).

SUMont on the other hand, appear suitable for intramuscular administration in calves. When N nanorings alone were administered to calves intranasally and intramuscularly, or intramuscularly alone, both regimens afforded similarly partial protection in a challenge with moderate clinical expression (Riffault et al. 2010). The enhanced protection induced by SUMont (in a severe challenge), compared to N nanorings alone, is likely attributable to the addition of HRSV proteins P and M2-1.

SUAbis induced limited protection when administered subcutaneously, but to the author's knowledge, there is no literature to suggest that the subcutaneous route is inherently unfavorable in cattle. Studies using subcutaneous administration of recombinant protein formulated with AbISCO-300 have successfully induced both humoral and cellular immune responses: immunization of cattle with recombinant bluetongue proteins, adjuvanted by AbISCO-300, induced neutralizing antibodies against the surface protein VP2, NS1- and NS2-specific T cell responses, and protection of cattle against bluetongue infection (Anderson et al. 2013; Anderson et al. 2014). Thus, the lack of immunogenicity of SUAbis is more likely due a sub-optimal combination of adjuvant and antigen, rather than to route of administration.

The two adjuvants used to formulate the subunit vaccines are reported to be capable of inducing similar immune responses, albeit through different modes of action (Lövgren Bengtsson et al. 2011; Aucouturier et al. 2001), which may also partially explain the difference in immunogenicity.

SUAbis was adjuvanted by AbISCO-300, which are reported to have similar adjuvant effects to that of classic ISCOMs, as discussed earlier (Lövgren Bengtsson et al. 2011), although these effects are likely enhanced by various degrees of antigen incorporation into classic ISCOMs. The immunogenicity of SUAbis would likely be improved by the addition of a full-length BRSV membrane protein, which is more apt to associate with ISCOMs, and may also promote uptake of ISCOM/antigen complexes by APCs (Lövgren Bengtsson et al. 2011; Villacres et al. 1998). The BRSV F protein may be a good candidate, as this protein was abundant in BRSV-ISCOMs (paper II).

SUMont contained Montanide ISA71^{VG} as adjuvant, a water-in-oil emulsion. The adjuvanting effects of emulsions are mediated through induction of general inflammation; presentation of antigen at the water-oil interface; enhancing cell internalization of antigen; as well as a depot effect (Aikawa et al. 1998; Aucouturier et al. 2001). SUMont induced a Th1-oriented T cell response, which is in agreement with previous studies using the same and similar adjuvants, as reviewed by Heegaard et al. (Heegaard et al. 2011), and

which probably largely explains the good level of protection observed in SUMont-immunized calves.

Therefore, Montanide ISA71^{VG} appeared more suitable than AbISCO-300 to adjuvant the subunit composition in paper IV, although the addition of full-length BRSV glycoproteins to the subunit composition might reverse this.

None of the subunit vaccines caused more than limited local and systemic adverse reactions following parenteral immunization, and live ΔSHrBRSV induced only nominal clinical signs in some individuals, following intranasal immunization. Furthermore, no exacerbation of clinical or histopathological disease was observed following challenge.

Transmission of ΔSHrBRSV to co-housed seronegative sentinel calves was excluded in paper IV. However, calves immunized with ΔSHrBRSV in paper IV had moderate levels of serum specific MDA and shed only marginal amounts of virus after vaccination. When calves with low levels of specific MDA were inoculated with either ΔSHrBRSV or wild-type recombinant BRSV, both groups shed comparable amounts of virus detected in nasal secretion (Taylor et al. 2014b). Further studies are needed to investigate the potential transmission of ΔSHrBRSV under field-like conditions, where vaccinated calves, with serum specific MDA titers ranging from none to high, are co-housed with seronegative animals.

Attenuation of the ΔSHrBRSV vaccine virus relies solely on gene deletion, which makes it more refractory to wild-type reversion, compared to live virus vaccines in which attenuation, fully or in part, rely on point mutations (Lin et al. 2006). However, although the risk that ΔSHrBRSV regains the SH gene might be considered negligible, there is also the remote possibility that recombinantly introduced intergenic sequences are transferred to BRSV field strains during co-infections (Spann et al. 2003). This highlights the need for further studies on the extent of viral shedding and transmission following ΔSHrBRSV immunization, and warrants investigations on the specific risk of genetic recombination between ΔSHrBRSV and BRSV field strains.

In conclusion, vaccines can induce protection against BRSV-infection through induction of several types of immune responses, which may be influenced by vaccine-composition, administration route and immunization regimen, as well as factors in the host, including level of specific MDA.

Despite undetectable immune responses before challenge, a single intranasal immunization with ΔSHrBRSV primed local T cells, as well as rapid production of neutralizing systemic and mucosal IgA antibodies following challenge five weeks later, and afforded calves almost complete protection in a severe challenge.

The good protection afforded by SUMont immunization was likely mediated through HRSV N, P and M2-1 specific T cell cross-reactive responses to corresponding BRSV proteins. Non-neutralizing antibodies induced by SUMont were directed against the internal proteins N, P and M2-1, and not against the BRSV F and G epitopes grafted onto N nanorings.

Further studies are required to investigate the duration of protective immune response induced by ΔSHrBRSV and SUMont. Priming and boosting using heterologous vaccines or routes are increasingly used to improve the duration of immunity (Blanco et al. 2014; Grunwald et al. 2014; ClinicalTrials.gov id NCT01805921), and is under evaluation using ΔSHrBRSV and a modified SUMont vaccine.

The DIVA characteristics of ΔSHrBRSV and SUMont need further investigation, through prolonged seromonitoring of vaccinated and sentinel animals that are exposed to BRSV, to ascertain that contagious animals develop anti-SH antibodies, and that animals with sufficient virological protection do not. Using the DIVA property, seromonitoring of calves and older animals in vaccinated herds could be used to detect changes in vaccine safety and efficacy, and outbreaks of BRSV despite vaccination. Detecting isotypes other than IgG_1 might circumvent the masking effect of MDA, when detecting anti-SH antibodies in a DIVA assay.

5.5 Thesis Summary and Conclusions

This thesis work started out with the evaluation of BRSV-ISCOMs, which induced strong humoral responses and a Th1-oriented T cell response in young calves with specific MDA, afforded calves with a high level of clinical and virological protection, and was suggested to be safe.

The protein content of BRSV-ISCOMs was characterized, and the immune responses in immunized calves were further investigated, to enable standardization of the formulation, and to facilitate formulation of a new vaccine using recombinant proteins, either classic ISCOMs or with mixed-in adjuvant. Protein content characterization identified BRSV proteins F, N, G, M, P, M2-1 and SH in BRSV-ISCOMs, and demonstrated that proteins F and N constituted approximately half of the protein content, with ~77 µg of F and ~17 µg of N per dose of BRSV-ISCOMs.

A BRSV calf infection model was refined and characterized, to facilitate the evaluation of the two subunit vaccines and the live recombinant vaccine. This BRSV infection model proved to be robust, with high clinical expression in calves with passive immunity.

Two recombinant subunit vaccines were designed, consisting of HRSV proteins N, P and M2-1, and epitopes from BRSV F and G, adjuvanted with ISCOMs matrices, AbISCO-300 (SUAbis) and the Montanide oil emulsion (SUMont), respectively, and were evaluated along with the live recombinant vaccine BRSV, ΔSHrBRSV.

ΔSHrBRSV induced almost complete clinical and virological protection, whereas SUMont immunization induced a high level of protection following challenge in the severe model. ΔSHrBRSV and SUMont appear to achieve this level of protective immunity through activation of different immunological pathways. ΔSHrBRSV-induced protection relied mainly on induction of neutralizing antibodies directed against viral surface protein, and local T cell responses in the airways. SUMont-induced protection appears to have been mainly mediated by circulating T cells, which were likely activated by HRSV N, P or M2-1 protein in the vaccine, but cross-reacted with corresponding BRSV protein.

Effective immunity in young calves depends on a strong and balanced immune response, which might be achieved by activating multiple arms of the immune system, by combining antigens that induce humoral and cellular immune response, and through appropriate adjuvant immunomodulation. Rapid and long lasting immunity in young calves might be accomplished by combining the rapid response induced by mucosal immunization, also shown to be less inhibited by maternal antibodies, with heterologous parenteral boosting, which may induce longer lasting immunity.

Although duration of immune responses was not investigated in this thesis, ΔSHrBRSV and SUMont may form a suitable base for the development of a BRSV vaccination regimen, alone or in combination, which could provide long lasting protective immunity in young calves with maternal serum antibodies. In addition, the DIVA characteristics of these vaccine candidates need further investigation.

At the time of writing, investigations are ongoing to evaluate the effect on duration of protective immunity from heterologous priming with ΔSHrBRSV, and boosting with a modified SUMont vaccine (without BRSV epitopes), compared to ΔSHrBRSV priming alone or with homologous boosting (Makabi-Panzu et al. 2014). That study will also investigate vaccine T cell priming in more detail, including priming of $CD8^+$ T cells, through the use of MHC-defined calves.

6 Future prospects

The challenge to develop safe and effective vaccines against respiratory syncytial virus infection early in life has resulted in considerable advances in vaccinology and in the understanding of both BRSV and HRSV. However, apart from the practical goal of safe and effective vaccines, there are also many aspects about these viruses and the virus/host interaction that need to be elucidated further.

For instance, the identification of a universal pre-infection immunological correlate of protection from BRSV infection could substitute calf infections when evaluating BRSV vaccines, and could in addition be used in the field to monitor expected vaccine-induced protection. Although pre-infection systemic and local neutralizing antibodies, as well as RSV specific T cells have individually been associated with protection, these cannot be used to predict the degree of protection following infection. The quest for a vaccine-induced balanced immune response will likely also require an aggregate correlate of protection, including several immunological factors.

I believe this quest must also involve further refinement of the methods used to investigate conventional immunological parameters, such as measuring $CD8^+$ T cell specific cytotoxic activity and B cell specific expansion after *ex vivo* restimulation, combined with flow cytometric analysis of mucosal homing markers. Furthermore, as *in vitro* virus neutralizing capacity of sera do not correlate with protection, and do not account for non-neutralizing protective antibodies (e.g. antibodies against the SH proteins), new ways to estimate the *in vivo* effectiveness of antibodies needs to be developed, and may entail a combination of several epitope-specific avidity ELISAs (such as a modified mAb 19 competitive ELISA). However, and more importantly, there are likely also factors in the bovine immune response and generation of immune memory to BRSV that we are not yet aware of, which may best be investigated by

hypotheses-generating *in vitro* and *in vivo* studies using transcriptomics and proteomics.

Another factor restricting BRSV research and the evaluation of new vaccine candidates has been the lack of a reproducible infection model, with field-like clinical signs and pathology in calves with passive BRSV-specific immunity. Similarly for HRSV, small animal models do not mimic disease seen in infants. The BRSV calf infection model and tools for post-infection monitoring presented in this thesis can serve as a solid foundation for future BRSV research, and certain aspects may be extrapolated to pathogenesis and immune responses in human infants infected with HRSV. Moreover, post-infection monitoring could be made even less subjective through incorporation of novel quantitative technology, such as: automated quantification of calf feed intake and activity; more accurate quantification and localization of lung lesions post-mortem using computed tomography; and improvement of pulmonary function measurements such as the forced oscillation technique in calves.

Nonetheless, the infection models used in this thesis work clearly demonstrated protective efficacy of three vaccine candidates: BRSV-ISCOMs, ΔSHrBRSV and SUMont.

BRSV-ISCOMs induced a high level of protection in calves with maternal antibodies, but would be difficult to produce consistently in commercial quantities, and have not been shown to be DIVA-compatible. On the other hand, classic ISCOMs produced using a recombinant deleted gene BRSV (e.g. ΔSHrBRSV, which can be propagated in high titers), would be DIVA-compatible. However, a recombinant subunit approach, using BRSV (or HRSV) proteins, in proportions identified in BRSV-ISCOMs, might better facilitate batch-to-batch consistency, potentially increase the vaccine purity and decrease production costs, as well as permit DIVA compatibility.

The recombinant subunit composition evaluated in this thesis work, using a subset of the proteins identified in BRSV-ISCOMs, induced a good level of protection in a severe challenge model, when adjuvanted by an oil emulsion (SUMont), which was mainly mediated by T cell responses directed against the internal HRSV proteins N, P and M2-1, and not the BRSV F and G epitopes.

The individual roles of the N, P and M2-1 proteins, and whether they are essential to attain the level of protection observed in this thesis wok, should be further investigated, and the subunit composition optimized regarding their individual quantities. The addition to the subunit composition of recombinant antigens that could induce neutralizing antibodies, would likely enhance

induced protection; particularly considering that SUMont immunization induced production of mucosal IgA antibodies, which would likely be effective if they were directed against viral surface proteins. For this purpose the fusion protein F, in full length, is a good candidate, as this protein is highly conserved between strains of BRSV, and between BRSV and HRSV (Valarcher et al. 2000), and may provide protection against a wide range of BRSV strains circulating in the field. In addition, the F protein contains epitopes recognized by bovine $CD8^+$ T cells and was abundant in BRSV-ISCOMs, which in turn were highly protective. Work to produce full-length recombinant BRSV or HRSV F proteins is currently ongoing, but outside the scope of this thesis work.

After optimization, a modified subunit composition might be better adjuvanted by a stable oil-in-water emulsion (Richards et al. 2004) or a water-in-oil-in-water emulsion (Dar et al. 2013), possibly in conjunction with TLR-agonists, to further enhance innate immune responses and promote maturation of antibodies (Delgado et al. 2009). On the other hand, if the optimized subunit composition contains only recombinant HRSV proteins, an oil-in-water emulsion licensed for use in children (Vesikari et al. 2011) would also be interesting to evaluate. Regardless of the final composition of subunits and adjuvant, this recombinant protein approach will require further safety evaluation before being ready for field evaluation.

ΔSHrBRSV, on the other hand, is almost ready for field evaluation. ΔSHrBRSV has been shown to be sufficiently clinically attenuated in calves with levels of serum specific MDA ranging from none to moderate (Taylor et al. 2014a; paper IV). However, although ΔSHrBRSV did not transmit to co-housed seronegative sentinel calves when calves with moderate levels of serum specific MDA were intranasally immunized in this thesis work (paper IV), gnotobiotic calves and calves with low levels of serum specific MDA shed vaccine virus in nasal secretion following combined intranasal/intratracheal immunization (Taylor et al. 2014a). Therefore, the only remaining concern, which needs further investigation, is whether conventional calves with varying levels of specific MDA would shed and transmit this genetically modified virus in the field following intranasal immunization.

ΔSHrBRSV could be very useful in the field, by inducing early acquired immunity in young calves with specific MDA, as demonstrated in this thesis work. However, intranasal immunization with a live vaccine may not provide long lasting immunity (Ellis et al. 2013), and homologous intranasal boosting might be less effective than boosting by a different route, or using a different

vaccine. For example, when calves with specific MDA were immunized intranasally with ΔSHrBRSV, either one or two times (with four weeks interval), and subsequently challenged with virulent BRSV eleven weeks after first vaccination, both groups shed similar amounts of virus in nasal secretion (Makabi-Panzu et al. 2014). In the same study, calves primed intranasally with ΔSHrBRSV and boosted intramuscularly with a reduced SUMont vaccine (only N, P and M2-1) shed less virus in nasal secretion following challenge, compared to calves only immunized with ΔSHrBRSV (one or two times) (Makabi-Panzu et al. 2014).

Therefore, a heterologous prime/boost regimen, using intramuscular boosting with ΔSHrBRSV or an optimized subunit formulation (with full-length protein F) might be more effective in providing long lasting sterilizing immunity, and should be investigated in future studies. Specifically, future evaluation studies of ΔSHrBRSV (i.n. + i.m.), an optimized subunit vaccine (i.m. + i.m.), or a heterologous prime/boost regimen should investigate post-vaccination and pre-infection correlates of protection (as previously mentioned), degree and duration of protection induced by each regimen, as well as safety of each regimen.

In addition, the usefulness of ΔSHrBRSV and an optimized subunit composition not including the SH protein (or a combined regimen) as DIVA vaccines need to be evaluated in prolonged serological studies of immunized and subsequently infected animals, with varying levels of passive immunity, to evaluate the ability of these vaccines to avert virus replication and prevent induction of anti-SH antibodies when vaccinated animals are exposed to the virus. If vaccine-induced virological protection fails, due to genetic changes in the virus or due to waning immunity in vaccinated animals, and animals become contagious, rising titers of anti-SH antibodies should indicate this.

Finally, an anti-SH antibody DIVA assay, able to identify significant anti-SH seroconversions in vaccinated animals and herds, needs to be validated. To avoid detection of colostrum-derived anti-SH antibodies in samples from calves, such an assay could detect antibody isotypes other than IgG_1, or herd seromonitoring could focus on older calves and heifers. In conjunction with other control measures, these DIVA vaccines and companion DIVA assay could be used to establish BRSV-free regions or herds, while simultaneously protecting unexposed animals and herds, with maintained ability to serologically monitor virus circulation as well as vaccine efficacy and safety.

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