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CHARACTERISATION OF EPITHELIAL CELLS INFECTION BY MORBILLIVIRUSES

By
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A thesis submitted in partial fulfillment of the requirements of
the degree of *Magister Scientiæ* (M.Sc.) in Immunology and Virology

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"You have to have confidence in your ability,
and then be tough enough to follow through."

- Rosalyn Carter

Summary

Measles (MeV) and *Canine distemper virus* (CDV) are closely related members of the genus *Morbillivirus* in the *Paramyxoviridae* family. They are transmitted by aerosol droplets, and cause a moderate to severe disease characterized by rash, fever, respiratory and gastrointestinal signs and immunosuppression in their respective hosts. The virus initially targets immune cells expressing its primary entry receptor: the signaling lymphocyte activation molecule (SLAM; CD150). After massive replication in lymphoid tissues, the virus spreads to epithelial tissues in organs throughout the body, which coincides with the appearance of clinical signs. The virus is finally released into the respiratory tract, and transmitted to a new host.

Recent studies have refined the current model of MeV pathogenesis, proposing that virus replication in epithelial cells is not necessary to initiate an infectious cycle or for viral dissemination, but required for shedding and transmission. To validate this new model, we took advantage of a more severe disease phenotype seen in CDV-infected ferrets. We hypothesized that the mechanism of interaction with the epithelial cell receptor is conserved between MeV and CDV, and that the same H protein region is involved in this interaction.

To test our hypothesis, we first introduced the mutations conferring an epithelial cell receptor (EpR)-blind phenotype from MeV H to the CDV H protein (V478S, P493S and Y539A). Protein transport to the cell surface was slightly reduced for one of the mutant H proteins, but nevertheless, they all retained wild-type fusion capacity in SLAM-expressing cells. We then produced a recombinant CDV expressing a H protein carrying the respective EpR-blind mutations. All mutant viruses replicated with similar efficacy as the wild-type CDV in SLAM-expressing cells, but poorly in canine and ferret epithelial cells, illustrating their inability to infect epithelial cell. The tropism and pathogenesis of the EpR-blind CDV was finally evaluated in ferrets. Infection with wild-type CDV causes a lethal disease in these animals, and recapitulates the clinical signs of measles in humans. Ferrets infected with the EpR-blind CDV had similar viral loads and experienced a similar extent of immunosuppression as wild-type infected animals. However, they did not show any signs of clinical disease such as rash, fever or weight

loss. More importantly, no virus was detected in the epithelial tissues of the trachea, lungs or bladder.

Taken together, our data from the ferret model supports and strengthens the previous MeV pathogenesis study in macaques by Leonard *et al.* We have shown that the infection of epithelial cells has little or no role in establishing infection and systemic spread, but rather contributes to the development of clinical disease, virus shedding and transmission to a new host. The implication of structurally conserved H protein residues in epithelial cells entry, further suggests that morbilliviruses share a common epithelial receptor. In this perspective, approaches that interfere with the entry into epithelial cells may constitute a novel strategy for controlling the transmission of these highly contagious viruses, and may therefore contribute to the global eradication of MeV.

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Veronika von Messling
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List of Abbreviations

ATCC	American Type Culture Collection
BrdU	5-bromo-2'-deoxyuridine
°C	degree Celsius
CD	cluster of differentiation
cDNA	complementary DNA
CDV	canine distemper virus
CypB	cyclophilin B
DAB	3, 3'-diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
d.p.i.	days post-infection
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EpR	epithelial cell receptor
F	fusion
FBS	fetal bovine serum
FtAEpC	ferret alveolar epithelial cells
GS	gene-start
GE	gene-end
H	hemagglutinin
h	hour
H&E	hematoxylin and eosin
IFN	interferon
IL	interleukin
IJ	intergenic junction
kDa	kilodalton
L	large

M	matrix
MDCK	Madin-Darby canine kidney
MeV	measles virus
min	minutes
mL	milliliter
M.O.I.	multiplicity of infection
mRNA	messenger RNA
N	nucleocapsid
nm	nanometer
nt	nucleotide
P	phosphoprotein
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PHA	phytohemagglutinin
p.i.	post-infection
PFA	paraformaldehyde
RNA	ribonucleic acid
RNP	ribonucleoprotein complex
RPV	rinderpest virus
RT	room temperature
SLAM	signaling lymphocytic activation molecule
S.O.C.	super optimal broth with catabolite repression
$TCID_{50}$	50% tissue culture infectious dose
µg	microgram
µL	microliter
vRdRp	viral RNA-dependent RNA polymerase

CHAPTER 1: Introduction

1.1 The Family *Paramyxoviridae* and the Genus *Morbillivirus*

1.1.1 Taxonomy, Phylogeny and Morphology

Non-segmented, single-stranded negative-sensed RNA viruses are classified in the order *Mononegavirales*. These viruses are organized into four families: *Bornaviridae*, *Filoviridae*, *Paramyxoviridae* and *Rhabdoviridae*. The family *Paramyxoviridae* is further divided into two sub-families: *Pneumovirinae* and *Paramyxovirinae*. The former sub-family has two genera: *Metapneumovirus* and *Pneumovirus*, and the latter has five genera: *Avulavirus*, *Henipavirus*, *Respirovirus*, *Rubulavirus* and *Morbillivirus* (King *et al.*, 2012).

The genus *Morbillivirus* consists of highly contagious pathogens that are notorious for their ability to induce a profound immunosuppression in their respective hosts (Lamb *et al.*, 2007). *Measles virus* (MeV) is the only morbillivirus known to cause disease in humans, whereas the other five members of the *Morbillivirus* genus are animal pathogens, including *Canine distemper virus* (CDV), *Cetacean morbillivirus* (CeMV), *Peste-des-petits-ruminants virus* (PPRV), *Phocine distemper virus* (PDV) and *Rinderpest virus* (RPV). Recently, a new paramyxovirus has been isolated in domestic cats, and sequence alignments of the viral genes suggest that this *Feline morbillivirus* (FmoPV) may belong to the *Morbillivirus* genus as well (Woo *et al.*, 2012). The host range for MeV is limited to humans, which are also the only natural reservoir for this virus. In contrast, CDV can infect a broad range of terrestrial and aquatic animals including dogs, ferrets, badgers, foxes, seals and different felines (Grachev *et al.*, 1989; Appel *et al.*, 1994; Barrett, 1999). Phylogenetic analysis has shown that the recently eradicated bovine morbillivirus, RPV, may be the archevirus of this genus, thereby suggesting that MeV is likely derived from RPV (Fig. 1.1) (Norrby *et al.*, 1985; Barrett, 1999; Sips *et al.*, 2007).

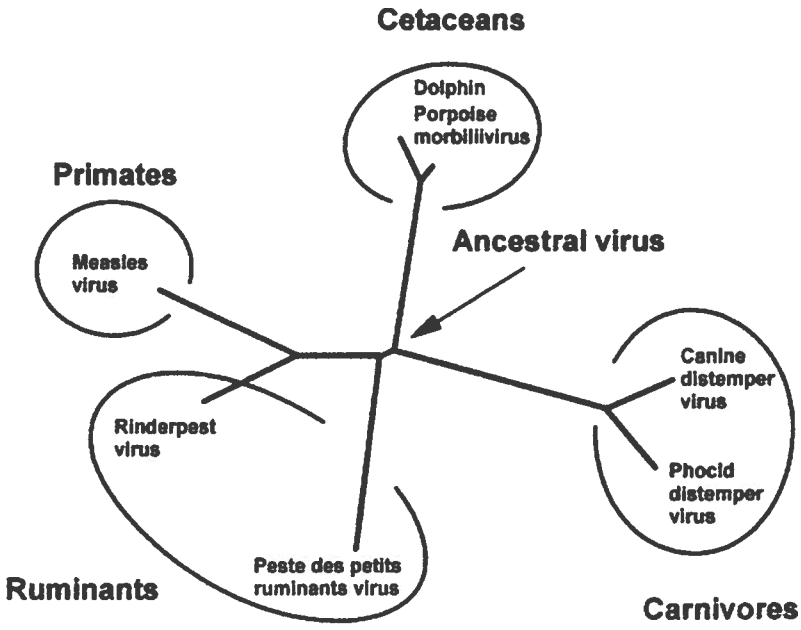

Figure 1.1) Phylogenetic tree illustrating the evolutionary relationship between morbilliviruses based on the sequence alignment of the P genes. The length of the branches corresponds to the mutational distance between the viruses, which diverge from a common morbillivirus ancestor indicated by the arrow. The encircled area represents the target host range of the different species. (Reprinted with permission from Barrett, 1999)

Figure 1.1) Arbre phylogénique représentant les liens de parenté entre les morbillivirus selon l'analyse des séquences du gène P. La longueur des branches est proportionnel à l'écart des mutations acquises entre chaque espèce de virus qui divergent à partir d'un morbillivirus ancestral commun. La région encerclée représente l'hôte ciblé par les espèces de virus. (Tiré de Barrett, 1999)

Morbilliviruses have a lipid envelope derived from the host cell membrane, and are generally spherical or pleomorphic with a diameter of 120-300 nm (Fig. 1.2A) (Lamb *et al.*, 2007). The lipid envelope harbours two transmembrane glycoproteins: the hemagglutinin (H) and fusion (F) proteins that give the appearance of spike-like projections by electron microscopy. The inner side of the viral envelope is coated with the matrix (M) proteins interconnecting the envelope glycoproteins to the nucleocapsid core, which contains the negative-stranded RNA genome encapsidated by nucleoproteins (N). In combination with phospho (P)- and large (L) proteins, which constitute the viral RNA-dependant RNA polymerase (vRdRp), the N protein-encapsidated viral genome forms the ribonucleoprotein complex (RNP).

1.1.2 Genome Organisation and Encoded Proteins

The morbillivirus genome is approximately 16,000 nucleotides (nt) in size, and encodes six non-overlapping, consecutive genes giving rise to eight proteins (Fig. 1.2B) (Griffin, 2007). The genes are organized from 3' to 5' as follow: N, P, M, F, H and L, with the P gene encoding two additional viral proteins, V and C. The 3' and 5' extremities of the genome are flanked by cis-acting elements, known as the leader and trailer sequences, which regulate transcription and replication. Each viral gene begins with a 3' gene-start (GS) sequence that initiates transcription and directs capping of the nascent mRNA, and ends with a 5' gene-end (GE) sequence that encodes a template of four to seven uridine residues for polyadenylation and signals transcription termination (Grdzelishvili *et al.*, 2005). All genes are separated by a three nt long, non-transcribed intergenic junction (IJ) located between the GE and the GS of the following gene. The morbillivirus genome follows the rule of six, which states that the total number of nucleotides must be in a multiple of six (6n+0) to achieve efficient replication (Calain *et al.*, 1993). This requirement is due to the binding of one N protein monomer to exactly six nucleotides, and only the fully N-encapsidated genome, not the naked RNA, functions as a template for replication (Lamb *et al.*, 2007). Viruses with genome lengths that do not obey the rule of six are impaired in replication and are eliminated, or additional nt can be inserted to restore the genome length in a multiple of six (Sidhu *et al.*, 1995).

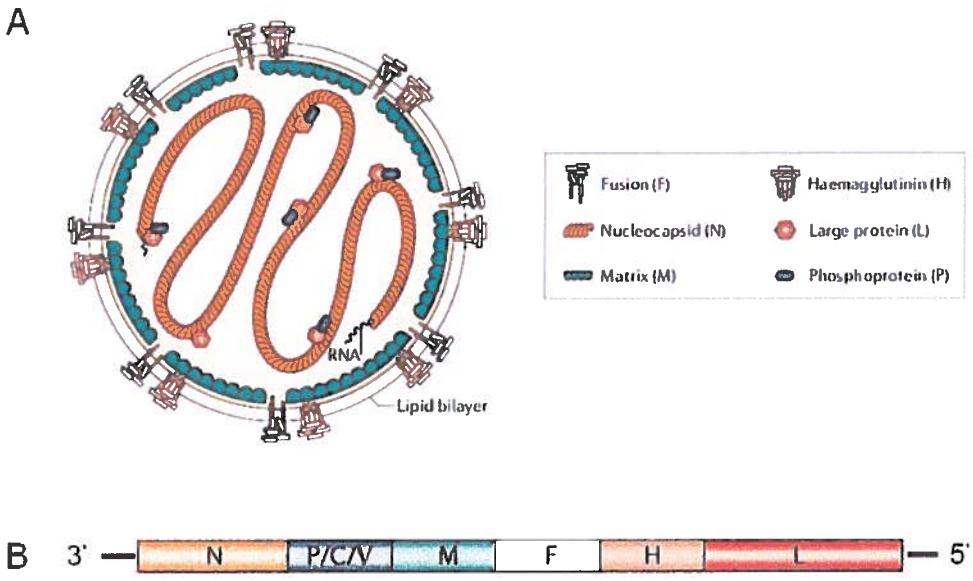

Figure 1.2) Morbillivirus structure and genome organisation. Schematic representation of (**A**) a virion consisting of a nucleocapsid core surrounded by a lipid envelope, and (**B**) the order of the six viral genes in the genome. (Reprinted with permission from Moss *et al.*, 2006)

Figure 1.2) La structure et l'organisation des gènes du morbillivirus. Représentation (**A**) d'une particule virale constituée de la nucléocapside recouverte d'une enveloppe lipidique, et (**B**) l'ordre des six gènes viraux codés dans le génome. (Tiré de Moss *et al.*, 2006)

1.1.3 Overview of Viral Proteins

1.1.3.1 Structural Proteins

Morbilliviruses have six structural proteins grouped into two functional units: N, P and L form the RNP, while M, H, and F are envelope proteins. The N protein is the most abundant and it encapsidates the viral RNA genome into a dense structure with left-handed helical symmetry, which can resist digestion by cellular proteases and nucleases (Liston *et al.*, 1997; Spehner *et al.*, 1997; Karlin *et al.*, 2002). The L protein is the catalytic subunit of the vRdRp, and has intrinsic 5' capping and methylation, and 3' polyadenylation activities (Grdzelishvili *et al.*, 2005). The P protein is the polymerase co-factor, which stabilises the interaction of the L protein with the RNA template (Horikami *et al.*, 1992; Kingston *et al.*, 2004). In addition, it binds to unassembled N protein monomers to mediate the encapsidation of newly synthesized RNA (Huber *et al.*, 1991; Spehner *et al.*, 1997). The M protein forms a paracrystalline array on the inner face of the virion's lipid envelope, and interconnects the cytoplasmic tails of the envelope glycoproteins, H and F, with the N proteins in the RNP (Iwasaki *et al.*, 2009). It thus plays a pivotal role in virus assembly and budding (Liljeroos *et al.*, 2011). Studies using siRNA have shown that the M protein also acts as a negative regulator of transcription and replication (Reuter *et al.*, 2006). The H and F proteins form the envelope glycoprotein complex that mediates virus entry. Their biological properties and functions will be discussed in sections 1.2.1 and 1.2.2.

1.1.3.2 Non-Structural Proteins

In contrast to the structural proteins, V and C are accessory proteins, which are dispensable for virus replication in cell culture, but are essential virulence factors (Takeuchi *et al.*, 2005; von Messling *et al.*, 2006; Devaux *et al.*, 2008). The V and C proteins counteract the host's antiviral defence mechanism by inhibiting type I interferon (IFN α/β) signaling (Tober *et al.*, 1998; Shaffer *et al.*, 2003; Takeuchi *et al.*, 2003). The V protein is produced by mRNA editing, whereby a single non-templated guanine (G)

nucleotide is inserted in the mRNA during transcription of the P gene (Cattaneo *et al.*, 1989). The N-terminus of the V protein is shared with the P protein, whereas its C-terminus forms a unique zinc finger-like cystein-rich domain (Liston *et al.*, 1994). The C protein is also derived from the P mRNA transcript, but translation is initiated at a different start codon giving rise to a smaller basic polypeptide (Bellini *et al.*, 1985). In addition to its role in the modulation of the cellular response to the infection, the C protein also promotes viral replication by modulating the activity of the vRdRp (Bankamp *et al.*, 2005).

1.1.4 Replication Cycle

1.1.4.1 Virus Entry

Morbillivirus entry into its target cell is coordinated by two envelope glycoproteins that each performs distinct functions. The H protein mediates the initial contact with the host cell by binding to a specific receptor molecule at the cell surface, which in turn triggers a series of structural rearrangements of the F protein leading to the fusion of the virus envelope with the plasma membrane (Navaratnarajah *et al.*, 2011). After merging the membranes, the RNP dissociates from the M proteins and is released into the cytoplasm where the replication cycle is initiated (Lamb *et al.*, 2007).

1.1.4.2 Transcription of Viral Genes and Genome Replication

Most of the current knowledge on transcription and replication of negative-stranded RNA viruses is based on studies with vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family as reviewed in Curran *et al.* (2008). RNA synthesis starts from the 3' end of the genome where a unique entry site gives the polymerase access to the buried RNA template (Emerson, 1982). The vRdRp scans the sequence, and initiates transcription at the first GS sequence and ends transcription at the GE sequence, giving rise to a functional mRNA transcript with 5' cap and 3' polyadenylation. After transcribing the first gene, the vRdRp remains attached to the template and travels across

the IJ region to re-starts transcription at the GS sequence of the gene downstream. All the subsequent genes are transcribed using this "start-stop" mechanism (Lamb *et al.*, 2007). However, the efficiency at which the vRdRp restarts transcription decreases as it moves further away from the 3' end of the template. This results in a gradient of transcription, whereby the N gene is the most transcribed and the L gene the least transcribed (Plumet *et al.*, 2005).

As the level of viral proteins increases in the infected cell, unassembled N protein monomers (N_0) interact with the P proteins on the vRdRp to switch its activity from transcription to replication. RNA synthesis now begins from the leader sequence at the extreme 3' end of the template and the nascent RNA chain is immediately encapsidated by N_0 (Blumberg *et al.*, 1981). The attachment of N_0 to this leader RNA modifies the activity of the vRdRp in such a way that all the downstream transcriptional control sequences of the genes are ignored, and a full-length positive-stranded copy, known as antigenome, is produced (Blumberg *et al.*, 1981). These antigenomes are found only in low numbers in the infected cell, and they serve uniquely as a template for the synthesis of the negative-stranded genomic RNA (Lamb *et al.*, 2007). The transcription and replication activities of the vRdRp are thus determined by the availability of N_0 in the infected cell (Plumet *et al.*, 2005; Lamb *et al.*, 2007). When the level is low, such as during the earlier stages of infection, the vRdRp is committed to transcription of the viral genes. As more N_0 becomes available, N_0-P complex enables encapsidation, thereby promoting the synthesis of full-length RNA chains. In morbillivirus-infected cells, nuclear and cytoplasmic inclusion bodies can be detected, which contain primarily N protein-encapsidated RNA (Griffin, 2007).

1.1.4.3 Assembly and Budding

The assembly and budding process is primarily coordinated by the M protein. As it accumulates in the cell, the M protein interacts with multiple proteins partners to transport the structural viral components to the budding sites at the plasma membrane (Runkler *et al.*, 2007). The M protein binds to the N proteins on the RNP for assembly

and regulating RNA synthesis (Runkler *et al.*, 2007; Iwasaki *et al.*, 2009; Liljeroos *et al.*, 2011). The envelope proteins are processed and transported via the host's secretory pathways, reaching the plasma membrane as a homotetrameric H and trimeric F glycoprotein complex (Navaratnarajah *et al.*, 2008). At the plasma membrane, the M protein associates to the inner leaflet of the lipid membrane, and interacts at the same time with the cytoplasmic tails of the envelope glycoproteins and the nucleoprotein core (Runkler *et al.*, 2007; Tahara *et al.*, 2007b). Once the viral components are assembled, the lipid bilayer is pinched off and infectious viral particles are released (Lamb *et al.*, 2007).

1.2 Envelope Glycoproteins

1.2.1 The Attachment Hemagglutinin (H) Protein

The morbillivirus H protein mediates receptor-binding to the target cell, which is a key determinant for viral tropism and pathogenesis (Tatsuo *et al.*, 2000a; von Messling *et al.*, 2001). H is a type II transmembrane glycoprotein (Fig. 1.3A). The extracellular domain is divided into a globular head domain and a stalk region, followed by a transmembrane domain and the N-terminus cytoplasmic tail. According to the MeV H protein crystal structure, the globular head domain consists of six β-propeller blades (B1-B6) arranged around a central axis, and each blade is composed of four anti-parallel β-strands (Fig. 1.3C) (Colf *et al.*, 2007; Hashiguchi *et al.*, 2007). The H protein undergoes several post-translational modifications, including N-glycosylation and the formation of disulfide bonds via two cysteine residues in the stalk domain to generate H homodimers (Plemper *et al.*, 2000). The N-glycan chains mask a large surface area of the globular head domain exposing only a limited region for receptor or antibody binding (Hashiguchi *et al.*, 2007). H homodimers may oligomerize into homotetramers via intermolecular bonds as they mature through the secretory pathway or upon binding with a receptor at the cell surface (Brindley *et al.*, 2010; Hashiguchi *et al.*, 2011). The mature H protein also exhibits a tilt towards the horizontal axis when oligomerized (Fig. 1.3B) (Hashiguchi *et al.*, 2007).

In contrast to other paramyxoviruses, the morbillivirus H protein lacks neuraminidase activity and interacts with cell surface proteins rather than sugar moieties (e.g. sialic acid). Three cellular receptors have so far been identified for MeV H: SLAM, CD46 and nectin-4, and which will be discussed in detail in section 1.3.

The receptor-binding sites on the MeV H protein for SLAM, CD46 and nectin-4 are all clustered to a recessed groove defined by blades 4 and 5 (B4 and B5) located on the lateral side of the β-propeller, and each cellular receptor binds to specific amino acids within that area of the H protein (Fig 1.4) (Masse *et al.*, 2004; Vongpunsawad *et al.*, 2004; Leonard *et al.*, 2008; Tahara *et al.*, 2008). The structural conformation of this groove is distinct from the corresponding region in the attachment protein of other paramyxoviruses, which may explain the ability of the morbillivirus H protein to recognize specific protein receptors (Leonard *et al.*, 2008; Santiago *et al.*, 2010; Hashiguchi *et al.*, 2011).

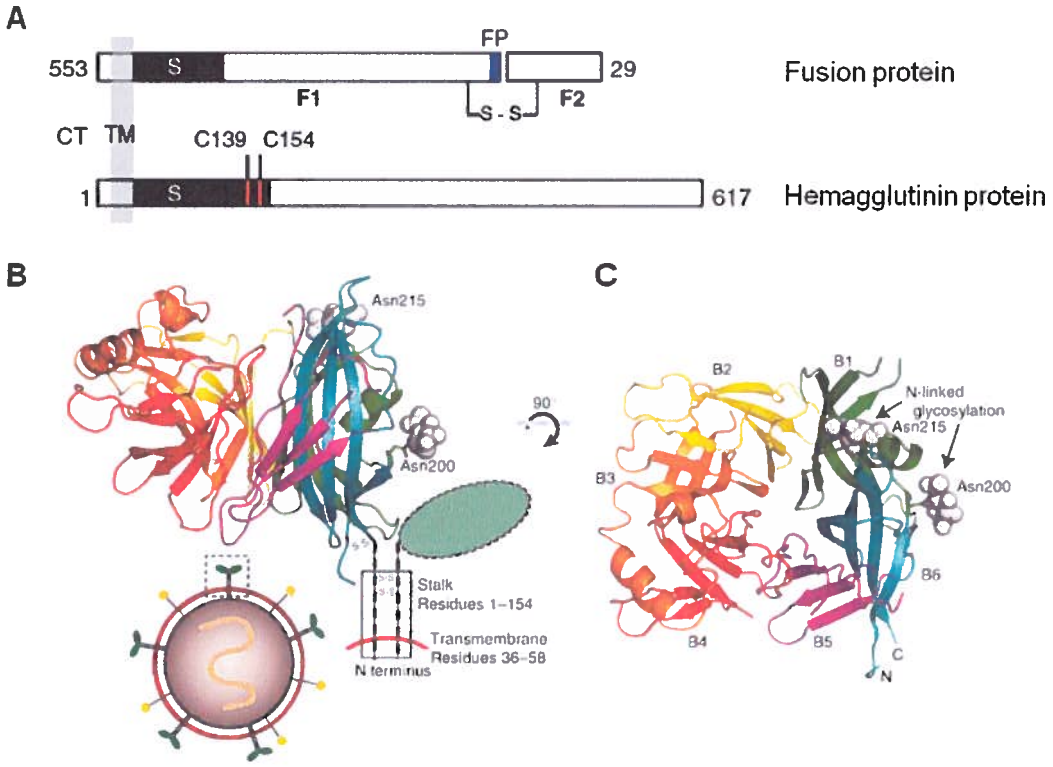

Figure 1.3) Morbillivirus envelope glycoproteins. (A) Schematic representation of the domains in the MeV F and H proteins, and **(B)** structural model of the homodimeric MeV H protein at the surface of a virion. The ribbon diagram illustrates the three-dimensional structure of the globular head domain with the predicted N-glycans represented by grey spheres. **(C)** A 90° rotation along the horizontal axis gives a view of the globular head domain from above revealing a six-bladded (B1-B6) β-propeller fold. (Reprinted with permissions from Navaratnarajah *et al.*, 2012 and Colf *et al.*, 2007)

Figure 1.3) Glycoprotéines de l'enveloppe morbillivirale. (A) Schéma représentant les domaines des protéines F et H, et **(B)** un modèle structural de la protéine H homodimérique du MeV à la surface d'un virion. La structure tridimensionnelle du domaine extracellulaire est représentée par ce diagramme en ruban, et les chaînes N-glycan sont illustrées par les sphères grises. **(C)** Une rotation de 90° sur l'axe horizontale donne une vue de dessus du domaine extracellulaire de la protéine H, d'où on perçoit la forme d'une hélice composée de six ailes béta (B1-B6). (Tiré de Navaratnarajah *et al.* 2012 et Colf *et al.*, 2007)

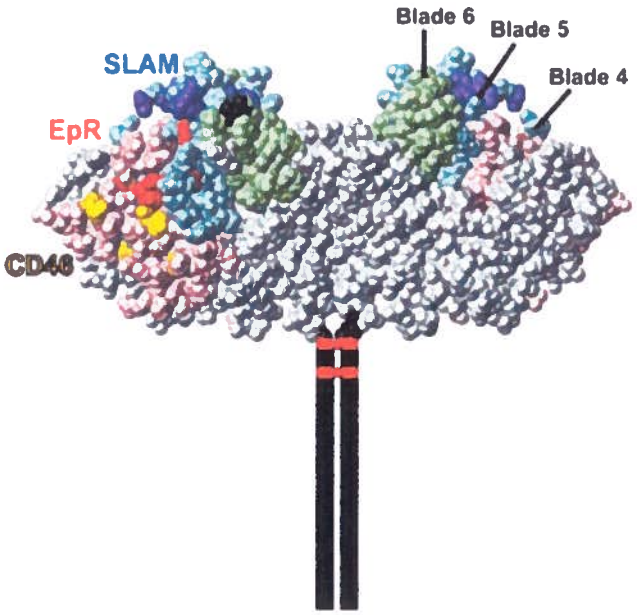

Figure 1.4) Mapping of the receptor-binding sites on the dimeric globular head domain of the MeV H protein. Two disulfide bonds are shown by the red horizontal bars, and the stalks are represented by vertical lines. The surface residues of β-propeller blades 4, 5, and 6 are shaded in pink, blue, and green, respectively. The specific amino acid residues that interact with SLAM, nectin-4 (EpR) and CD46 are coloured in dark purple, red and yellow, respectively. All these residues cluster to a recessive groove formed by β-propeller blades 4 and 5, located on the side of the globular head domain of the H protein. (Reprinted with permission from Cattaneo, 2010)

Figure 1.4) Cartographie des sites de liaisons des récepteurs cellulaires sur la protéine H du MeV. Le domaine de la tête globulaire en dimère est représenté dans ce modèle moléculaire trois-dimensionnel. Les ponts disulfures sont illustrés par les barres horizontales en rouges, et les domaines de la tige sont représentés par les lignes verticales. Les hélices béta 4, 5, et 6 sont indiquées en rose, bleu et vert, respectivement, et les résidus d'acide aminés interagissant avec SLAM, nectin-4 (EpR) ou CD46 sont illustrés en violets, rouges et jaunes, respectivement. Tous ces résidus se regroupent à l'intérieure d'une région formée par les hélices béta 4 et 5 sur le coté latéral de la protéine H. (Tiré de Cattaneo, 2010)

1.2.2 The Fusion (F) Protein

The F protein interacts with the H protein and mediates the fusion of the virus envelope with target cell plasma membrane in a pH-independent manner (Lamb *et al.*, 2007). F is a type I transmembrane glycoprotein (Fig 1.3A), which is translated as an inactive precursor, glycosylated and assembled into a homotrimer, F_0, in the endoplasmic reticulum (Lamb *et al.*, 2007). F_0 is then activated in the trans-golgi network by a ubiquitous intracellular protease, furin, which cleaves F_0 into the mature disulfide-linked F_1 and F_2 complex (M. Watanabe *et al.*, 1995). The F_1 subunit is anchored to the cell membrane, and has a hydrophobic stretch of amino acids representing the fusion peptide (FP) at its N-terminus and followed by two conserved hydrophobic heptad repeats (HRA and HRB), which are essential to its fusion activity (Buckland *et al.*, 1992). The smaller F_2 subunit is linked to F_1 by the disulfide bond, and harbours three N-glycans for protein processing and transport to the cell surface (Lamb, 1993; Hu *et al.*, 1995).

Membrane fusion is an irreversible process. Initially in a metastable conformation, F undergoes several conformational changes upon binding of H to its cellular receptor to expose the FP (Navaratnarajah *et al.*, 2011). The FP is then inserted into the opposing membrane, and the F protein finally adopts a stable post-fusion conformation, resulting in formation of the fusion pore and subsequent membrane fusion (Navaratnarajah *et al.*, 2011). In cell culture, co-expression of H and F proteins in the presence of a cellular receptor leads to cell-to-cell fusion, and the formation of giant multinucleated cells, known as syncytia (Lamb *et al.*, 2007).

1.3 Morbillivirus Cellular Receptors

1.3.1 SLAM (CD150)

The signaling lymphocyte activation molecule (SLAM or CD150) is used by all morbilliviruses to infect immune cells (Griffin, 2007). SLAM was originally identified as the receptor for MeV, and further studies have shown that CDV, RPV, and other morbilliviruses also use SLAM proteins of their respective host species (Tatsuo *et al.*, 2001; Baron, 2005). This receptor was identified by functional expression cloning, whereby a non-susceptible cell line was transfected with a cDNA library of clones expressing surface molecules from a MeV-susceptible lymphoid cell line, B95a. The clone expressing SLAM was the only one that conferred virus entry, replication and formation of syncytia for both wild-type and vaccine MeV (Tatsuo *et al.*, 2000b). The molecule SLAM belongs to the superfamily of immunoglobulin expressed on immature thymocytes, activated T- and B-lymphocytes, mature dendritic cells (DCs), macrophages and platelets. SLAM has two extracellular domains, V and C2, and a cytoplasmic tail that interacts with SLAM-associated proteins (SAP) and EWS/FLI1 activated transcript 2 (EAT2) for signal transduction (Veillette *et al.*, 2006).

The key residues on the MeV H protein that mediate either direct contact or maintain structural conformations for SLAM-binding are: I194, D505, D507, D530, R533, F552, and P554 (Vongpunsawad *et al.*, 2004; Hashiguchi *et al.*, 2007). All these residues clusters to a common receptors-binding region located on the side of the β-propeller as described above (Fig. 1.4). Binding sites for canine SLAM were also mapped on the corresponding structural interface of CDV H protein at positions D526, T527, S528, R529, Y547, F548 and R552 (von Messling *et al.*, 2005). Mutation at multiple residues on CDV H was required to completely abolish SLAM-dependent cell entry, whereas introducing a single mutation at the MeV H residues above was sufficient to generate a SLAM-blind phenotype, which is a H protein that is unable to bind to SLAM (Vongpunsawad *et al.*, 2004; von Messling *et al.*, 2005).

1.3.2 CD46

In addition to SLAM, the molecule CD46 also serves as an entry receptor, but only for the vaccine and laboratory-adapted strains of MeV (Dorig *et al.*, 1993; Naniche *et al.*, 1993a; Erlenhofer *et al.*, 2002). Although CD46 was the first receptor identified for MeV, its role as an entry receptor was not fully established yet, because unlike the vaccine strains, most clinical isolates from measles patients were unable to grow efficiently in $CD46^+$ cell lines (Kobune *et al.*, 1990; Tatsuo *et al.*, 2000b; Ono *et al.*, 2001). In addition, MeV strains propagated in $SLAM^+$ lymphoid cell lines were shown to retain their virulence in experimentally infected monkeys, whereas those propagated in a $SLAM^-/CD46^+$ Vero cell line accumulated attenuating mutations (Enders *et al.*, 1960; van Binnendijk *et al.*, 1994; Auwaerter *et al.*, 1999; Manchester *et al.*, 2000). These findings suggested that wild-type and virulent MeV strains preferentially uses SLAM and not CD46 as receptor *in vivo*. CD46, or membrane cofactor protein (MCP), is ubiquitously expressed on all nucleated human and monkey cells. It is a type I glycoprotein with an extracellular domain composed of four short consensus repeats (SCR 1-4), followed by a transmembrane region and a cytoplasmic tail. CD46 is a regulator of complement activation, and vaccine MeV H-CD46 binding leads to a downregulation of the latter from the cell surface (Seya *et al.*, 1989; Naniche *et al.*, 1993b; Bartz *et al.*, 1996; Galbraith *et al.*, 1998). A recent structural model of CD46 complexed with MeV H shows that SCR 1 and 2 on the membrane-distal domain of CD46 mediate contact with the H globular head domain (Devaux *et al.*, 1996; Santiago *et al.*, 2010).

Adaptation to CD46 can occur when a wild-type MeV is propagated in $SLAM^-/CD46^+$ Vero cells, whereby the H protein acquires several mutations that enable the interaction with CD46 (Buckland *et al.*, 1997). The most prominent mutations that confer CD46-binding are substitutions from asparagine to tyrosine at position 481 (N481Y), or serine to glycine at 564 (S564G) (Nielsen *et al.*, 2001; Li *et al.*, 2002). Additional mutations that strengthen the binding affinity to CD46 are: N390I, N416D, T446S, T484N and E492G (Fig. 1.4) (Seki *et al.*, 2006; Tahara *et al.*, 2007a; Santiago *et al.*, 2010). Hemagglutination is the result of H binding to CD46 expressed on monkey

erythrocytes, and it is a characteristic property of vaccine and laboratory-adapted MeV strains only (Buckland *et al.*, 1997).

1.3.3 Nectin-4

Nectin-4 (also known as poliovirus-like receptor 4; PVLR-4) is the epithelial cell entry receptor for MeV. Two independent research groups recently identified nectin-4 by a large scale screening of surface molecules expressed by MeV-permissive human pulmonary epithelial cell lines and breast adenocarcinoma (Mühlebach *et al.*, 2011; Noyce *et al.*, 2011). Nectin-4 belongs to the superfamily of immunoglobulin-like molecules, which form cellular adhesions at the basolateral side of polarized epithelial cells (Reymond *et al.*, 2001). It is a type I transmembrane protein, composed of three extracellular immunoglobulin domains (V, C and C), a transmembrane region and a cytoplasmic tail. Nectin-4 mRNAs are mainly detected in human placenta, and also found at lower levels in the trachea, skin, lungs, prostate and stomach (Brancati *et al.*, 2010). The over-expression of nectin-4 is often associated with breast, lung and ovarian cancers, which makes it a potential biomarker for tumorgenesis (Fabre-Lafay *et al.*, 2007; Takano *et al.*, 2009; Derycke *et al.*, 2010). In addition, real-time PCR and immunohistology analysis show a correlation between the level of N and nectin-4 in the trachea and lungs tissues of cynomologus macaque infected with wild-type MeV (Mühlebach *et al.*, 2011). The residues on the MeV H protein that supports virus entry in well-differentiated epithelial cells are located at positions L482, F483, P497, Y541 and Y543, which are all located within the same receptor-binding region as described above for SLAM and CD46 (Fig. 1.4) (Leonard *et al.*, 2008; Tahara *et al.*, 2008; Takeda, 2008).

1.3.4 Other Receptors

Two C-type lectins: dendritic cell-specific intercellular adhesion molecule 3-grabbing non-intergrin (DC-SIGN) and Langerin have been shown to mediate attachment but not entry into dendritic cells (de Witte *et al.*, 2006; van der Vlist *et al.*, 2011). Both molecules interact with N-glycans on the wild-type and vaccine MeV H glycoproteins,

but this interaction alone does not trigger membrane fusion. Binding to DC-SIGN can enhance virus entry into DCs possibly by reducing the distance between the viral and host membranes, thereby facilitating subsequent SLAM-binding. Alternatively, the DCs may capture infectious MeV particles using DC-SIGN or Langerins and then migrate to the local lymph node to transmit the virus to T-lymphocytes (van der Vlist *et al.*, 2011).

CD147 (or EMMPRIN) has also been proposed by Watanabe *et al.* to be an entry receptor in epithelial and neuronal cells *in vitro* (2010). Virus entry via CD147 is mediated by cyclophilin B (CypB), which is a cellular protein embedded in viral envelope (A. Watanabe *et al.*, 2010). Binding of CD147 to CypB triggers membrane fusion by a mechanism that is independent of the H glycoprotein. CD147 is a transmembrane glycoprotein expressed on various human cell types, including epithelial and neuronal cells (Yurchenko *et al.*, 2001). CypB is believed to be incorporated into the envelope of new MeV virions through interaction with the N protein (A. Watanabe *et al.*, 2010). This study provides evidence that CD147-CypB acts as a functional receptor for MeV entry, but its role *in vivo* remains to be determined.

1.4 Morbillivirus Pathogenesis

1.4.1 Infection Cycle

Morbilliviruses are highly contagious pathogens that are transmitted via aerosol droplets (Griffin, 2007). As illustrated by this model of MeV infection in humans, an infectious particle comes in contact with the host via the upper respiratory tract, and targets DCs and alveolar macrophages in the mucosa (Fig. 1.5A) (de Swart *et al.*, 2007; Lemon *et al.*, 2011). After crossing the respiratory epithelium, the infected cells migrate to the regional lymph nodes, where the virus establishes its initial site of replication by infecting SLAM-expressing activated B- and T-lymphocytes (de Swart *et al.*, 2007; de Vries *et al.*, 2010). These MeV-infected lymphocytes enter the blood stream, spreading the virus throughout the lymphatic system including lymph nodes, spleen, thymus and the Peyer's patches (Fig.1.5B) (de Swart *et al.*, 2007; de Vries *et al.*, 2010). The massive

virus replication in these lymphoid tissues causes a severe immunosuppression, defined by a reduced number of leukocytes and an inhibition of lymphocyte proliferation, which renders the host susceptible to secondary bacterial and viral infections (Schneider-Schaulies *et al.*, 2009). As the viral load reaches its peak, MeV spreads throughout the body by infecting endothelial and epithelial cells in the gastrointestinal tract, liver, kidney, bladder, skin and lungs (Fig. 1.5C) (de Swart *et al.*, 2007; de Vries *et al.*, 2010; Ludlow *et al.*, 2010). The apparition of the clinical signs coincides with the beginning of host's immune response to the viral replication. This phase is characterized by fever, cough, coryza, conjunctivitis, gastrointestinal signs and maculopapular rash (de Swart *et al.*, 2007; de Vries *et al.*, 2010). Infected lymphocytes reach the sub-mucosal tissues in the respiratory tract, and transmit the virus to the respiratory epithelium where viral replication is sustained in epithelial cells (de Vries *et al.*, 2010; Ludlow *et al.*, 2010). New infectious particles are shed from the apical side of the respiratory epithelium (Leonard *et al.*, 2008). The infected individuals cough or sneeze, releasing infectious particles as secretions or aerosol, and transmit the virus to a new susceptible host (Fig. 1.5D).

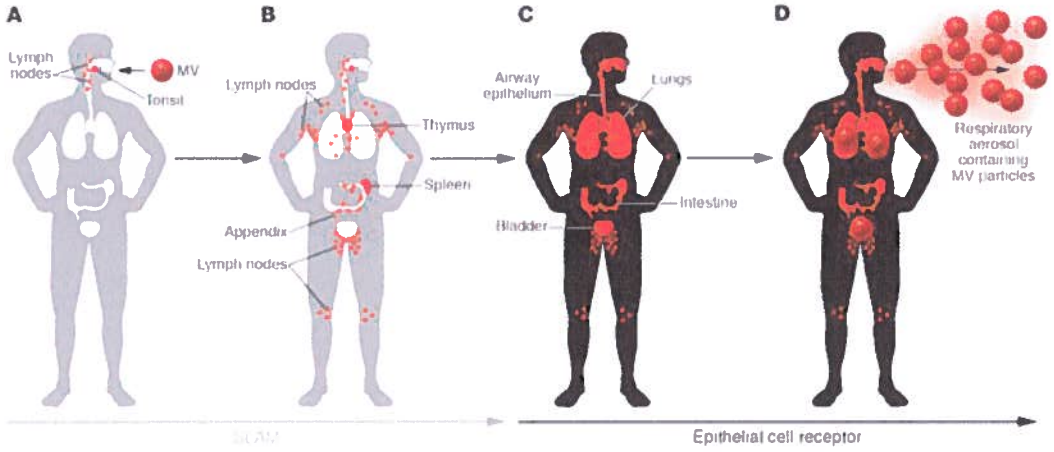

Figure 1.5) Morbillivirus pathogenesis and receptor usage. **(A, B)** Graphic of an individual contracting MeV via the respiratory tract, and subsequent spread of the virus through lymphoid organs by interacting with the cellular receptor SLAM. **(C, D)** As the viral load reaches its peak, MeV disseminates to epithelial cells in various organs via the epithelial receptor, and new infectious particles are released into the environment. (Reprinted with permission from Takeda, 2008)

Figure 1.5) La pathogenèse morbillivirale et l'utilisation des récepteurs. **(A, B)** Un graphique illustrant un individu qui contracte MeV par la voie respiratoire, suivi pas la dissémination du virus dans les organes lymphoïdes à l'aide du récepteur cellulaire SLAM. **(C, D)** Lorsque la charge virale atteint son point culminant, MeV se diffuse aux cellules épithéliales dans les divers organes en interagissant avec le récepteur épithélial, et des nouvelles particules infectieuses sont libérées dans l'environnement. (Tiré de Takeda, 2008)

1.5 Animal Models for Studying Morbillivirus Pathogenesis

Macaques are the most appropriate model for studying MeV pathogenesis. Rhesus and cynomolgus macaques (*Macaca mulatta* and *Macaca fascicularis*, respectively) are Old World monkeys that are naturally susceptible to infection with wild-type MeV. They develop a mild disease that reproduces the full spectrum of clinical signs of MeV infection in humans (Blake *et al.*, 1921a; El Mubarak *et al.*, 2007; de Vries *et al.*, 2010). Animals that recover from the acute infection develop a strong humoral and cellular immunity specific to MeV, and are protected from subsequent re-infections (Blake *et al.*, 1921b). Macaques are extensively used in pre-clinical studies for investigating the interference of maternal antibodies with MeV vaccination, testing alternative routes of vaccine administration, and for the development of a new generation of vaccines (van Binnendijk *et al.*, 1997; Stittelaar *et al.*, 2002). New World monkeys, such as the squirrel monkeys (*Saimiri sciureus*) and marmosets (*Saguinus mystax*), have a higher sensitivity to MeV compared to macaques, and develop a more severe and often lethal disease (van Binnendijk *et al.*, 1995). Since marmosets develop encephalitis upon intracranial inoculation of MeV, they are sometimes used for neurovirulence studies, and the development of MeV as an oncolytic agent has led to an increasing use of squirrel monkeys as a pre-clinical model to evaluate biodistribution and neurotoxicity (Albrecht *et al.*, 1981; Myers *et al.*, 2007; Russell *et al.*, 2009).

In addition to primates, different rodent models have been developed. Cotton rats (*Sigmodon hispidus*) are naturally susceptible to MeV wild-type and vaccine strains (Wyde *et al.*, 1999). However, MeV-infected cotton rats do not reproduce the same pathology observed in humans, and the virus replication is restricted to the respiratory tract with limited replication in lymphoid tissues (Tober *et al.*, 1998; Pfeuffer *et al.*, 2003). Nevertheless, studies in these animals have contributed to our understanding of the role of the MeV V protein in viral replication and virus-induced immunosuppression (Niewiesk *et al.*, 1997; Tober *et al.*, 1998). Mice have been genetically engineered to express the entry receptors for MeV, human SLAM and CD46, making them susceptible to infection. Transgenic mouse lines expressing either one or both receptors with

ubiquitous or cell-type specific expression pattern are available (Rall *et al.*, 1997; Lawrence *et al.*, 1999; Hahm *et al.*, 2003). CD46-transgenic mice are often used to study neurological disease, since intracranial inoculation of young mice with a laboratory strain produces infectious particles and induces severe encephalitis (Rall *et al.*, 1997; Lawrence *et al.*, 1999). The permissiveness of SLAM-transgenic mice has been further modified to increase susceptibility and permissiveness by cross-breeding with a line that is defective in type I IFN response. These animals are able to reproduce the characteristic lymphotropism and immunosuppression associated with MeV infection in natural hosts (Mrkic *et al.*, 1998; Ohno *et al.*, 2007).

CDV, a close relative of MeV, naturally infects diverse species in the order *Carnivora*, and dogs and ferrets are the most common animal models used to study morbillivirus pathogenesis. CDV infection in dogs leads to canine distemper, which reproduces a similar spectrum of clinical and neurological signs as acute measles in humans (Appel, 1969). However, the severity and course of disease are highly variable in these animals (Appel, 1969; Summers *et al.*, 1984).

Domestic ferrets (*Mustela putorius furo*) are more sensitive to wild-type CDV infections than dogs, and also develop all the clinical signs of measles, including rash around the mouth, chin and abdomen (Fig. 1.6). All ferrets infected with wild-type strains die within 2 weeks, often due to septicemia (von Messling *et al.*, 2003; von Messling *et al.*, 2004). In addition, neurotropic CDV strains lead to dissemination to the central nervous system, and cause neurological signs characteristic of acute encephalitis and meningitis (Rudd *et al.*, 2006; Bonami *et al.*, 2007; Ludlow *et al.*, 2012). The lack of ferret-specific reagents for the characterization of the immune responses remains one of the major disadvantages of this animal model. However, in addition to the antibodies targeting ferret immunoglobulin chains and pro-inflammatory cytokines, there are increasing number of commercial antibodies against a variety immunological markers that can cross-react with ferret epitopes, as presented in the review by Pillet *et al.* (2009; Rudd *et al.*, 2010). The genomic sequences of ferret inflammatory cytokines and

chemokines can be obtained from Genbank, and the sequencing of the ferret genome is currently in progress (Svitek *et al.*, 2007; Danesh *et al.*, 2008; 2011-2012).

The generation of enhanced green fluorescent protein (EGFP)-expressing wild-type MeV and CDV has contributed importantly to the characterization of morbillivirus pathogenesis (Duprex *et al.*, 1999; Hashimoto *et al.*, 2002). Viral dissemination can be followed over the course of an infection, with green fluorescence being detected in lymphoid tissues, digestive and respiratory tracts and the skin of the animals (Duprex *et al.*, 2002; von Messling *et al.*, 2003; von Messling *et al.*, 2004; de Vries *et al.*, 2010).

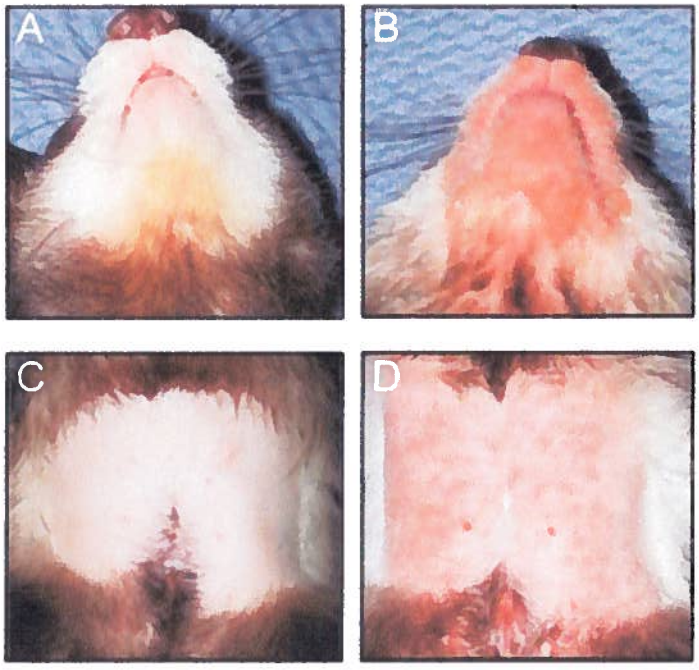

Figure 1.6) Characterization of morbillivirus pathogenesis using ferrets (*Mustela putorius furo*) as an animal model. Photographs of ferrets sacrificed at day 12 post-infection with a wild type (**B, D**) or attenuated (**A, C**) recombinant CDV strain. The skin rash appears, which appears over the chin, mouth and abdomen of the animal infected with wild-type CDV, is one of the hallmarks of morbillivirus infections. (Reprinted with permission from von Messling *et al.*, 2003)

Figure 1.6) Caractérisation de la pathogenèse morbillivirale à l'aide du furet (*Mustela putorius furo*) comme modèle animal. Photographies des furets sacrifiés au jour 12 post-infection qui sont infectés par une souche sauvage (**B, D**) ou atténuée (**A, C**) du CDV recombinant. Les éruptions cutanées apparaissant sur le menton, la bouche et l'abdomen de l'animal infecté par le virus du type sauvage sont des traits caractéristiques d'une infection par les morbillivirus. (Tiré de von Messling *et al.*, 2003)

1.6 The Role of Epithelial Cell Infection in Morbillivirus Pathogenesis

Although SLAM-expressing lymphocytes are the first targets during morbillivirus infection, histopathoglogical analysis of epithelial tissues from measles patients and experimentally-infected macaques indicates an important contribution of epithelial cell infection to pathogenesis (de Vries *et al.*, 2010; Moss *et al.*, 2012). However, the mechanisms by which morbillivirus cross the respiratory epithelium and the cell types involved in this process remained to be characterized.

As most respiratory viruses, morbilliviruses enter the host via the upper respiratory tract, but studies have shown that the earliest target cells are $SLAM^{+}$ DCs and alveolar macrophages in the mucosal surface, indicating that the virus crosses the respiratory epithelium without an initial round of replication in epithelial cells (Lemon *et al.*, 2011). Using an EGFP-expressing MeV, de Vries *et al.* have shown that the epithelial cells in the respiratory epithelium of rhesus macaques are infected only after the onset of viremia, and not during the earlier stage of infection (2010). Furthermore, Leonard *et al.* generated a recombinant virus that carries mutations in the H protein conferring an epithelial cell receptor (EpR)-blind phenotype (2008). Rhesus macaques infected with this EpR-blind MeV were immunosuppressed, but no virus was detected in the broncho-aveolar lavage samples from the infected animals, suggesting that epithelial cell infection is required for virus shedding (Leonard *et al.*, 2008). Wild-type MeV efficiently infects well-differentiated columinar epithelial cells via the basolateral side, where nectin-4 is expressed, and new virions are only detected on the apical side (Leonard *et al.*, 2008; Ludlow *et al.*, 2010). Based on these recent findings, an adjusted model for MeV pathogenesis has been proposed, with the replication in epithelial cells being required during virus egress and transmission, rather than during the initial infection steps.

1.7 Hypothesis and Research Objectives

Since the introduction of a live attenuated MeV vaccine in the 1960's and the implementation of a universal immunization program by the World Health Organization (WHO), MeV-associated morbidity and mortality rates have dramatically declined (Moss *et al.*, 2012). However, MeV remains the leading cause of vaccine-preventable deaths in children under 5 years old in the developing countries due to malnourishment, weak immune systems, and the lack of logistical and financial resources (WHO, 2011). Finding ways to reduce the transmissibility of this highly contagious virus will thus contribute to the on-going efforts to reduce and possibly eradiate measles across the globe.

Currently, the most comprehensive model of morbillivirus pathogenesis is based on MeV infection of non-human primates, rhesus and cynomolgus macaques, as summarized above. However, MeV generally induces a mild and short-lived disease in these animals, and the viral replication is mostly limited to immune tissues. It is thus difficult to assess the role of epithelial cell infection for pathogenesis in this animal model. In contrast, CDV causes a severe and lethal disease in ferrets with massive infection of the epithelia. These animals are highly susceptible to CDV and succumb to the disease within 2-5 weeks post-infection. In addition, EGFP-expressing CDV strains are available, allowing direct assessment of viral spread during the course of an infection.

The goal of this research project was to characterize the CDV H protein residues that interact with the canine epithelial receptor, and to determine the contribution of epithelial cell infection to CDV pathogenesis in ferrets. Given the conserved usage of the receptor SLAM among morbilliviruses, we hypothesized that the mechanism of interaction with the epithelial receptor is also conserved between MeV and CDV, and that the same H protein region is involved in this interaction. To test our hypothesis, we established three objectives:

1. Identify H protein residues involved in epithelial cell entry;
2. Generate an EpR-blind CDV;
3. Characterize the pathogenesis of the EpR-blind CDV in ferrets.

CHAPTER 2: Publication

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Canine Distemper Virus Epithelial Cell Infection is Required for Clinical Disease but not for Immunosuppression

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Text: 3,053 words

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Contribution

I contributed to the *in vitro* and *in vivo* experiments of this research project, including the generation and characterisation of the recombinant CDVs, the animal work, the immunohistochemical and immunofluorescence stainings and the preparation of the figures in this manuscript.

Abstract

To characterize the importance of infection of epithelial cells for morbillivirus pathogenesis, we took advantage of the severe disease caused by canine distemper virus (CDV) in ferrets. To obtain a CDV unable to enter epithelial cells but retaining the ability to enter immune cells, we transferred to its attachment (H) protein two mutations shown to interfere with the interaction of measles virus H with its epithelial receptor, human nectin-4. As expected for an epithelial receptor (EpR)-blind CDV, this virus infected dog and ferret epithelial cells inefficiently, and did not cause cell fusion or syncytia formation. On the other hand, the EpR-blind CDV replicated in cells expressing canine signaling lymphocyte activation molecule (SLAM), the morbillivirus immune cell receptor, with similar kinetics as wild type CDV. While ferrets infected with wild type CDV died within 12 days after infection after developing severe rash and fever, animals infected with the EpR-blind virus showed no clinical signs of disease. Nevertheless, both viruses spread rapidly and efficiently in immune cells, causing similar levels of leukopenia and inhibition of lymphocyte proliferation activity, two indicators of morbillivirus immunosuppression. Infection was documented in airway epithelia of ferrets infected with wild type CDV, but not in animals infected with the EpR-blind virus, and only animals infected with wild type CDV shed virus. Thus epithelial cell infection is necessary for clinical disease and efficient virus shedding but not immunosuppression.

Introduction

Morbilliviruses are highly contagious and can cause severe disease in their respective hosts. *Measles virus* (MeV), which infects humans and certain non-human primates, is generally associated with mild to moderate clinical signs (5), while the closely related *canine distemper virus* (CDV) infects a broad range of carnivores and causes severe and frequently lethal disease (2). Upon aerosol transmission, these viruses initially target signaling lymphocyte activation molecule (SLAM; CD150)-expressing immune cells in the respiratory tract (6, 11), followed by dissemination throughout the lymphatic system (4, 32). SLAM proteins from different host species serve as receptors for multiple morbilliviruses (31), and the amino acid residues involved in the interaction of the MeV and CDV attachment (H) proteins with SLAM are structurally conserved (33, 37). The importance of immune cell targeting for the establishment of morbillivirus infection is illustrated by the complete attenuation of SLAM binding-defective MeV and CDV *in vivo* despite their ability to infect and replicate in non-immune cells as efficiently as wild type viruses *in vitro* (12, 35).

In contrast, the role of epithelial cell tropism in morbillivirus pathogenesis is less clear. Infection of epithelia coincides with the development of the characteristic rash and respiratory and gastrointestinal signs of disease, and the extent of dissemination correlates with severity of clinical signs (15, 32). MeV H protein residues critical for infection of epithelial cells have been previously identified (13, 29), and these residues are important for binding to nectin-4, the recently identified MeV epithelial receptor (17, 19). Pathogenesis of a nectin4-blind MeV in Rhesus macaques was similar to the parental wild type strain, although no virus shedding into the airways was observed (13). However, since MeV-induced disease in monkeys was generally mild and short-lived, it was difficult to assess how relevant infection of epithelial tissues is for disease and pathogenesis.

Because of the more severe disease caused by CDV in ferrets, this animal model is ideal to characterize the morbillivirus pathogenesis mechanisms (24). A recent study demonstrated that several of the amino acids that are involved in nectin4-binding of MeV H are also important for CDV epithelial cell entry (10, 13), indicating that, similar to

immune cell entry via SLAM, the mechanism of epithelial cell infection may be conserved among morbilliviruses. We therefore transferred mutations conferring the nectin4-blind phenotype to MeV H into the H protein of a lethal CDV strain. After *in vitro* characterization of the H proteins carrying mutant H proteins, we generated a candidate epithelial receptor (EpR)-blind virus and confirmed that it was unable to infect canine and ferret epithelial cells. The pathogenesis and tropism of this virus was then characterized in ferrets.

Materials and methods

Cells and transfections. Vero cells constitutively expressing canine SLAM (VerodogSLAMtag) (34), 293 (ATCC #CRL-1573), Madin-Darby canine kidney (MDCK; ATCC #CCL-34), and ferret alveolar epithelial cells (FtAEpCs; 9) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS). For the biochemical analysis of recombinant proteins, 12-well plates of VerodogSLAMtag cells were transfected at approximately 90% confluency as described previously (26, 27). Briefly, each well was transfected with 2 μg each of the H expression pCG-H5804Pzeo (33) or the respective mutant H plasmids, and a plasmid expressing either the MeV Edmonston (3) or CDV 5804P F protein using Turbofect (Fermentas). Fusion activity was evaluated after 24 h. Alternatively, cells were lysed in 200 μL of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Complete; Roche) 16 h after transfection. The lysate was centrifuged at 14,000 rpm for 15 min at 4°C and stored at -20°C.

Production of EpR/nectin4-blind CDV and MeV H proteins. To generate EpR-blind CDV H proteins, site-directed mutagenesis was performed on the plasmid pCG-H5804PZeo (33) to introduce the mutations V478S, P493S, and Y539A, as well as the double mutation P493S/Y539A. These mutations correspond to those reported by Leonard et al (13), taking into account the four additional amino acid present in the MeV H upstream of that region (33). The nectin4-blind MeV H protein expression plasmids L482S, P497S, and Y543A, and P497S/Y543A were generated by site-directed mutagenesis of the plasmid pcDNA3.1-MeV H_{IC323} (13, 30).

To generate the recombinant virus p5804Pe$H_{EpR\text{-}blind}$, the P497S/Y543A double mutation was first introduced in a subgenomic plasmid containing the region between the *Bsr*GI and *Asc*I restriction sites. Using these restriction sites, the fragment was then transferred into the full-length p5804PeH plasmid, which expresses EGFP from an additional open reading frame located between the H and polymerase genes (32). All plasmids were verified by sequencing.

Recovery of the recombinant virus and growth kinetics. The recombinant EpR-blind CDV was recovered by transfecting semi-confluent 293 cells with 6 μg of p5804PeH$_{EpR\text{-}blind}$ and a combination of 0.5, 0.1, 0.5, and 0.7 μg MeV N, P, polymerase (L), and T7 RNA polymerase expression plasmids (1, 14), respectively, using Turbofect (Fermentas). Three days later, the transfected cells were transferred onto VerodogSLAMtag cells seeded at 80-90% confluency in 10-cm dishes. Individual syncytia were inoculated on fresh VerodogSLAMtag cells. Stocks of all viruses used in this study were produced in VerodogSLAMtag cells. Titers were determined by limiting dilution and expressed as 50% tissue culture infectious dose ($TCID_{50}$).

For growth kinetics, VerodogSLAMtag, MDCK, and FtAEpC cells were plated in 24-well plates and infected with multiplicities of infection of 0.1 or 0.01. For VerodogSLAMtag cells, cells were scraped in the supernatant and harvested daily for 4 days, for MDCK and FtAEpC cells, samples were harvested every second day for 8 days. Titers were determined by limited dilution method and expressed as $TCID_{50}$. Phase contrast and fluorescence pictures were taken on the days of harvest.

Cell surface biotinylation and Western blot analysis. For cell surface biotinylation, transfected cells were incubated for 20 min at 4°C with EZ-Link NHS-LC-LC-Biotin (Pierce) at a concentration of 2 mM in PBS, and then washed with 100 mM glycine in phosphate-buffered saline (PBS, Invitrogen) to quench excess biotin. The cells were then lysed with RIPA buffer with protease inhibitor, and a 20 μl aliquot of each sample was directly mixed with an equal volume of 2X SDS-gel loading buffer (SDS-GLB) containing 10% β-mercaptoethanol and stored at -20°C. The remaining lysate was mixed with immobilized Protein G-PLUS beads (Santa Cruz) and a rabbit anti-peptide antisera raised against the cytoplasmic tail (Hcyt) of the respective H protein (36). After agitation overnight at 4°C, samples were washed three times, mixed with 1X SDS-GLB, and heated at 75°C for 5 min. The immunoprecipitated proteins were separated in parallel on SDS-PAGE gels and transferred to PVDF membranes. Total cellular expression of recombinant H proteins was detected using the respective Hcyt antiserum in combination with a peroxidase (HRP)-labeled secondary antiserum, while biotinylated proteins were detected using a streptavidin-HRP conjugate (Pierce) and visualized using the ECL^{+}

Chemiluminescence kit (GE Healthcare). Bands were quantified using Molecular Imaging software (Kodak).

***In vivo* characterization of recombinant viruses.** Ferrets (*Mustela putoris furo*), 16 weeks and older (Marshall Farms) and without antibodies against CDV, were used for all studies. All animal experiments were approved by the Animal Care and Use Committee of the INRS-Institut Armand-Frappier. Groups of five or eight ferrets were infected intranasally with 10^5 $TCID_{50}$ of the parental 5804PeH or 5804PeH$_{FpR\text{-}blind}$, respectively. Body temperature and clinical signs were recorded daily and scored based on a 0-1-2 scale with 0 representing no change, 1 representing moderate and 2 severe disease. Blood samples were collected from the jugular vein under general anesthesia twice weekly for the first two weeks and weekly thereafter. Cell-associated viremia in peripheral blood mononuclear cells (PBMC) was quantified by limiting dilution method (35), and the percentage of EGFP-expressing cells was determined by flow cytometry. The extent of PBMC proliferation inhibition was assessed by stimulation of Ficoll-purified PBMCs with 1 μg/mL phytohemagglutinin (PHA) and subsequent detection of BrdU incorporation into proliferating cells using the Cell Proliferation ELISA BrdU kit (Roche). Wild type virus infection results include the data obtained from three previously published animals (27). Two animals of each group were sacrificed on day 7 and three on day 12 post-infection, to assess dissemination and to harvest tissues for histological analysis. On day 12, throat swabs were collected in 0.5 mL OptiMEM (Invitrogen) with double concentrated penicillin and streptomycin (Invitrogen), and urine was collected aseptically from the bladder with a needle and syringe. Virus titers were quantified by limited dilution method and expressed as $TCID_{50}$/ml.

Immunohistochemistry of ferret tissues. For immunohistochemistry staining, paraffin sections were deparaffinized and rehydrated following standard immunohistochemistry protocols. Endogenous peroxidase was quenched with 0.3% H_2O_2 in PBS for 12 min. After blocking with a 1:50 dilution of goat serum in PBS, the primary anti-CDV N monoclonal antibody (CDV-NP, VMRD) was added for 1 h at room temperature, followed successively by a biotinylated goat anti-mouse secondary antibody

and a peroxidase-labeled streptavidin conjugate (Vector Laboratories) for 45 min each at room temperature. Infected cells were visualized using 3,3'-diaminobenzidine (DAB) substrate (Sigma) and slides were counterstained with hematoxylin.

For the detection of different cell types, cryosections of paraformaldehyde-fixed OTC-embedded tissues were thawed, permeabilized with 0.1% Triton-X100 in PBS for 10 min, and blocked with 1:50 diluted goat serum in PBS for 30 min. The slides were then incubated with either a CD3 (sc-20047, Santa Cruz) or a pan-cytokeratin (C2562, Sigma) antibody for 1 h, followed by a goat-anti mouse AlexaFluor 568-labeled secondary antiserum (Invitrogen). The slides were counter stained with 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) and mounted in Vectamount (Vector Laboratory).

Results

The CDV H P493S/Y539A double mutant is EpR-blind while retaining wild type SLAM-mediated fusion efficacy. To generate an EpR-blind CDV, the MeV IC323 and CDV 5804P H proteins were aligned, and the mutations V478S, P493S, and Y539A, which correspond to the mutations L482S, P497S, and Y543A that confer the nectin4-blind phenotype in MeV H (13), were introduced in a CDV H expression plasmid (Fig. 1A). Two of these positions, V478 and Y539, have recently been identified independently as EpR-relevant in a CDV H protein alanine scan (10), thereby validating our approach. Alteration of position 493 resulted in a 70% reduction of surface expression (Fig. 1B), as detected by cell surface biotinylation. A similar reduction in surface expression was observed for the corresponding MeV P497S mutant (Fig. 1C), as well as the CDV and MeV H proteins carrying the double mutations (P493S/Y539A for CDV H and P497S/Y543A for MeV H, Fig. 1B and C), indicating that the proline residue at position 493/497 is important for H protein transport. However, none of the mutations significantly affected the level of cell-to-cell fusion in VerodogSLAMtag cells when the respective H proteins were co-expressed with either the MeV Edmonston (3) or CDV 5804P fusion (F) protein (Fig. 1D).

Since none of the carnivore epithelial cell lines used for the fusion assay revealed syncytia formation when wild type or mutant CDV H proteins were co-expressed with the F protein, it was not possible to assess the extent of fusion support of the recombinant H proteins outside the viral context. We thus introduced the individual point mutations as well as the P493S/Y543A double mutation into the CDV wild type strain 5804PeH. This virus expresses the enhanced green fluorescent protein (EGFP) gene from an additional open reading frame located between the H and polymerase genes (32), allowing direct visualization of infected cells. While each of the individual mutations resulted in a reduction of epithelial cell infection efficiency (data not shown), only the double mutant previously identified in the MeV system was unable to infect epithelial cells and thus chosen for further characterization.

The wild type and 5804PeH$_{EpR\text{-}blind}$ viruses replicated to similar maximum titers in VerodogSLAMtag cells (Fig. 2A, left panel). Both viruses induced syncytia of

comparable sizes (Fig. 2C), illustrating that the reduced cell surface expression associated with the P497S mutation did not affect fusion efficiency or overall viability in the context of SLAM-mediated infection. In contrast, only the wild type virus replicated efficiently in MDCK and FtAEpC cells (Fig. 2A and B). Even at a tenfold higher MOI, 5804PeH$_{EpR\text{-}blind}$ resulted in only few infected foci and titers slightly above detection levels at some of the time points (Fig. 2B, D, and E). Thus, the transfer of the mutations involved in MeV interaction with nectin-4 to CDV results in the expected EpR-blind phenotype.

5804PeH$_{EpR\text{-}blind}$ causes severe immunosuppression but no clinical disease. To assess the importance of spread to epithelial tissues for morbillivirus, ferrets were infected intranasally with 10^5 TCID$_{50}$ of the parental 5084PeH or 5804PeH$_{EpR\text{-}blind}$. In contrast to 5804PeH-infected animals, which developed severe rash and fever and died within 12 days after infection, the EpR-blind virus did not elicit fever and caused no clinical signs of disease (Fig. 3A and B). Fluorescent imaging revealed few weakly GFP-expressing spots around the mouths and eyes as only indication of 5804PeH$_{EpR\text{-}blind}$ infection, while the wild type virus resulted in extensive fluorescence of all mucosal surfaces and caused the typical skin rash (32; data not shown). Despite these differences in pathogenesis, both viruses resulted in similar kinetics of cell-associated viremia (Fig. 3C) with 70-90% of PBMCs infected 7 days after inoculation (Fig. 3D), indicating that the epithelial cell receptor-blind virus was not affected in its ability to infect immune cells and spread through the lymphatic system. All animals also developed strong leukopenia, resulting in an 80% reduction in leukocytes at day 10 after infection (Fig. 3E). Finally, infection with both viruses resulted in 75% inhibition of lymphocyte proliferation activity (Fig. 3F). Thus, the values of these two indicators of morbillivirus immunosuppression (7, 24) were equivalent for the first 10 days of infection. Even though recovery of white blood cell count and lymphocyte proliferation activity in 5804PeH$_{EpR\text{-}blind}$-infected animals correlated to a certain extent with virus clearance, pre-infection levels were not reached during the duration of the experiment.

To investigate the importance of epithelial cell infection for shedding, three additional animals were infected with each virus, and throat swabs and urine were collected on day 12 after infection. While high titers were detected in samples of wild

type-infected animals, no virus was found in samples from the EpR-blind virus group (Fig. 3G). Thus, the EpR-blind CDV remains immunosuppressive, but does not cause disease and is not shed.

5804PeH$_{EpR\text{-}blind}$ is not detected in epithelial tissues. Immunohistochemical staining of paraformaldehyde-fixed paraffin-embedded tissue sections from animals sacrificed on day 7 or 12 after infection using a CDV nucleoprotein-specific monoclonal antibody revealed similar infection levels in the intestinal lymph node (Fig. 4A-D) and other immune tissues (data not shown). In contrast, CDV-positive epithelial cells were only found in tissues from animals infected with wild type virus (Fig. 4E-P), with infection levels increasing over time. Occasional infected cells detected in epithelial tissues of EpR-blind virus-infected animals were all of lymphoid morphology (Fig. 4J and L, arrows), and staining of paraformaldehyde-fixed cryosections, which maintain EGFP expression in infected cells, with antibodies for T cells or epithelial cells confirmed that most infected cells were indeed immune cells (Fig. 5). Taken together, these results suggest that infection of epithelial tissues is essential for clinical disease and shedding, but has minimal if any influence on immunosuppression.

Discussion

After transmission by aerosol, morbilliviruses cross the respiratory epithelium to reach immune tissues via infected immune cells, most likely macrophages or dendritic cells (6, 11, 13, 32). Nevertheless, since it is conceivable that epithelial cell infection sustains virulence in late infection stages, we sought to confer an EpR-blind phenotype to an extremely virulent CDV strain that is lethal for ferrets. Towards this, we transferred the MeV H P497S/Y543A double mutation, which results in a nectin4-blind MeV (13, 17), into the CDV 5804PeH H protein. The resulting recombinant virus efficiently infected and replicated in SLAM-expressing Vero cells, but was severely impaired in its ability to infect canine or ferret epithelial cell lines. In ferrets the EpR-blind CDV was initially indistinguishable from the wild type virus, reaching similar infection levels and causing severe leukopenia and inhibition of lymphocyte proliferation. However, the virus was completely attenuated, and there was no shedding. Immunohistochemistry staining confirmed the EpR-blind phenotype *in vivo*, indicating that epithelial cell infection is critical for clinical morbillivirus disease.

Immune cell infection alone is sufficient to induce prolonged immunosuppression. Severe acute and in many cases long lasting immunosuppression is a hallmark of morbillivirus infections (7). During the acute phase, morbillivirus immunosuppression is characterized by an often dramatic leukopenia and an inability of PBMCs to proliferate upon stimulation (18, 25, 28), and the extent of both parameters correlates with disease severity (21, 28, 38). However, while leukocyte counts rapidly recover after virus clearance (16, 22), the inhibition of lymphocyte proliferation frequently persists beyond clinical recovery (8, 38). The wild type levels of leukopenia and inhibition of lymphocyte proliferation we saw in animals infected with the EpR-blind virus now demonstrates that acute immunosuppression is primarily a consequence of immune cell infection and can occur independently from clinical disease. Our findings are supported by the leukopenia and inhibition of lymphocyte proliferation observed in knock-in mice expressing a murine SLAM with a human V domain (20), which carries the MeV H-interacting residues (23), since MeV is de facto EpR-blind in these animals

due to the lack of appropriate receptors in other than the SLAM-expressing immune cells. The prolonged inhibition of lymphocyte proliferation activity in the absence of clinical disease in ferrets infected with the EpR-blind CDV further suggests that this virus provides a unique opportunity to investigate mechanisms underlying prolonged morbillivirus immunosuppression and its impact on susceptibility to secondary infections.

Epithelial cell infection is not required for the initial infection and dissemination but essential for clinical disease and shedding. Consistent with a previous report of a nectin4-blind MeV reaching wild type titers in PBMCs from infected Rhesus monkeys (13), the EpR-blind CDV replicated with a wild type kinetic in ferret PBMCs and attained similar infection levels. Taken together with the lack of viremia and disease progression associated with SLAM-blind viruses (12, 35), these results further illustrate that epithelial cell infection plays little to no role in the initial stages of morbillivirus pathogenesis.

In wild type and nectin4-blind MeV infected Rhesus macaques, similar rash frequency and severity, but no other clinical signs, were reported (13). In contrast, we observed only a few EGFP-expressing spots at the lips and eyes of ferrets infected with the EpR-blind virus, while the wild type virus was associated with severe rash. Since studies with EGFP-expressing MeV in cynomolgus macaques demonstrated that rash represents infected immune cells in the skin rather than infected skin cells (4), the occasionally observed EGFP-positive foci in EpR-blind ferrets may thus likely be attributed to immune cell infection, while the more severe manifestation seen in wild type-infected animals reflects spread to epithelial skin cells. We previously reported a correlation between the onset of rash and clinical signs and spread to epithelial tissues (32), and septicemia due to disruption of epithelial barriers in the intestine has long been considered the most likely immediate cause of death for CDV-infected ferrets. The results presented here clearly establish epithelial cell infection as cause of CDV and likely general morbillivirus signs of clinical disease. Furthermore, the absence of virus in tracheal aspirates of monkeys (13) and throat swabs and urine of ferrets infected with EpR-blind viruses suggests that epithelial infection is necessary for shedding and ultimately spread to new hosts. Therapeutic interventions that aim to interrupt the

dissemination to epithelial tissues may thus not only protect from clinical disease but also prevent transmission.

Morbillivirus H protein residues important for epithelial cell infection are conserved. Epithelial cell infection to varying extent is an integral part of morbillivirus pathogenesis (7). Several of the MeV H protein amino acids involved in binding to the recently discovered epithelial cell receptor nectin-4 (13, 17, 19, 29) were found to also be essential for CDV epithelial cell infection in an independent alanine scan of the corresponding CDV H protein region (10). Here we show that introduction of the two key mutations into the H protein of a wild type CDV strain resulted in an EpR-blind phenotype *in vitro* and *in vivo*. While the role of nectin-4 as a receptor for CDV remains to be demonstrated, the involvement of the same H protein residues in MeV and CDV epithelial cell infection strongly suggest that the epithelial cell receptor, similar to the immune cell receptor SLAM (31), is conserved among morbilliviruses.

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Figure 2.1

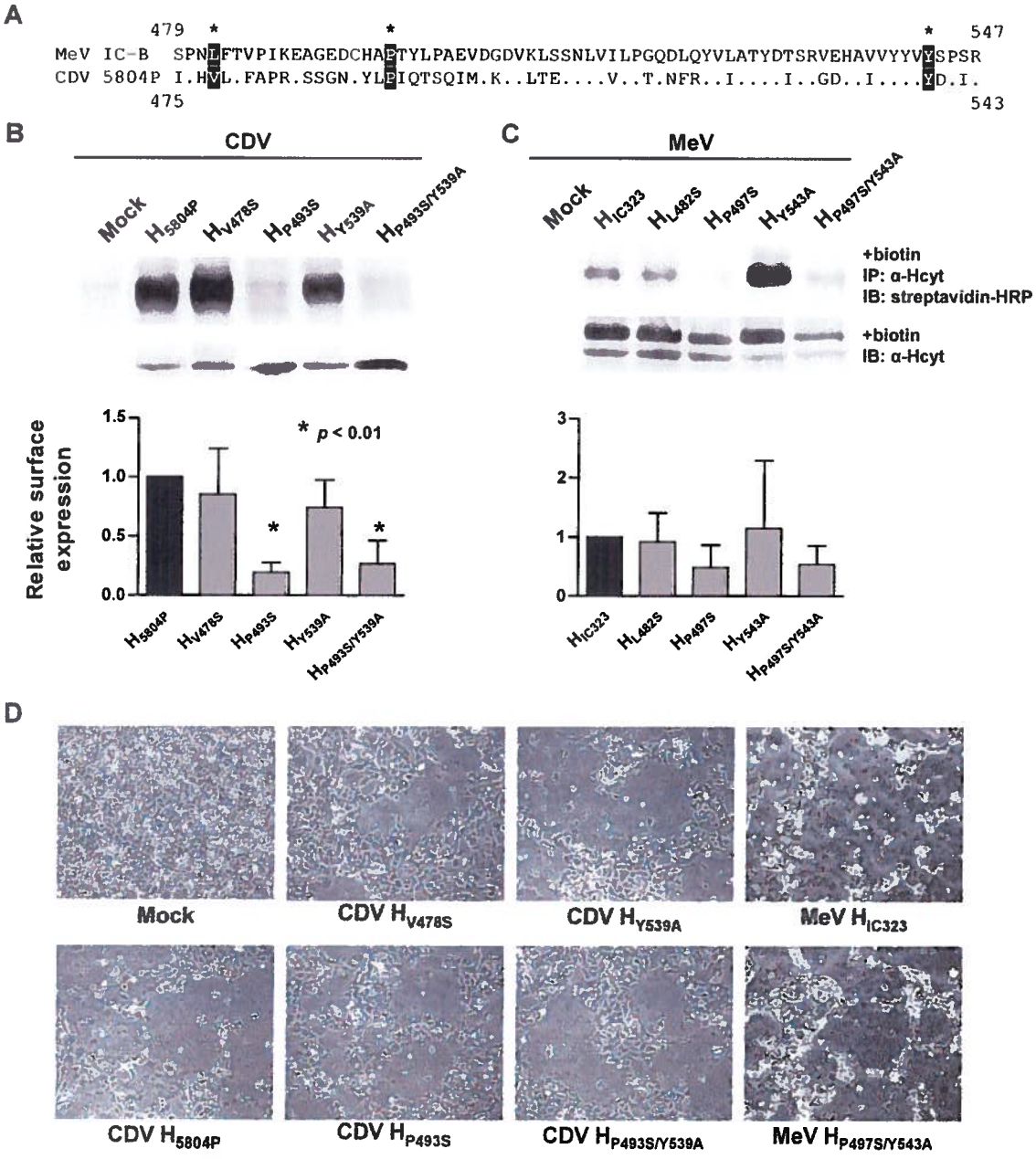
A
479 * * * 547
MeV IC-B SPNLFTVPIKEAGEDCHAPTYLPAEVDGDVKLSSNLVILPGQDLQYVLATYDTSRVEHAVVYYVYSPSR
CDV 5804P I.HVL.FAPR.SSGN.YLPIQTSQIM.K..LTE....V..T.NFR..I....I..GD..I....YD.I.
475 543
B
CDV
Mock
H5804P
HV478S
HP493S
HY539A
HP493S/Y539A
C
MeV
Mock
HIC323
HL482S
HP497S
HY543A
HP497S/Y543A
+biotin
IP: α-Hcyt
IB: streptavidin-HRP
+biotin
IB: α-Hcyt
Relative surface expression
1.5
1.0
0.5
0.0
* p < 0.01
*
*
H5804P
HV478S
HP493S
HY539A
HP493S/Y539A
3
2
1
0
HIC323
HL482S
HP497S
HY543A
HP497S/Y543A
D
Mock
CDV HV478S
CDV HY539A
MeV HIC323
CDV H5804P
CDV HP493S
CDV HP493S/Y539A
MeV HP497S/Y543A

Figure 2.1) Transport and function of mutant H proteins. (**A**) Amino acid alignment of the MeV IC-B H protein and the corresponding region of the CDV 5804P H protein. Residues identified by Leonard *et al.* (13) (L482, P497, and Y543) as important for interaction of MeV H with nectin-4 are highlighted with asterisks above the residues. The corresponding residues V478, P493, and Y539 were mutated in 5804P H. (**B** and **C**) Cell surface biotinylation of CDV and MeV H proteins. Cells transfected with expression plasmids for CDV (**B**) or MeV (**C**) parental and mutant H proteins were labelled with NHS-LC-LC-biotin, immunoprecipitated with an antiserum recognizing either the MeV H or CDV H protein cytoplasmic tail, and detected with a streptavidin-HRP conjugate. Western blots of cell lysates from the same experiment were analyzed for total H protein expression. The relative surface expression of each protein was calculated by normalizing the biotinylation or Western blot signals to the signal obtained for the respective parental H protein. The relative surface expression represents the ratio between the normalized biotinylation signal and the normalized Western blot signal for each replicate. Average relative surface expression levels were calculated from four independent experiments and are shown as bar graphs below the blots. (**D**) Cell-cell fusion observed for wild type and mutant CDV or MeV H proteins. Confluent monolayers of VerodogSLAMtag cells were transfected with either the respective H protein and the corresponding CDV or MeV F protein expression plasmids. Pictures were taken 24 h post-transfection at 100-fold magnification.

Figure 2.2

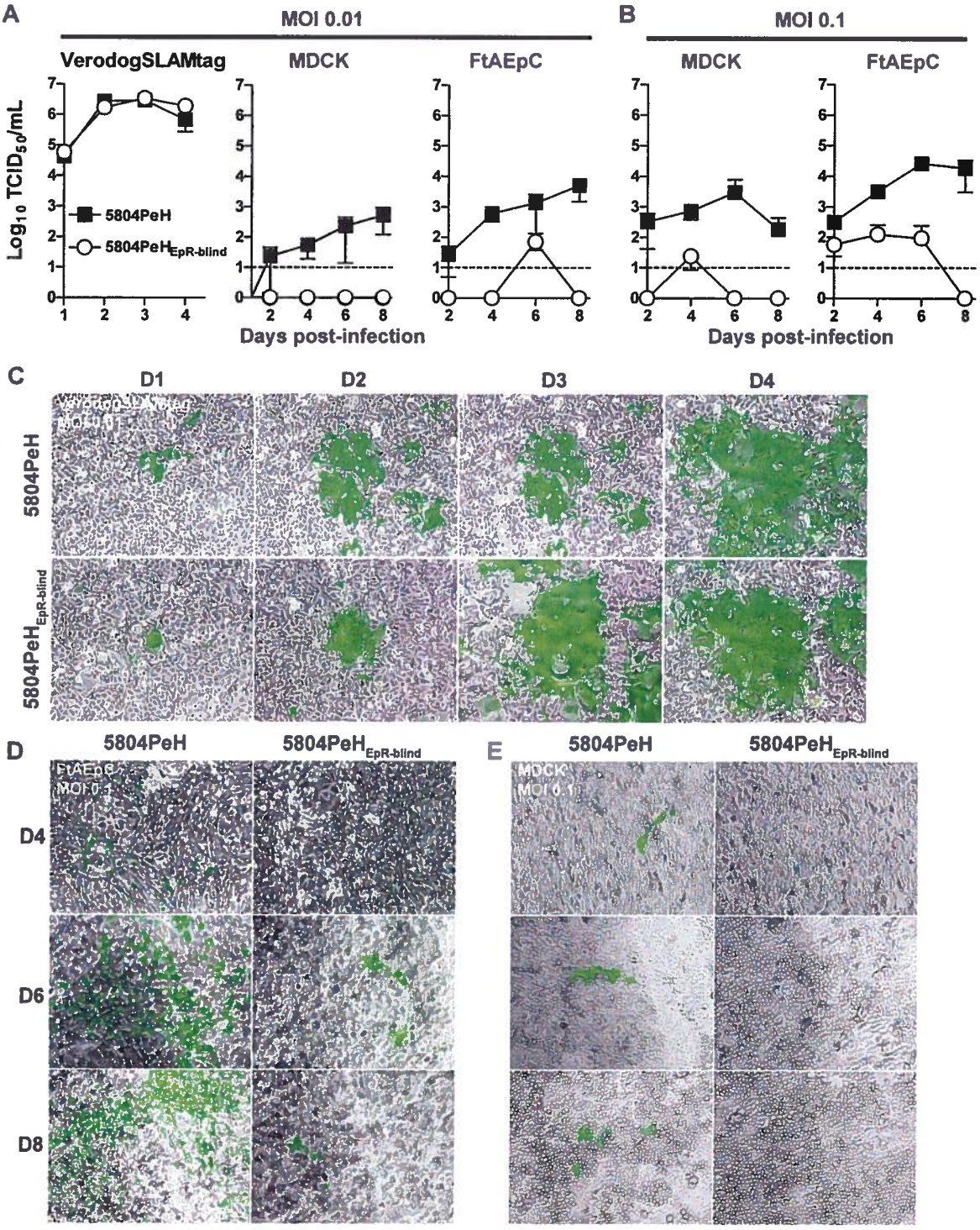
A
MOI 0.01
VerodogSLAMtag
MDCK
FtAEpC
Log10 TCID50/mL
5804PeH
5804PeHEpR-blind
Days post-infection
B
MOI 0.1
MDCK
FtAEpC
Days post-infection
C
D1
D2
D3
D4
5804PeH
5804PeHEpR-blind
D
5804PeH
5804PeHEpR-blind
FtAEpC
MOI 0.1
D4
D6
D8
E
5804PeH
5804PeHEpR-blind
MDCK
MOI 0.1

Figure 2.2) Characterization of recombinant viruses. (**A** and **B**) Growth kinetics in VerodogSLAMtag, MDCK, and FtAEpC cells. Cells were infected with a MOI of (**A**) 0.01 or (**B**) 0.1 and samples were collected daily for 4 days for VerodogSLAMtag cells or every second day for 8 days for epithelial cell lines. The cell-associated virus titer was determined by limited dilution method and expressed as 50% tissue culture infectious doses ($TCID_{50}$). The average values of 4 experiments are shown, and error bars indicate the standard deviation. The dotted line represents the detection limit of the assay. (**C-E**) Syncytium formation in (**C**) VerodogSLAMtag infected with a MOI of 0.01 and (**D**) MDCK and (**E**) FtAEpC cells infected with a MOI of 0.1. Photographs were taken at the indicated time points using fluorescence excitation and phase contrast at 100-fold magnification. An overlay of both photographs is shown.

Figure 2.3

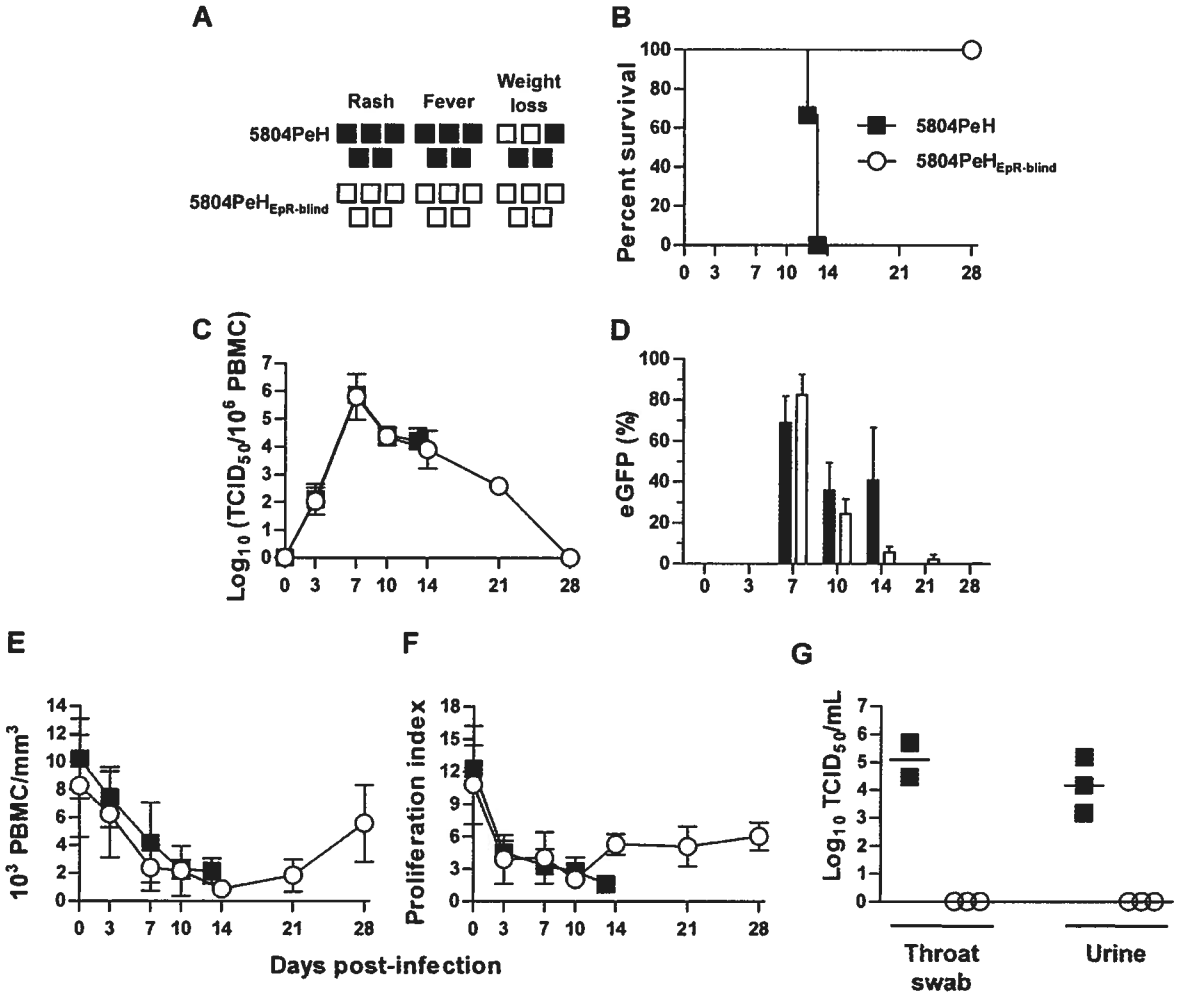
A
Rash
Fever
Weight loss
5804PeH
5804PeH$_{EpR-blind}$
B
Percent survival
5804PeH
5804PeH$_{EpR-blind}$
C
Log$_{10}$ (TCID$_{50}$/10^6 PBMC)
D
eGFP (%)
E
10^3 PBMC/mm^3
F
Proliferation index
Days post-infection
G
Log$_{10}$ TCID$_{50}$/mL
Throat swab
Urine

Figure 2.3) Pathogenesis of recombinant CDVs in ferrets. Groups of five and eight animals were infected intranasally with 10^5 $TCID_{50}$ of either the parental strain 5804PeH or the recombinant virus $5804PeH_{EpR\text{-}blind}$, respectively. The data of three 5804PeH-infected animals published previously (16) were also included, resulting in groups of eight animals for each virus. Only the five animals followed until day 12 after infection or death, respectively, are shown in panels **A** and **B**. (**A**) Clinical scores observed for wild type- and $5804PeH_{EpR\text{-}blind}$-infected animals. The five animals followed until day 12 after infection or death, respectively, are shown for. Each square represents one animal. White squares indicate no changes, and black squares indicate severe rash, fever above 40°C, and weight loss above 10%. (**B**) Survival of the animals infected with the different viruses. Death of an animal is indicated by a step down on the curve. (**C**) The course of cell-associated viremia is shown as the $\log_{10}$ of the virus titer per 10^6 PBMCs, and (**D**) the percentage of infected PBMCs was quantified by flow cytometry. (**E**) Total leukocyte counts from infected animals, shown as 10^3 leukocytes per mm^3. (**F**) *In vitro* proliferation activity of lymphocytes from infected animals. Days post-infection are indicated on the *x* axes of the graphs, and error bars represent standard deviations. (**G**) Quantification of virus shedding in epithelial tissues on day 12 after infection. Titers are expressed as $TCID_{50}$/mL. Each symbol represents one animal, and geometric means are indicated by horizontal black lines for each group.

Figure 2.4

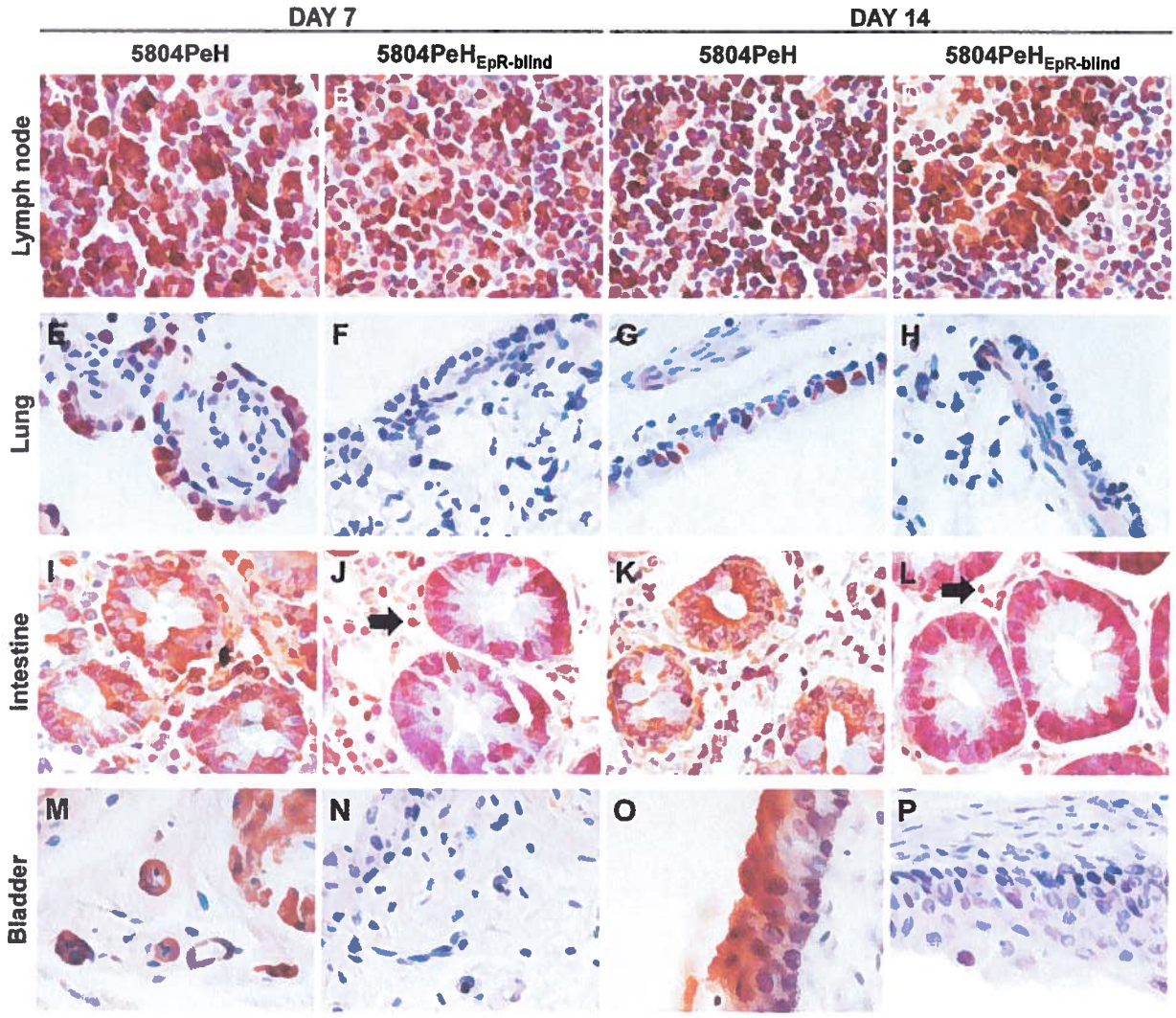
DAY 7
DAY 14
5804PeH
5804PeH EpR-blind
5804PeH
5804PeH EpR-blind
Lymph node
Lung
Intestine
Bladder
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 2.4) Immunohistochemical detection of CDV in ferret lymphatic and epithelial tissues. (**A-D**) Lymph node sections from animals sacrificed at either day 7 (**A** and **B**) or 12 (**C** and **D**) post-inoculation infected with either 5804PeH (**A** and **C**) or 5804PeH$_{EpR\text{-}Blind}$ (**B** and **D**). (**E-H**) Lung sections from animals sacrificed at either day 7 (**E** and **F**) or 14 (**G** and **H**) post-infection infected with either 5804PeH (**E** and **G**) or 5804PeH$_{EpR\text{-}blind}$ (**F** and **H**). (**I-L**) Intestinal sections from animals sacrificed at either day 7 (**I** and **J**) or 14 (**K** and **L**) post-infection infected with either 5804PeH (**I** and **K**) or 5804PeH$_{EpR\text{-}blind}$ (**J** and **L**). Black arrows in panels **J** and **L** indicate infected cells that are of lymphoid morphology. (**M-P**) Bladder sections from animals sacrificed at either day 7 (**M** and **N**) or 14 (**O** and **P**) post-infection infected with either 5804PeH (**M** and **O**) or 5804PeH$_{EpR\text{-}blind}$ (**N** and **P**). All pictures were taken at 1000-fold magnification under oil immersion.

Figure 5

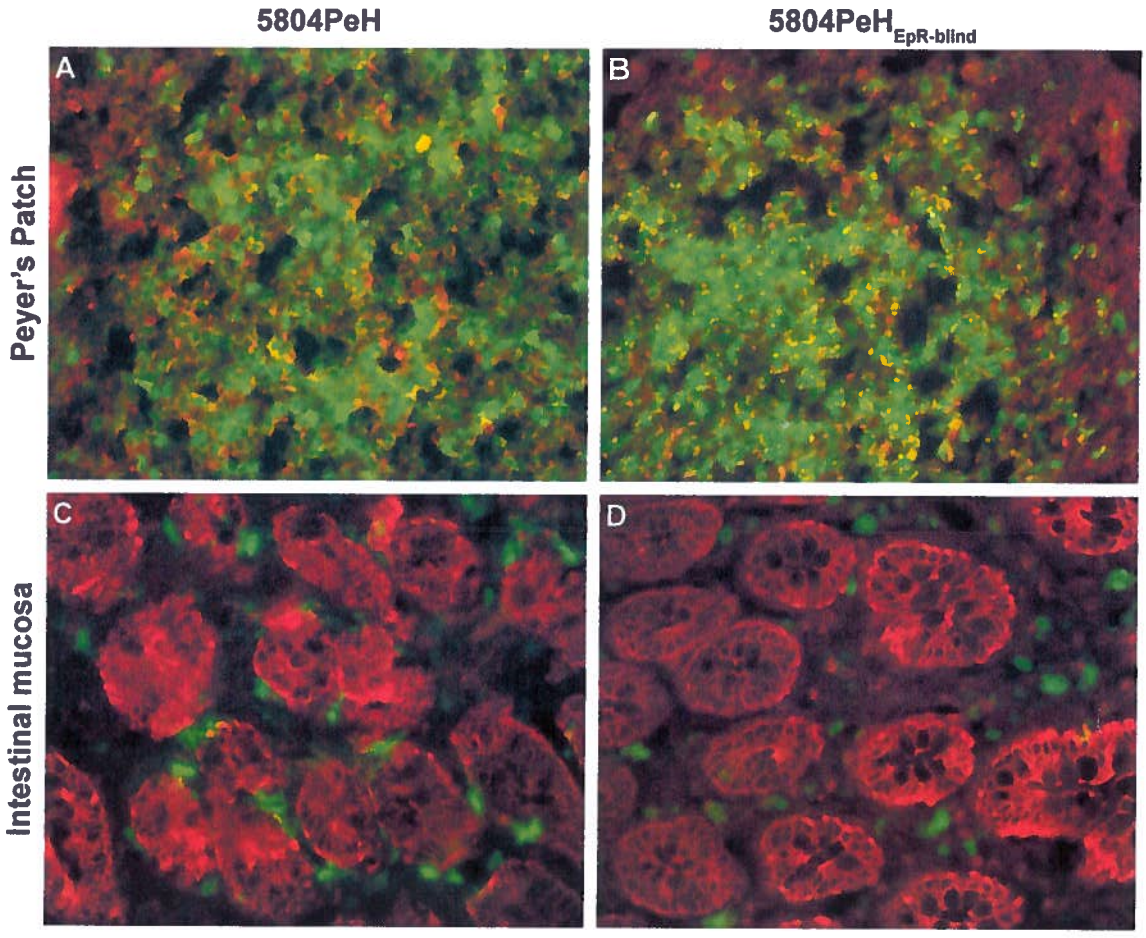

Figure 2.5) Immunohistochemical staining of infected immune and epithelial cells. Paraformaldehyde-fixed cryosections of Peyer's patches and small intestine harvested on day 7 after infection with 5804PeH (**A** and **C**) or 5804PeH$_{EpR\text{-}blind}$ (**B** and **D**) were stained with markers for T cells (**A** and **B**; CD3, sc-20047, Santa Cruz) or cytokeratin (**C** and **D**; C2562, Sigma) followed by an AlexaFluor 568-labeled secondary antiserum. Nuclei were counterstained with DAPI.

CHAPTER 3: Discussion and Conclusions

Characterization of the CDV H protein domain involved in infection of epithelial cells. The three CDV H residues (V478, P493 and Y539) supporting entry into canine and ferret epithelial cells identified here correspond exactly to the MeV H residues interacting with the recently identified human epithelial cells receptor, nectin-4 (Leonard *et al.*, 2008; Mühlebach *et al.*, 2011). An alanine-scanning mutagenesis of the residues located in a receptor-binding surface of the H protein from a neurovirulent CDV strain, A75/17, revealed a total of eleven residues that support cell fusion in primary canine keratinocytes (Langedijk *et al.*, 2011). The authors also verified that none of the EpR-blind mutations affected the overall structure of the H protein by testing the binding of antibodies to conformation-specific epitopes (Langedijk *et al.*, 2011). In addition to V478 and Y539, three of the identified residues, L479, I494 and Y537, are in close structural proximity to the EpR-binding amino acids identified in our study (Leonard *et al.*, 2008; Tahara *et al.*, 2008; Langedijk *et al.*, 2011; Sawatsky *et al.*, 2012).

Although the residue P493 is highly conserved among morbilliviruses, Langedijk *et al.* excluded it from their analysis, because of its predicted low surface exposure on a structural model of the CDV H protein, making it thus unlikely to be available for receptor-binding. Instead, they mutated the neighbouring surface-exposed residue I494, which also resulted in an EpR-blind phenotype (Langedijk *et al.*, 2011). The characterisation of our H protein mutants revealed that the surface expression of the P493S mutant was indeed impaired (Sawatsky *et al.*, 2012). Reduction in H protein surface expression may thus contribute to an EpR-blind phenotype, suggesting that multiple H proteins are required to stabilize the interaction with the epithelial receptor.

The predicted three-dimensional structure of CDV H indicates that the EpR-binding site is located on the side of the globular head domain, which overlaps with the region for SLAM-binding, but each cellular receptor interacts with distinct residues in that region (von Messling *et al.*, 2005; Zipperle *et al.*, 2010; Langedijk *et al.*, 2011). Given the conserved usage of the immune receptor SLAM among morbilliviruses, our observation that the same residues in MeV and CDV H are involved in EpR-binding

suggests that the identity of the canine epithelial cells receptor may also be the adherens junction protein, nectin-4, or a closely related protein.

The infection of epithelial cells is not necessary for virus-induced immunosuppression, but it is required for the development of clinical disease and virus shedding. At the early stage of infection, the virus primarily replicates in SLAM-expressing immune cells, and thereby leading to a decrease in leukocytes and a profound immunosuppression (de Swart *et al.*, 2007; Schneider-Schaulies *et al.*, 2009; Lemon *et al.*, 2011). As the virus load reaches its peak, the apparition of clinical signs such as fever and skin rash coincides with the infection of epithelial cells throughout the body (von Messling *et al.*, 2004; de Swart *et al.*, 2007; de Vries *et al.*, 2010; Ludlow *et al.*, 2010). In a recent study on MeV pathogenesis, rhesus macaques infected with an EpR-blind MeV were immunosuppressed, but no virus was detected in the respiratory tract of these animals, suggesting that epithelial cell infection is required for virus shedding (Leonard *et al.*, 2008). Although macaques reproduce the full spectrum of clinical disease as MeV in humans, the severity of the disease is generally mild and short-lived, thus making it difficult to assess the contribution of epithelial cells infection for pathogenesis in these animals. In contrast, ferrets develop an acute disease upon infection with wild-type CDV and develop extensive skin rash and redness on the chin, mouth and abdomen, thus making it a more sensitive model for the characterization of epithelial cells infection (von Messling *et al.*, 2003; von Messling *et al.*, 2004). We also observed that ferrets infected with an EpR-blind CDV had similar viral loads and experienced a similar extent of immunosuppression as wild-type infected animals (Sawatsky *et al.*, 2012). However, they did not show any signs of clinical disease such as rash, fever or weight loss. More importantly, no virus was detected in the epithelial tissues of the trachea, lungs or bladder (Sawatsky *et al.*, 2012). Taken together, we have shown that epithelial cell infection has little or no role in establishing the initial infection and in systemic spread, but rather contributes to the development of clinical disease, virus shedding and transmission to a new host (Sawatsky *et al.*, 2012).

The epithelium lining in the gastrointestinal and respiratory tracts is an important physical protection barrier against opportunistic pathogens. Secondary infections that take advantage of the host's immunosuppressed state contribute potentially to morbillivirus-associated mortalities. Gastroenteritis, pneumonia and septicemia are common causes of death in wild-type CDV-infected ferrets (von Messling *et al.*, 2003). While ferrets infected with an EpR-blind CDV also experienced severe viremia and profound immunosuppression by day 7 post-infection, they did not develop clinical signs and eventually cleared the virus and survived the infection (Sawatsky *et al.*, 2012). The observed lack of infected epithelial cells in the gastrointestinal and respiratory tracts at any of the time points analyzed suggests that virus replication in the epithelial cells plays an important role in disrupting the junctions of the intestinal or respiratory epithelium, thereby destroying the integrity of this protective barrier and giving access to opportunistic pathogens. The resulting secondary infections may then lead to severe complications and even death.

Abolishing the interaction with the epithelial cells receptor may be a novel strategy to reduce the transmission of morbilliviruses. MeV is one of the most infectious airborne pathogens. A single infected person can transmit the virus to two or three susceptible individuals, and the chain of infection can then be sustained and amplified through a population with low immunity, which results in outbreaks (Moss *et al.*, 2006). Thus, antiviral drugs that interfere with epithelial cells entry represent a novel strategy to interrupt transmission of this highly contagious virus. Blocking virus entry via its natural receptors also has particular importance in the development of MeV-based oncolytic therapies (Cattaneo, 2010). Viruses can be engineered to target a specific receptor expressed by cancerous cells, and the specificity is increased when the interaction with the natural receptors, SLAM and nectin-4, is abolished (Schneider *et al.*, 2000; Cattaneo, 2010). Moreover, a virus that does not bind to nectin-4 will not be shed and there is thus no risk of transmission to susceptible individuals, increasing its safety profile.

CHAPTER 4: Résumé (French summary)

4.1 Introduction

4.1.1 La famille *Paramyxoviridae* et le genre *Morbillivirus*

Les virus à ARN simple brin non-segmenté de polarité négative appartiennent à l'ordre *Mononegavirales*. Ces virus sont organisés en quatre familles : *Bornaviridae*, *Filoviridae*, *Paramyxoviridae* et *Rhabdoviridae*. La famille *Paramyxoviridae* se divise en deux sous-familles: *Pneumovirinae* et *Paramyxovirinae*. La première contient deux genres : *Metapneumovirus* et *Pneumovirus*, et la seconde en contient cinq : *Avulavirus*, *Henipavirus*, *Respirovirus, Rubulavirus* et *Morbillivirus* (King *et al.*, 2012).

Le genre *Morbillivirus* est composé agents pathogènes hautement contagieux qui sont connus pour leurs capacités à induire une immunosuppression sévère chez leurs hôtes respectifs (Lamb *et al.*, 2007). Le virus de la rougeole (*Measles virus*; MeV) est le seul morbillivirus pouvant causer une maladie chez l'humain, alors que les cinq autres membres du genre *Morbillivirus* sont des agents pathogènes chez les animaux, incluant le virus de la maladie de Carré (*Canine distemper virus;* CDV), le morbillivirus des cétacés (*Cetacean morbillivirus;* CeMeV), le virus de la Peste-des-petits-ruminants (*Peste-des-petits-ruminants virus*; PPRV), le virus de la maladie de Carré des phoques (*Phocine distemper virus;* PDV) et le virus de la peste bovine (*Rinderpest virus;* RPV). Récemment, un nouveau paramyxovirus a été isolé chez le chat domestique, et l'alignement des séquences des gènes viraux suggère que *Feline morbillivirus* (FmoPV) appartiendrait aussi au genre *Morbillivirus* (Woo *et al.*, 2012). Le spectre d'hôte du MeV est limité aux humains, et ces derniers sont aussi le seul réservoir naturel pour ce virus. En revanche, le CDV peut infecter un large éventail d'animaux terrestres et aquatiques, y compris les chiens, furets, blaireaux, renards, phoques et plusieurs espèces de grands félins (Grachev *et al.*, 1989; Appel *et al.*, 1994; Barrett, 1999). Les analyses phylogénétiques et immunologiques indiquent que le RPV, qui a été récemment éradiqué, était le plus proche parent du MeV et ces études suggèrent aussi que le RPV représente

l'archétype du genre *Morbillivirus* (Fig. 1) (Norrby *et al.*, 1985; Barrett, 1999; Sips *et al.*, 2007).

L'enveloppe de la particule virale des morbillivirus est dérivée de la cellule hôte qui prend une forme sphérique ou pléomorphique avec un diamètre de 120 à 300 nm (Fig. 2A) (Lamb *et al.*, 2007). La couche lipidique est associée à deux glycoprotéines virales transmembranaires : la protéine de l'attachement (H) et la protéine de la fusion (F) qui ressemblent à des structures pointilleuses en microscopie électronique. La paroi du coté interne de la particule virale est revêtue de la protéine de la matrice (M) reliant les glycoprotéines de l'enveloppe à la nucléocapside. La nucléocapside est composée du génome d'ARN encapsidé par les nucléoprotéines (N). La protéine de la polymérase (L) et la phosphoprotéine (P) forment ensemble le complexe de réplication d'ARN viral (vRdRp), qui s'associe ensuite à la nucléocapside donnant lieu à un complexe ribonucléique (RNP).

4.1.2 L'organisation génomique

Le génome morbillivirale contient approximativement 16,000 nt, et il comporte six gènes codant pour huit protéines (Fig. 1.2B) (Griffin, 2007). La séquence des gènes viraux est organisée du 3' au 5' tel que suit : N, P, M, F, H et L. Le gène P code pour deux protéines supplémentaires : V et C. Les extrémités 3' et 5' du génome sont délimitées par des séquences régulatrices pour la transcription et réplication, nommées séquences *leader* et *trailer*. Chaque gène viral débute par une séquence 3' (*gene start*; GS) qui initie la transcription et induit l'ajout d'une coiffe au transcrit d'ARN messagers (ARNm) viraux. Chaque gène se termine par une séquence 5' (*gene end*; GE) qui code pour une séquence de polyadénylation de quatre à sept uridines, suivi d'un signal de fin de transcription (Grdzelishvili *et al.*, 2005). Tout les gènes sont séparés par une région intergénique constitué de la séquence GE, d'une jonction intergénique (*intergenic junction*; IJ) de trois nt, non-transcrit, et en aval du GS gène suivant.

La longueur du génome morbillivirale est définie par la règle de six qui stipule que le nombre total de nucléotides doit être un multiple de six (6n +0) pour qu'il y ait une réplication efficace (Calain *et al.*, 1993). Cette nécessité est due au fait qu'un monomère de la protéine N se lie exactement à six nucléotides, et seul le génome entièrement encapsidé, et non l'ARN nu, fonctionne comme matrice pour la réplication. Les virus ayant un génome d'une longueur qui ne respecte pas la règle de six sont incapables de se répliquer efficacement (Sidhu *et al.*, 1995).

4.1.3 Survol des protéines virales

4.1.3.1 Protéines structurales

Les morbillivirus expriment six protéines virales regroupées en deux unités fonctionnelles : les protéines N, P et L forment le complexe RNP, et les protéines M, H et F sont associées à l'enveloppe virale. La protéine N est le composant majeur de la nucléocapside qui entoure l'ARN génomique viral donnant lieu à une structure en forme d'hélice, et cette structure peut résister à la digestion par les protéases et nucléases cellulaires (Liston *et al.*, 1997; Spehner *et al.*, 1997; Karlin *et al.*, 2002). La protéine L est l'unité catalytique du complexe vRdRp. Elle effectue les modifications post-transcriptionelles tel que l'ajout de la coiffe au 5' et la polyadenylation au 3' des ARNm (Grdzelishvili *et al.*, 2005). La protéine P est le co-facteur de la polymérase qui stabilise ce dernier sur l'ARN parental (Horikami *et al.*, 1992; Kingston *et al.*, 2004). De plus, la protéine P interagit avec la forme monomérique de la protéine N pour l'encapsidation des brins d'ARN nouvellement synthétisés (Huber *et al.*, 1991; Spehner *et al.*, 1997). La protéine M tapisse la paroi côté interne de l'enveloppe virale et interconnecte les queues cytoplasmiques des glycoprotéines de l'enveloppe, H et F, avec la protéine N du RNP (Iwasaki *et al.*, 2009). Elle joue donc un rôle essentiel dans l'assemblage du virus et le bourgeonnement. Des études ont montré que la protéine M agit également comme un régulateur négatif de la transcription et de la réplication (Reuter *et al.*, 2006). Les protéines H et F forment des complexes de glycoprotéines à la surface de l'enveloppe qui

sont impliquées dans l'attachement et l'entrée du virus dans l'hôte. Leurs propriétés et fonctions biologiques seront discutées d'avantage dans la section 4.1.4.

4.1.3.2 Protéines non-structurales

Les protéines accessoires V et C ne sont pas nécessaire à la réplication du virus en culture cellulaire, mais elles sont toutefois des facteurs de virulence essentielles à la pathogenèse (Takeuchi *et al.*, 2005; von Messling *et al.*, 2006; Devaux *et al.*, 2008). Ces protéines agissent contre le mécanisme antivirale de l'hôte en inhibant la voie de signalisation des interférons de type I (IFN α/β) (Tober *et al.*, 1998; Shaffer *et al.*, 2003; Takeuchi *et al.*, 2003). La protéine V est produite par une modification post-transcriptionelle, le *mRNA editing*, qui implique l'insertion d'un nucléotide guanine dans l'ARNm lors de la transcription du gène P (Cattaneo *et al.*, 1989). La séquence d'acide aminé de la partie N-terminal de la protéine V est identique à celle de la protéine P, alors que celle du C-terminal forme un doigt de zinc qui est riche en cystéine (Liston *et al.*, 1994). La protéine C est dérivée de l'ARNm de la protéine P, mais elle est synthétisée à partir d'un autre codon d'initiation (Bellini *et al.*, 1985). En plus de son rôle dans la modulation de la réponse immunitaire à l'infection, la protéine C favorise la réplication virale en modulant l'activité de la vRdRp (Bankamp *et al.*, 2005)

4.1.4 Glycoprotéine de l'enveloppe

4.1.4.1 La protéine d'attachement hémagglutinine (H)

La protéine H médie l'attachement du virus à la cellule hôte en se fixant aux récepteurs cellulaires. La spécificité de la protéine H pour son récepteur détermine le tropisme cellulaire et la pathogenèse du virus (Tatsuo *et al.*, 2000a; von Messling *et al.*, 2001). La protéine H est une glycoprotéine de type II (Fig. 1.3A). Elle porte un domaine extracellulaire constitué d'une tête globulaire et d'une tige, suivi par une région transmembranaire et une queue cytoplasmique en N-terminal. La structure tridimensionnelle de la protéine H du MeV suggère que le domaine globulaire est en

forme d'hélice à six lames (B1-B6), et chaque lame est composée de quatre feuillets béta antiparallèles (Fig. 1.3C) (Colf *et al.*, 2007; Hashiguchi *et al.*, 2007). La maturation de la protéine H s'effectue dans le réticulum endoplasmique et l'appareil de Golgi, où les chaînes des N-glycans et des ponts disulfures y sont ajoutés pour produire un homodimère (Plemper *et al.*, 2000). Les chaînes des N-glycans masquent une grande superficie du domaine globulaire et il expose qu'une petite région pour permettre la liaison des récepteurs cellulaires et des anticorps (Hashiguchi *et al.*, 2007). L'homodimère de la protéine H peut aussi s'oligomériser en homotétramère par des liaisons intramoléculaires (Brindley *et al.*, 2010; Hashiguchi *et al.*, 2011). La forme mature de la protéine H à la surface cellulaire est inclinée vers l'axe horizontal lorsque ce dernier s'oligomérise en dimère ou tétramère (Fig. 1.3B) (Hashiguchi *et al.*, 2007).

Contrairement aux autres paramyxovirus, la protéine H des morbillivirus ne contient pas d'activité neuraminidase et elle se lie à des acides aminés plutôt que des molécules de sucres (ex.: acide sialique) (Griffin, 2007). À ce jour, trois récepteurs cellulaires ont été identifiées pour MeV H : SLAM, CD46 et nectine-4, ils seront discutés en détail dans la section 4.1.5.

Les sites de liaison de la protéine H aux récepteurs cellulaires sont tous regroupés dans une région commune formée par les lames 4 et 5 (B4 et B5) située sur le coté latéral du domaine globulaire, et chaque récepteur cellulaire se lie à des acides aminés spécifiques à l'intérieur de cette région (Fig 1.4) (Masse *et al.*, 2004; Vongpunsawad *et al.*, 2004; Leonard *et al.*, 2008; Tahara *et al.*, 2008). De plus, la conformation structurale de cette région est distincte de celle de la protéine d'attachement des autres paramyxovirus, ce qui expliquerait la capacité de la protéine H des morbillivirus à reconnaître uniquement des récepteurs protéiques (Leonard *et al.*, 2008; Santiago *et al.*, 2010; Hashiguchi *et al.*, 2011).

4.1.4.2 La protéine de fusion (F)

La protéine F permet la fusion de l'enveloppe virale avec la membrane plasmique de la cellule cible suite à l'attachement de la protéine H à son récepteur (Lamb *et al.*, 2007). Cette glycoprotéine de type I est d'abord synthétisée sous forme inactive (F_0) dans le réticulum endoplasmique, elle est ensuite glycosylée et s'oligomérise en homotrimère (Fig 1.3A) (Lamb *et al.*, 2007). La protéine F est activée par le clivage dans l'appareil de Golgi de la forme F_0 en deux sous-unités (F_1 et F_2) via la protéase intracellulaire furine (Watanabe *et al.*, 1995). La sous-unité F_1 est ancrée à la membrane lipidique, et dispose d'un tronçon hydrophobe d'acides aminés constituant le peptide de fusion (*fusion peptide*; FP) et de deux heptades hydrophobes conservés HRA et HRB, qui sont essentiels à son activité de fusion (Buckland *et al.*, 1992). La plus petite unité F_2 est liée à F_1 par un pont disulfure et porte trois chaînes N-glycans qui assurent le bon fonctionnement et le transport de la protéine à la surface cellulaire (Lamb, 1993; Hu *et al.*, 1995).

La fusion membranaire est un processus irréversible. Le mécanisme de fusion des morbillivirus est pH-indépendant. La fixation de la protéine H sur son récepteur cellulaire entraine un changement de conformation chez la protéine F. Ce dernier se replie en plusieurs autres conformations exposant le FP qui est ensuite inséré dans la membrane opposée. Cela mène à la formation d'un pore de fusion et au fusionnement des membranes lipidiques (Navaratnarajah *et al.*, 2011). En culture cellulaire, la co-expression des protéines H et F en présence d'un récepteur cellulaire mène à la fusion des cellules avoisinantes et à la formation de cellules géantes multinucléées, nommées syncytia (Lamb *et al.*, 2007).

4.1.5 Récepteurs cellulaires

4.1.5.1 SLAM (CD150)

Signaling lymphocyte activation molecule (SLAM ou CD150) est le récepteur universel utilisé par tous les morbillivirus pour infecter les cellules immunitaires (Griffin, 2007). Suite à l'identification de SLAM comme récepteur d'entrée du MeV, il a été

démontré que CDV, RPV ainsi que les autres morbillivirus utilisent également les protéines SLAM de leurs espèces respectives (Tatsuo *et al.*, 2001; Baron, 2005). Le criblage des protéines de surface provenant d'une lignée lymphoïde susceptible à l'infection par MeV a permis d'isoler le récepteur d'entrée des cellules immunitaires. Le clone exprimant la molécule SLAM a permit l'entrée et la réplication des virus du type sauvage et de la souche vaccinale (Tatsuo *et al.*, 2000b). SLAM appartient à la superfamille des immunoglobulines exprimée par les thymocytes immatures, les cellules T et B activées, les cellules dendritiques (*dendritic cells*; DC) activées, les macrophages et les plaquettes. SLAM a deux domaines extracellulaires, V et C2, et une queue cytoplasmique qui interagit avec les protéines associées au SLAM (SLAM-*associated proteins*; SAP) et EWS/FLI1 *activated transcript* 2 (EAT2) pour la transduction de signal intracellulaire (Veillette *et al.*, 2006).

Les résidus clés de la protéine H du MeV qui médié l'entrée du virus via SLAM sont : I194, D505, D507, D530, R533, F552 et P554 (Vongpunsawad *et al.*, 2004; Hashiguchi *et al.*, 2007). Tous ces résidus sont regroupés dans une région commune située sur le côté latéral de l'hélice béta tel que décrit ci-dessus (Fig. 1.4). Les sites de liaison pour le SLAM canin ont été cartographiés sur l'interface correspondante de la protéine H du CDV aux positions : D526, T527, S528, R529, Y547, F548 et R552 (von Messling *et al.*, 2005). La mutation de plusieurs de ces résidus de CDV H est nécessaire pour complètement abroger l'entrée du virus via le récepteur SLAM, alors que l'introduction d'une seule mutation dans le MeV H est suffisante pour générer un phénotype SLAM-*blind* (von Messling *et al.*, 2005).

4.1.5.2 CD46

En plus de SLAM, le CD46 peut également servir comme récepteur d'entrée, mais seulement pour la souche vaccinale du MeV et celle adaptée en laboratoire (Dorig *et al.*, 1993; Naniche *et al.*, 1993a; Erlenhofer *et al.*, 2002). Bien qu'il soit le premier récepteur identifié pour MeV, la pertinence de CD46 en tant que récepteur d'entrée n'était pas entièrement fondé puisqu'il a été observé que la plupart des isolats cliniques provenant de

patients atteints de rougeole sont incapables de se répliquer dans les lignées cellulaires exprimant seul le CD46 (Kobune *et al.*, 1990; Tatsuo *et al.*, 2000b; Ono *et al.*, 2001). De plus, les souches MeV propagées dans des lignées cellulaires lymphoïdes $SLAM^+$ conservent leur virulence chez les macaques, alors que celles propagées dans les cellules Vero $SLAM^-/CD46^+$ accumulent des mutations atténuantes (Enders *et al.*, 1960; van Binnendijk *et al.*, 1994; Auwaerter *et al.*, 1999; Manchester *et al.*, 2000). Ces résultats suggèrent que les souches virulentes de type sauvage du MeV utilisent préférentiellement SLAM et non CD46 comme récepteur *in vivo*.

CD46 (*membrane cofactor protein*; MCP) est exprimé de façon ubiquitaire par toutes les cellules nucléées d'origine humains et de singes. Il s'agit d'une glycoprotéine de type I avec un domaine extracellulaire comprenant quatre séquences conservées (*short consensus repeat*; SCR 1-4), suivi par une région transmembranaire et une queue cytoplasmique. CD46 est un régulateur de l'activation du complément, et l'interaction entre H et CD46 entraîne une régulation à la baisse de ce récepteur de la surface de la cellule (Seya *et al.*, 1989; Naniche *et al.*, 1993b; Bartz *et al.*, 1996; Galbraith *et al.*, 1998). Un modèle récent de la structure de CD46 complexé avec H indique que le SCR 1 et 2 du CD46 est en contact avec le domaine globulaire de H (Devaux *et al.*, 1996; Santiago *et al.*, 2010).

L'adaptation à CD46 peut se produire lorsqu'une souche de type sauvage du MeV est propagée dans des cellules Vero $SLAM^-/CD46^+$, où la protéine H acquiert progressivement plusieurs mutations qui permettent l'interaction avec CD46 (Buckland *et al.*, 1997). Les mutations les plus pertinentes sont les substitutions de l'asparagine à la tyrosine à la position 481 (N481Y), ou de la serine à la glycine à la position 564 (S564G) (Nielsen *et al.*, 2001; Li *et al.*, 2002). D'autres mutations qui renforcent l'affinité de liaison au CD46 sont : N390I, N416D, T446S, T484N et E492G (Fig. 1.4) (Seki *et al.*, 2006; Tahara *et al.*, 2007; Santiago *et al.*, 2010).

4.1.5.3 Nectine-4

La nectine-4 (*poliovirus-like receptor* 4; PVLR-4) est le récepteur d'entrée du MeV dans les cellules épithéliales. Deux groupes de chercheurs indépendants ont récemment identifié nectine-4 par criblage à grande échelle des molécules de surface exprimées par des lignées de cellules épithéliales des poumons et du sein qui sont permissives à l'infection par MeV (Muhlebach *et al.*, 2011; Noyce *et al.*, 2011). Nectine-4 appartient à la superfamille des immunoglobulines qui permet la formation des jonctions serrées dans les cellules épithéliales polarisées (Reymond *et al.*, 2001). Il s'agit d'une protéine transmembranaire de type I, composé de trois domaines immunoglobuline extracellulaires (V, C et C), une région transmembranaire et une queue cytoplasmique. L'ARNm de la nectine-4 est principalement détecté dans le placenta humain mais aussi dans la trachée, la peau, les poumons, la prostate et l'estomac à des moindres niveaux (Brancati *et al.*, 2010). La surexpression de nectine-4 est souvent associée à des cancers du sein, des poumons et des ovaires, ce qui rend la nectine-4 comme un potentiel biomarqueur de la tumorigenèse (Fabre-Lafay *et al.*, 2007; Takano *et al.*, 2009; Derycke *et al.*, 2010). De plus, les analyses de PCR en temps réel et d'immunohistologie démontrent une corrélation entre le niveau de la protéine virale N et la nectine-4 dans les tissus de la trachée et des poumons de macaques infectés par le MeV du type sauvage (Muhlebach *et al.*, 2011). Les résidus de la protéine H de MeV qui permettent l'entrée du virus dans les cellules épithéliales polarisées sont: L482, F483, P497, Y541 et Y543. Ils se regroupent tous dans la même région qui fixe SLAM et CD46 (Fig. 1.4) (Leonard *et al.*, 2008; Tahara *et al.*, 2008; Takeda, 2008).

4.1.6 Pathogenèse morbillivirale

Les morbillivirus sont des agents pathogènes hautement contagieux qui sont transmis par aérosol (Griffin, 2007). Une particule infectieuse entre en contact avec l'hôte via le tractus respiratoire supérieur et cible les DCs et les macrophages alvéolaires dans les muqueuses (Fig. 1.5A) (de Swart *et al.*, 2007; Lemon *et al.*, 2011). Les cellules infectées franchissent l'épithélium respiratoire et migrent vers les ganglions lymphatiques, où le virus établie sa première phase de réplication dans les lymphocytes B

et T activés exprimant SLAM (de Swart *et al.*, 2007; de Vries *et al.*, 2010). Ces lymphocytes porteurs de virus se disséminent dans la circulation sanguine pour se rendre dans les autres ganglions lymphatiques, la rate, le thymus et les plaques de Peyer (Fig.1.5B) (de Swart *et al.*, 2007; de Vries *et al.*, 2010). Le virus se réplique de façon massive dans ces tissus lymphoïdes provoquant une immunosuppression profonde caractérisée par une réduction du nombre de leucocytes et une inhibition de la prolifération lymphocytaire. L'hôte est ainsi plus susceptible à des infections secondaires bactérienne et virale (Schneider-Schaulies *et al.*, 2009). L'augmentation de la charge virale mène par la suite à la dissémination du virus dans les tissus endothéliales et épithéliales du tractus gastro-intestinal, du foie, des reins, de la vessie, de la peau et des poumons (Fig. 1.5C) (de Swart *et al.*, 2007; de Vries *et al.*, 2010; Ludlow *et al.*, 2010). L'apparition des signes cliniques coïncide avec le début de la réponse immunitaire de l'hôte qui est caractérisée par une forte fièvre, de la toux, du coryza, des conjonctivites et des éruptions cutanées (de Swart *et al.*, 2007; de Vries *et al.*, 2010). Les lymphocytes infectés atteignent les muqueuses de la voie respiratoire et le virus se réplique dans les cellules épithéliales (de Vries *et al.*, 2010; Ludlow *et al.*, 2010). De nouvelles particules infectieuses sont relâchées par le côté apical de l'épithélium respiratoire (Leonard *et al.*, 2008). Le virus est dispersé dans l'environnent et transmis à un nouvel hôte (Fig. 1.5D).

4.1.7 Le rôle de l'infection des cellules épithéliales dans la pathogenèse morbillivirale

Les premières cibles morbillivirales sont les DCs et les macrophages alvéolaires exprimant SLAM qui se retrouvent à la surface des muqueuses respiratoires (Lemon *et al.*, 2011). À l'aide de virus exprimant une protéine fluorescente verte (EGFP), des études ont montré qu'en effet les cellules épithéliales de la voie respiratoire sont infectées seulement après l'infection des tissus immunitaires, et pas lors de la phase précoce de l'infection (de Vries *et al.*, 2010; von Messling *et al.*, 2004). De plus, Leonard *et al.* ont construit un virus recombinant exprimant un mutant de la protéine H qui est incapable de reconnaître le récepteur des cellules épithéliales (*epithelial receptor-blind*; EpR-blind) (2008). Les macaques rhésus infectés par EpR-blind MeV ont développé une immunosuppression similaire à celle qu'observée lors d'une infection avec un virus de

type sauvage. Par contre, aucun virus n'a été détecté dans les échantillons de lavage broncho-alvéolaire des animaux infectés, ce qui suggère que l'infection des cellules épithéliales est nécessaire pour libérer le virus dans la voie respiratoire mais n'est pas impliquée dans la suppression de la réponse immunitaire (Leonard *et al.*, 2008). De plus, il a été démontré que MeV infecte efficacement les cellules épithéliales polarisées des poumons via le coté basolatéral, où la nectine-4 est exprimée, et de nouveaux virions sont relargués uniquement du côté apical (Leonard *et al.*, 2008; Ludlow *et al.*, 2010). Ces résultats ont permis de réajuster le modèle de la pathogenèse du MeV, où le relâchement du virus et la transmission nécessite préalablement l'infection des cellules épithéliales.

4.2 Hypothèse et objectifs de recherche

L'introduction d'un vaccin vivant atténué et la mise en place d'un programme de vaccination universelle par l'Organisation mondiale de la Santé (OMS) ont permis de diminuer la morbidité et la mortalité associée à MeV de manière drastique (Moss *et al.*, 2012). Malgré cela, MeV reste la principale cause de décès chez les enfants de moins de 5 ans due à la naïveté du système immunitaire de ces jeunes enfants, à la malnutrition et au manque de ressources logistiques et financières dans les pays en voie de développement (WHO, 2011). Réduire la transmissibilité de ces virus hautement contagieux contribuera ainsi aux efforts visant à contrôler et à éradiquer MeV à travers le monde.

Les études sur la pathogenèse morbillivirale sont principalement basées sur l'infection des primates non-humain, tel que les macaques rhésus et cynomolgus. Cependant, MeV induit généralement une maladie bénigne et de courte durée chez ces animaux, et la réplication virale se limite essentiellement aux tissus immunitaires. Il est donc difficile d'évaluer la contribution de l'infection des cellules épithéliales dans la pathogenèse dans ce modèle animal. En revanche, CDV provoque une grave maladie chez les furets qui est souvent accompagnée d'une infection massive de l'épithélium. Ces animaux sont très sensibles à l'infection par le CDV et succombent à la maladie dans les 2 à 3 semaines suivant l'infection. De plus, un CDV recombinant exprimant la protéine EGFP est disponible et permet une évaluation visuelle de la propagation du virus au cours de l'infection.

Le but de ce projet de recherche est de caractériser les résidus de protéine H du CDV qui interagissent avec le récepteur épithéliale (EpR) canin, et de déterminer la contribution de l'infection des cellules épithéliales dans la pathogenèse du CDV chez les furets. Étant donné que l'utilisation du récepteur SLAM est hautement conservée parmi les morbillivirus, nous avons émis l'hypothèse que le mécanisme d'interaction avec le EpR est également conservé entre MeV et CDV, et aussi que la même région de la

protéine H est impliquée dans cette interaction. Pour tester notre hypothèse, nous avons établi trois objectifs:

1. Identifier les résidus de la protéine H impliqués dans l'entrée des cellules épithéliales
2. Produire un CDV EpR-blind
3. Caractériser la pathogenèse du CDV EpR-blind chez les furets

4.3 Résumé de l'article

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Canine Distemper Virus Epithelial Cell Infection is Required for Clinical Disease but not for Immunosuppression

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Afin de caractériser l'importance de l'infection des cellules épithéliales dans la pathogenèse morbillivirale, nous nous sommes servis du modèle d'infection du CDV chez le furet qui reproduit une maladie similaire à celle induite par le MeV chez les humains. Afin d'obtenir un virus recombinant du CDV incapable d'entrer dans les cellules épithéliales mais qui conserve sa capacité d'infecter les cellules immunitaires, nous avons transféré deux mutations dans la protéine d'attachement H qui interfèrent dans l'interaction du MeV avec le EpR humain, la nectine-4. Le CDV recombinant incapable de reconnaitre le EpR canin (*epithelial receptor-blind*; EpR-blind) infecte inefficacement les cellules épithéliales de chien et de furet, ne cause pas de fusion cellulaire et ne produit pas de syncytia. D'autre part, le CDV EpR-blind se réplique dans les cellules exprimant le récepteur immunitaire des morbillivirus, SLAM, avec une cinétique comparable à celle du virus de la souche de type sauvage. Les furets infectés par la souche de type sauvage du CDV meurent de 12 à 14 jours post-infection après l'apparition des signes cliniques tels que la fièvre et les éruptions cutanées, alors que les

furets infectés par le CDV EpR-blind ne montrent aucun signe clinique associé à la maladie. Toutefois, ces deux virus se propagent rapidement et efficacement dans les cellules immunitaires, induisant des niveaux similaires de leucopénie et d'inhibition de la prolifération lymphocytaire qui sont des marqueurs d'immunosuppression. L'infection des cellules épithéliales de la voie respiratoire a été démontrée chez des furets infectés par le CDV de type sauvage, mais pas chez ceux qui ont été infectés par le CDV EpR-blind, et seuls les furets infectés par le virus de type sauvage ont de nouvelles particules virales relâchées dans leurs voies respiratoires. Par conséquent, l'infection des cellules épithéliales est nécessaire à la progression de la maladie et au relâchement du virus dans la voie respiratoire, mais n'est pas impliquée dans l'immunosuppression.

4.4 Discussions et conclusion

Caractérisation du domaine de la protéine H impliqué dans l'infection des cellules épithéliales. Nous avons identifié dans notre étude trois résidus (V478, P493 et Y539) de la protéine H du CDV qui sont impliqués dans l'entrée du virus dans les cellules épithéliales de chien et de furet. Ceux-ci correspondent aux même acides aminés de la protéine H du MeV qui interagissent avec le récepteur des cellules épithéliales, nectine-4 (Leonard *et al.*, 2008; Muhlebach *et al.*, 2011). De plus, un autre groupe de chercheurs ont identifié onze résidus de la protéine H d'une souche neurovirulente du CDV, A75/17, qui permet la fusion des cellules primaires de kératinocytes canins (Langedijk *et al.*, 2011). À l'aide d'anticorps reconnaissant les épitopes de conformation, les auteurs ont montré qu'aucune de ces mutations EpR-blind n'affectaient la structure globale de la protéine H (Langedijk *et al.*, 2011). En plus des résidus V478 et Y539, Langedijk *et al.* proposent trois autre résidus : L479, I494 et Y537 qui sont tous localisés à proximité des acides aminés qui interagissent avec le EpR canin (Leonard *et al.*, 2008; Tahara *et al.*, 2008; Langedijk *et al.*, 2011; Sawatsky *et al.*, 2012).

Bien que le résidu P493 soit hautement conservé parmi les morbillivirus, Langedijk *et al.* l'a exclu de leur analyse puisqu'un modèle structural de CDV H indique que cet acide aminé est dissimulé à l'intérieur du domaine globulaire. Sa disponibilité pour la liaison au récepteur est donc peu probable. En revanche, le résidu avoisinant, I494, qui est exposé à la surface a été muté et a donné lieu à un phénotype EpR-blind (Langedijk *et al.*, 2011). La caractérisation de nos mutants de la protéine H a révélé qu'effectivement l'expression du mutant P493S à la surface cellulaire est à la baisse (Sawatsky *et al.*, 2012). Une réduction des protéines de surface pourrait donc contribuer à ce phénotype EpR-blind, et ce qui suggère que l'interaction avec le récepteur épithélial nécessite plusieurs protéines H pour la stabilisation.

La structure tridimensionnelle du CDV H indique que l'emplacement des résidus pour la liaison au EpR est situé sur le côté du domaine globulaire, où se fixe SLAM, mais les acides aminés employés par chaque récepteur cellulaire sont distincts de l'un à l'autre (von Messling *et al.*, 2005; Zipperle *et al.*, 2010; Langedijk *et al.*, 2011). Puisque l'utilisation du récepteur immunitaire SLAM est conservée chez les morbillivirus et que

les mêmes résidus du MeV et CDV H sont également impliqués dans la liaison au EpR, ceci suggèrent fortement que l'identité du récepteur des cellules épithéliales canin pourrait être une protéine apparentée à la protéine de jonction adhérentes, nectine-4.

L'infection des cellules épithéliales n'est pas requise pour l'induction de l'immunosuppression virale, mais elle est nécessaire au développement de la maladie et au relâchement du virus dans le tractus respiratoire. Dans la phase précoce de l'infection, le virus se réplique principalement dans les cellules immunitaires exprimant SLAM induisant une leucopénie et une inhibition de la prolifération lymphocytaire, et causant ainsi une profonde immunosuppression (de Swart *et al.*, 2007; Schneider-Schaulies *et al.*, 2009; Lemon *et al.*, 2011). Dès que la charge virale a atteint son point culminant, l'apparition des signes cliniques tel que la fièvre et les éruptions cutanées coïncident avec l'infection de cellules épithéliales dans tous les organes du corps (von Messling *et al.*, 2004; de Swart *et al.*, 2007; de Vries *et al.*, 2010; Ludlow *et al.*, 2010). Dans une étude récente sur la pathogenèse du MeV, des macaques rhésus infectés par MeV EpR-blind étaient immunosupprimés, mais aucun virus n'a été détecté dans la voie respiratoire de ces animaux, suggérant que l'infection des cellules épithéliales est nécessaire au relâchement du virus (Leonard *et al.*, 2008).

Bien que les macaques reproduisent les mêmes signes cliniques que la rougeole chez l'homme, la gravité de la maladie est généralement bénigne et de courte durée, ce qui rend difficile d'évaluer la contribution de l'infection des cellules épithéliales dans la pathogenèse de ces animaux. En revanche, les furets développent une maladie aiguë lors de l'infection par le CDV de type sauvage et ces animaux développent des rougeurs et éruptions cutanées sur le menton, la bouche et l'abdomen, ce qui en font un modèle plus sensible pour la caractérisation de l'infection des cellules épithéliales (von Messling *et al.*, 2003; von Messling *et al.*, 2004). Nous avons observé que les furets infectés avec un CDV EpR-blind avaient des charges virales et un niveau d'immunosuppression similaire aux animaux infectés par le CDV de type sauvage (Sawatsky *et al.*, 2012). Cependant, ils ne présentent pas de signes cliniques associés à la maladie tels que la perte de poids, la fièvre ou les éruptions cutanées. Enfin, aucun virus n'a été détecté dans les tissus épithéliaux de la trachée, des poumons ou de la vessie (Sawatsky *et al.*, 2012). En

résumé, nous avons montré que l'infection des cellules épithéliales a peu ou aucun rôle dans l'établissement initial de l'infection et dans la dissémination systémique du virus, mais elles contribuent plutôt au développement des signes cliniques de la maladie, au relâchement du virus dans la voie respiratoire et à la transmission à un nouvel hôte (Sawatsky *et al.*, 2012).

Les épithéliums intestinaux, gastriques et des voies respiratoires représentent une barrière de protection importante contre les agents pathogènes opportunistes. Les infections secondaires qui surviennent lors de la phase immunosuppressive contribuent potentiellement à la mortalité associée aux infections morbillivirales. La gastro-entérite, la pneumonie et la septicémie sont des causes fréquentes de décès chez les furets infectés par le CDV de type sauvage (von Messling *et al.*, 2003). Bien que les furets infectés avec CDV EpR-blind ont une virémie et ont développé une profonde immunosuppression au jour 7 post-infection, aucun signes cliniques n'a été observé chez ces animaux et ils ont survécu à l'infection (Sawatsky *et al.*, 2012). L'absence de virus dans les épithéliums intestinaux, gastriques et des voies respiratoires suggère que la réplication du virus dans les cellules épithéliales sert à perturber les jonctions de l'épithélium, détruisant ainsi l'intégrité de cette barrière de protection ce qui donne accès à des agents pathogènes opportunistes. Les infections secondaires qui en résultent peuvent alors conduire à des graves complications et même la mort.

Abolir l'interaction avec le récepteur des cellules épithéliales représente une nouvelle stratégie visant à réduire la transmission du morbillivirus. MeV est très contagieux, puisqu'une personne infectée peut transmettre le virus à deux ou trois autres individus susceptibles, et la chaîne d'infection peut alors être soutenue et amplifiée davantage au sein d'une population à faible immunité, ce qui déclenche des épidémies (Moss *et al.*, 2006). Ainsi, les médicaments antiviraux qui interfèrent avec l'entrée du virus dans les cellules épithéliales représentent une nouvelle stratégie pour interrompre la transmission de ce virus hautement contagieux. Ceci a également une implication particulière dans le développement des thérapies oncolytiques basés sur MeV (Cattaneo, 2010). Les virus peuvent être conçus pour cibler un récepteur spécifiquement exprimé par

les cellules cancéreuses, et cette spécificité est augmentée lorsque les interactions avec les récepteurs naturels, SLAM et nectine-4, sont abrogées (Schneider *et al.*, 2000; Cattaneo, 2010). En outre, un virus qui ne se lie pas à la nectine-4 ne sera pas relâché et il ne pose donc aucun risque de transmission à des personnes susceptibles, le rendant encore plus sécuritaire.

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APPENDIX II: Additional Contribution

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

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Adherens Jonction Protein Nectin-4 is the Epithelial Receptor for Measles Virus

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Contribution

1 contributed to the histology work including organ processing, tissue sectioning, immunofluorescent stainings and took pictures of the tissue sections presented in the figure 4 ofthis manuscript.

Résumé de l'article

Le virus de la rougeole (MeV) est un agent pathogène transmit par aérosol qui infecte plus de 10 millions d'enfants par année entraînant approximativement 120,000 décès. L'infection de l'épithélium respiratoire était reconnue comme étant l'étape qui précède la dissémination virale. Cependant, des recherches ont récemment montré que MeV cible initialement les monocytes et les cellules dendritiques à l'aide du récepteur SLAM (CD ISO). Ces cellules infectées traversent l'épithélium respiratoire et transportent le virus vers les tissus lymphatiques où il se réplique intensément. Le mécanisme de sortie du MeV à travers l'épithélium respiratoire est encore inconnu. Grâce aux analyses des protéines de surface exprimées par des lignées de cellules épithéliales humaines permissives à MeV, les auteurs ont identifié nectine-4 *(poliovints-receptor-/ike-4;* PVRL-4) comme récepteur cellulaire potentiel qui participerait à la relâche du virus dans la voie respiratoire. Cette protéine de jonction d'ancrage de la superfamille des immunoglobulines interagit avec le domaine extracellulaire de la protéine d'attachement virale H avec une haute affmité. Nectine-4 permet aussi l'entrée et la propagation de MeV dans une couche de cellules épithéliales polarisées lorsque ce dernier est infecté par le coté basolatéral. La molécule nectine-4 est régulée à la baisse dans les cellules épithéliales et dans la trachée respiratoire des macaques infectés par MeV. La plupart des virus respiratoires doivent interagir avec un récepteur pour pénétrer dans un hôte, alors qu'ici les auteurs proposent que MeV se lie à nectine-4 pour quitter l'hôte par la voie respiratoire. Nectine-4 est aussi un marqueur cellulaire pour plusieurs types de cancers, et a une grande implication dans plusieurs études cliniques d'oncolyse basées sur MeV présentement en cours.