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MEASLES VIRUS INFECTION

Interplay between virus and host cells

by

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To my family

ABSTRACT

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Measles virus infection – Interplay between virus and host cells
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Measles, caused by measles virus (MV), is a highly contagious viral disease causing severe respiratory infection and a typical rash. Despite the availability of a protective vaccine, measles is still the leading vaccine-preventable cause of childhood mortality worldwide. The high mortality associated with the disease is mainly due to an increased susceptibility to secondary infections during the period of immunosuppression that continues for several weeks after recovery.

The present study was undertaken to elucidate the role of cytoskeletal components in the regulation of MV infection. The most interesting finding was that MV replication was activated in unstimulated peripheral blood mononuclear cells (PBMC) when globular actin was converted into the filamentous form with jasplakinolide. This provides a new aspect in our understanding of MV infection in PBMC.

In the second part of the thesis we investigated MV-induced structural changes of cellular nuclear matrix, which is a proteinaceous framework of the nucleus similar to the cytoskeleton in the cytoplasm. We showed that cleavage of nuclear markers was virus-specific and a general caspase inhibitor rescued MV-infected cells from cell death.

Furthermore, we studied MV-induced innate immune mechanisms in lung epithelial and endothelial cells. Our results showed that MV infection resulted in activation of the double stranded RNA (dsRNA) binding molecules melanoma differentiation-associated gene 5 (mda-5), retinoic acid inducible gene I (RIG-I), and toll-like receptor 3 (TLR3) gene expression, followed by high expression of antiviral cytokine mRNA.

Keywords: measles virus (MV), actin filaments, microtubules, PBMC, innate immunity, mda-5, RIG-I, TLR, NuMA, lamins, caspase

TIIVISTELMÄ

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Viruksen ja solun välinen vuorovaikutus tuhkarokkovirusinfektiossa
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Tuhkarokko, jonka aiheuttaa tuhkarokkovirus, on lapsuusajan tarttuva virustauti ja sen yleisimmät oireet ovat kuumeinen hengitystieinfektio ja tyypillinen ihottuma. Vaikka suojaava rokote onkin olemassa, tuhkarokko on edelleen maailmanlaajuisesti merkittävä pienten lasten kuolinsyy. Korkea kuolleisuus johtuu usein akuutin infektion jälkeisessä immunosuppressiovaiheessa saaduista jälkitaudeista.

Tämän tutkimuksen tarkoitus oli selvittää solun tukirankarakenteiden merkitystä tuhkarokkovirusinfektion säätelyssä. Aktiinifilamenteillä on tärkeä merkitys viruksen lisääntymisessä. Mielenkiintoinen löydös oli, että aktiinifilamenttien stabiloiminen jasplakinolidilla aktivoi viruksen lisääntymisen lepäävissä perifeerisen veren mononukleaarisoluissa, joissa replikaatio muuten on hyvin rajoittunut.

Tutkimuksen toisessa osassa selvitimme tuhkarokkovirusinfektion aiheuttamia muutoksia tuman rakenteellisessa perusaineessa. Osoitimme, että tumaproteiineja pilkotaan virusspesifisesti ja käyttämällä yleistä kaspaasi-inhibiittoria pilkkoutuminen estyi ja solut säästyivät apoptoosilta.

Lisäksi tutkimme tuhkarokkoviruksen indusoiman synnynnäisen immuniteetin mekanismeja epiteeli- ja endoteelisoluissa. Tuloksemme osoittavat, että tuhkarokkovirusinfektio aikaansai kaksisäikeiseen RNA:han sitoutuvien molekyylien mda-5 (melanoma differentiation-associated gene 5), RIG-I (retinoic acid inducible gene I) ja TLR3 (toll-like receptor 3) geenien ekspression aktivoitumisen, joka johti antiviraalisten sytokiinien korkeaan lähetti RNA:n tuottoon.

Avainsanat: tuhkarokkovirus, aktiinifilamentit, mikrotubulus, mononukleaariset solut, synnynnäinen immuniteetti, mda-5, RIG-I, TLR3, NuMA, lamiinit, kaspaasi

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I to IV.

I Berghäll H, Wallen C, Hyypiä T, and Vainionpää R (2004). Role of cytoskeleton components in measles virus replication. *Archives of Virology* 2004 May;149:891-901.

II Berghäll H, and Vainionpää R. Formation of filamentous actin activates measles virus replication in unstimulated peripheral blood mononuclear cells. *Submitted as short communication*

III Taimen P, Berghäll H, Vainionpää R, and Kallajoki M (2004). NuMA and nuclear lamins are cleaved during viral infection - inhibition of caspase activity prevents cleavage and rescues HeLa cells from measles virus-induced but not from rhinovirus 1B-induced cell death. *Virology* 3201:85-98.

IV Berghäll H, Siren J, Sarkar D, Julkunen I, Fisher PB, Vainionpää R, and Matikainen S (2006). The interferon-inducible RNA helicase, mda-5, is involved in measles virus-induced expression of antiviral cytokines. *Microbes and Infection* 88:2138-2144.

ABBREVIATIONS

ATCC	American type culture collection
CARD	caspase recruitment domain
CNS	central nervous system
ER	endoplasmic reticulum
F	fusion protein
F-actin	filamentous actin
FCS	fetal calf serum
G-actin	globular actin
H	hemagglutinin protein
HRP	horseradish peroxidase
HRV	human rhinovirus
HUVEC	human umbilical vein endothelial cells
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
JAK	Janus kinase
jaspla	jasplakinolide
L	large protein
lat-A	latrunculin-A
M	matrix protein
mda-5	melanoma differentiation-associated gene 5
m.o.i.	multiplicity of infection
MV	measles virus
N	nucleoprotein
NF-κB	nuclear factor-κB
nt	nucleotide
noc	nocodazole
NuMA	nuclear mitotic apparatus protein
P	phosphoprotein
PARP-1	poly(ADP-ribose) polymerase-1
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PFU	plaque forming unit
PHA	phytohemagglutinin
PRR	patter recognition receptor
RIG-I	retinoic acid inducible gene I
SLAM	signal lymphocyte activation molecule
SSPE	subacute sclerosing panencephalitis
STAT	signal transducer and activator of transcription
TCID	tissue culture infective dose
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	terminal deoxynucleotidyl transferase (TdT)–mediated dUTP Nick-End Labelling
z-VAD-FMK	benzyloxylcarbonyl-Val-Ala-Asp-fluoromethylketone

1. INTRODUCTION

Measles is a typical childhood disease causing severe respiratory infection and a rash. In industrialized countries, where it has been possible to keep vaccination coverage high, measles is very rare, but world wide it remains a leading cause of preventable death. The disease can be very severe especially for malnourished children and those with a weakened immune system. The high mortality associated with measles is mainly due to secondary infections during the profound period of immune suppression continuing for weeks after recovery. The World Health Organization (WHO) estimates that more than 20 million people are affected by measles annually and still nearly 250 000 deaths occur, the majority being children. Globally measles mortality has decreased by 68% during this decade and the global average for routine measles immunization coverage was 80%. The next goal is to reduce deaths by 90% from 2000 levels by 2010 (WHO, 2007).

Measles is a highly contagious acute illness caused by measles virus (MV), a member of the Morbillivirus genus in the subfamily of *Paramyxovirinae* of the *Paramyxoviridae* family. It is a human disease with no known animal reservoir. Measles virus is an enveloped, negative-sense RNA virus replicating entirely in the cytoplasm of the host cell. It normally propagates in the cells of the back of the throat and the lungs and spreads through the lymph nodes to the blood and several organs in the body replicating mainly in endothelial and epithelial cells, as well as in monocytes and macrophages. Ever since the finding of the first evidence of the MV-induced immune suppression one hundred years ago (von Pirquet, 1908) many studies have focused on the mechanisms underlying it, but so far these are yet not completely understood. Infection of the cells of the immune system, the differentiation stage of the host cell, and apoptosis have all been suggested to play a role in this clinically relevant phase after the acute disease. In this thesis, the interplay between MV and the host cell have been studied in order to gain a better understanding of the complex nature of MV infection.

2. REVIEW OF THE LITERATURE

2.1. Measles virus

2.1.1. Structure of measles virus

Measles virus (MV) is an enveloped negative-sense RNA virus belonging to the Morbillivirus genus in the subfamily of *Paramyxovirinae* of the *Paramyxoviridae* family. The virions are pleomorphic in shape with a size range of 100-300 nm in diameter. An envelope containing virus specific glycoprotein spikes and lipids derived from the plasma membrane of the host cell surrounds the nucleocapsid core. The lack of neuraminidase activity of the envelope glycoproteins is unique for morbilliviruses distinguishing them from the other members of the subfamily.

The genome of MV is a 15894-nucleotide-long nonsegmented, single-stranded RNA with negative polarity (Fig. 1). At the 3' end is a short leader sequence and at the 5' end a trailer sequence, which have no coding capacity and are believed to function as regulators of transcription and replication. The 3' noncoding region is thought to contain a recognition site for the RNA polymerase complex (Horikami & Moyer, 1991), which sequentially initiates transcription of the viral genes. New data suggests that it is also involved in triggering the early interferon response when recognized by the recently described RNA helicase retinoic acid inducible gene I (RIG-I, Plumet et al., 2007). Following the leader sequence are the six nonoverlapping genes encoding for the structural proteins N, P, M, F, H, and L. In addition, the P gene encodes for two nonstructural proteins, C and V (Griffin, 2007).

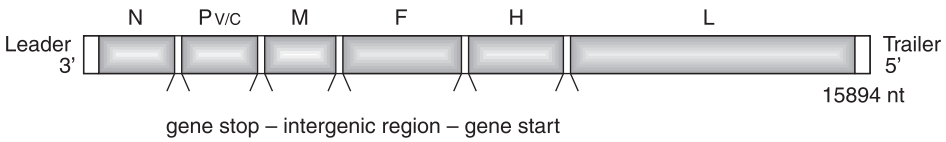

Figure 1. Schematic presentation of the measles virus RNA genome.

In the viral particle (Fig. 2), as well as in infected cells, the genomic RNA is encapsidated by the nucleoprotein (N), phosphoprotein (P), and large protein (L) forming the viral ribonucleocapsid structure. The P and L proteins together form the RNA-dependent RNA polymerase needed for both mRNA transcription and genome replication. The L protein functions as the catalytic component of the polymerase, whereas the P protein binds to L and N proteins and RNA to form the replicase complex. In addition to the phosphoprotein, the P gene also encodes for two nonstructural proteins C and V not found in the virion.

These are believed to regulate transcription and replication and interact with cellular proteins possibly modulating the intracellular environment (Liston et al., 1995, Tober et al., 1998). The envelope surrounding the nucleocapsid carries the transmembrane hemagglutinin (H) and fusion (F) glycoproteins, and the interior of the envelope is lined by the matrix (M) protein mediating the contact between the nucleocapsid (Hirano et al., 1992, 1993) and the glycoproteins (Cathomen et al., 1998b, Naim et al., 2000, Spielhofer et al., 1998). The F protein and the receptor binding H protein are both needed for fusion and entry (Cattaneo & Rose, 1993, Wild et al., 1991, Zhang et al., 2005). Fusion of the infected cells is a typical feature of all paramyxoviruses and the F protein is the central mediator of this process. It is synthesized as an inactive precursor protein F_0 which, after glycosylation, is transported to the plasma membrane and cleaved by a proteolytic cleavage involving host cell protease furin (Bolt & Pedersen, 1998, Watanabe et al., 1995) to yield the activated disulphide-linked F_1 and F_2 proteins. F_2 has all the predicted glycosylation sites (Alkhatib et al., 1994, Hu et al., 1995), whereas the new amino terminus of F_1 contains the region suggested to be important for the fusion process (Richardson et al., 1986). The cell-to-cell fusion gives rise to the formation of multinuclear giant cells, the syncytia, and this is at least partially regulated by the M protein (Cathomen et al., 1998a, 1998b). The syncytia give the typical cytopathic effect of MV infection. They are metabolically active and have been proposed to contribute to antiviral immune response in epithelial cells and mature dendritic cells since the fusiogenic activity of MV was shown to correlate with type I interferon (IFN) production (Herschke et al., 2007).

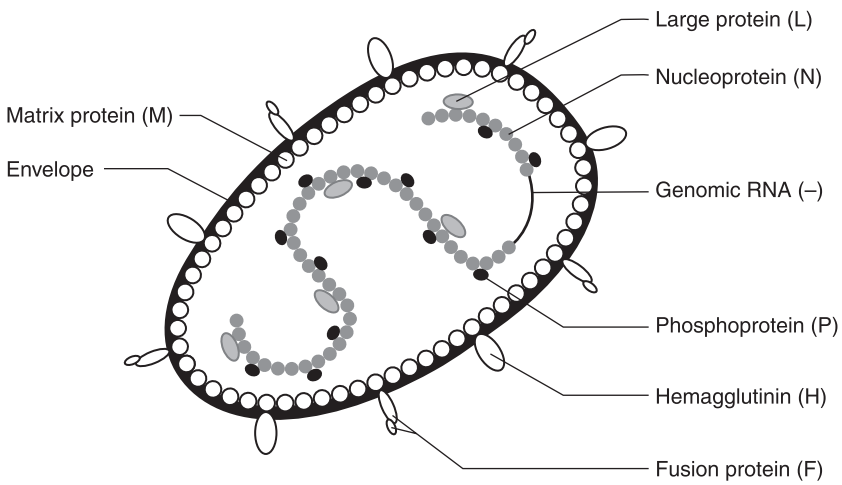

Figure 2. Structure of MV. The nucleocapsid structure is formed by the genomic RNA encapsidated by the N, P and L proteins. M protein is found at the inner surface of the lipid envelope, which also carries the glycoproteins F and H.

2.1.2. Measles virus life cycle

As for all members in the *Paramyxoviridae* family, MV replication occurs entirely in the cytoplasm of the host cell (Fig. 3). Upon receptor recognition and attachment, the envelope is fused to the cell membrane and the nucleocapsid is released to the cytoplasm and replication can begin. The genomic RNA of negative-sense RNA viruses has two functions: to serve as a template for primary transcription of mRNAs and as a template for synthesis of the antigenome (+) strand. For both purposes it uses its own viral RNA polymerase. mRNA synthesis is initiated at the N gene start signal and the genes are each separated by a trinucleotide intercistronic junction, CUU (Dowling et al., 1986, Rima et al., 1986). The polymerase ends transcription at the stop signal and restarts after the junction at the start signal of the following gene, thereby synthesizing the capped and polyadenylated monocistronic mRNAs, which subsequently are translated. The P gene is an exception since it not only produces the full-length copy P mRNA but also the C and V mRNAs, by alternative translational initiation at an overlapping reading frame and by a process of RNA editing, respectively (Bellini et al., 1985, Cattaneo et al., 1989). The efficiency of transcription is weaker towards the 5´end as the polymerase occasionally detaches from the template at the junctions, resulting in substantially less L mRNA as compared to N mRNA (Cattaneo et al., 1987a, 1987b, Ray & Fujinami, 1987). Protein synthesis begins early on after virus infection and is apparently required for genome replication.

For replication of the (-) genome, the viral polymerase must ignore the stop signals at the gene boundaries to produce a full-length complementary copy called antigenome. The only function of this (+) intermediate strand is to serve as a template to new genomic RNA. There have been no structural differences noted in the viral polymerase working as a transcriptase to generate mRNAs compared to functioning as a replicase to generate nascent genomic RNA. This nascent RNA is immediately encapsidated by N protein. Transition from mRNA transcription to RNA replication seems to be dependent on the availability of unassembled N protein, suggesting a straightforward self-regulatory mechanism (Horikami & Moyer, 1995, Wild & Buckland, 1995) but the details for these events, however, still remain unclear.

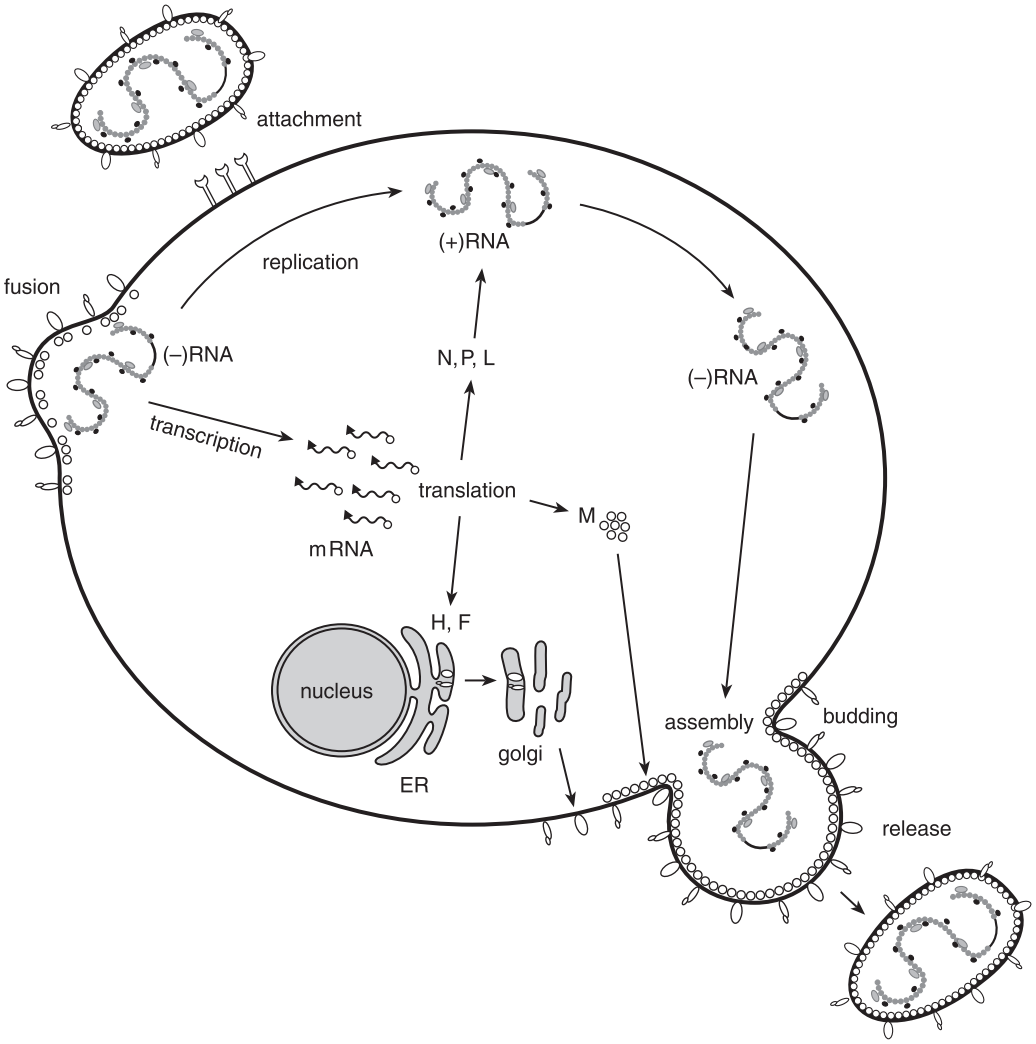

Figure 3. Schematic presentation of MV life cycle. MV replication takes place entirely in the cytoplasm of the host cell. Incoming virus recognizes receptors on the cell surface and following attachment the envelope is fused with the plasma membrane to release the nucleocapsid with negative sense RNA. This genomic (-) RNA serves as a template both for primary transcription of mRNAs and for synthesis of the antigenome (+) strand using its own viral RNA polymerase for both purposes. Nascent RNA is encapsidated by N, P, and L proteins to form new nucleocapsid structures. H and F proteins are transported to the ER for glycosylation, to the Golgi apparatus where F is cleaved and further to the plasma membrane. The M protein lines the inner side of the plasma membrane and is believed to be essential in the assembly process by attracting the glycoproteins as well as the nucleocapsids. New virions are released by a budding process.

The final assembly of the virus particle takes place at the plasma membrane. The glycoproteins H and F are glycosylated in the endoplasmic reticulum (ER). They are then assisted by several folding enzymes and molecular chaperones of the host cell to undergo conformational maturation (Plemper et al., 2001) before transported out of the ER to

the Golgi apparatus, where F_0 is cleaved and H undergoes oligosaccharide modification (Watanabe et al., 1995, Wild & Buckland, 1995). From the Golgi the glycoproteins are transported to the plasma membrane where they anchor by hydrophobic amino acid residues thereby leaving the cytoplasmic tails to attract M protein. In infected cells M protein forms a continuous layer on the inner surface of the plasma membrane and interacts with the cytoplasmic domains of the glycoproteins (Cathomen et al., 1998b, Spielhofer et al., 1998) as well as with the nucleocapsids (Hirano et al., 1993) to initiate virus assembly and the budding process. M proteins are believed to play a major role in this, yet not fully understood, assembly process by directing the newly synthesized nucleocapsids to the plasma membrane. The current model suggests that mature nucleocapsids accumulate in inclusion bodies and later in infection all viral components, the nucleocapsids, the M protein and the glycoproteins, gather at the plasma membrane where budding occurs. Recently, it was suggested that the nucleocapsids are cotransported to the plasma membrane as a complex with M protein, sufficient accumulation of M protein being a prerequisite (Runkler et al., 2007). Cytoskeleton structures are likely to be involved in the transportation of the different viral components and the new virus particles obtain the lipid envelope when they are released from the host cell by budding.

2.1.3. Infection and pathogenesis

Measles virus causes an acute, highly contagious childhood infection that spreads through the respiratory route. Symptoms begin to appear 10 to 12 days after exposure to the virus. The first symptoms are fever followed by cough, runny nose, conjunctivitis and small white spots inside the cheeks (Koplik's spots). The maculopapular rash develops in a few days, usually first on the face and upper neck spreading downward over the next three days, whereafter it starts to fade in the same order and recovery is followed by lifelong immunity to reinfection. Transmission of the virus can occur already four days before the onset of the rash. The high mortality associated with MV infections is mainly due to secondary infections during immunosuppression. Serious complications include blindness, encephalitis, severe diarrhea, ear infections and pneumonia, which is one of the most frequent life-threatening complications associated with measles. The most severe, but relatively rare complication (approx. 1/10 000 cases according to recent estimations, Bellini et al., 2005) is subacute sclerosing panencephalitis (SSPE). This is a fatal degenerative disease of the central nervous system (CNS) that has an average latency period of 7-10 years after infection leading to death 1-3 years after onset of symptoms. The reason for why MV establishes a persistent infection causing SSPE in some individuals is not known, but obtaining the original MV infection at an early age seems to be one risk factor and patients are more often male. Genetic material derived from brain tissue of SSPE patients has shown that

only wild-type MV, never measles vaccine, can cause the fatal disease (Bellini et al., 2005, Halsey et al., 1980, Jin et al., 2002, Zilber et al., 1983).

After the initial infection is established in the upper respiratory tract, the virus is transported to the regional lymph nodes and enters the lymphoid organs and tissues, which are prominent sites of replication. During the ensuing viremia the virus is spread to a large number of organs including the skin, conjunctiva, kidney, lung, gastrointestinal tract and genital mucosa (Griffin, 2007). Currently two host cell molecules have been identified as MV receptors, namely CD46 (Dörig et al., 1993, Naniche et al., 1993) and the human signaling lymphocyte activation molecule (SLAM; also known as CD150, Erlenhöfer et al., 2001, Hsu et al., 2001, Tatsuo et al., 2000). CD46 is a transmembrane glycoprotein involved in complement regulation and it is expressed on all nucleated cells in humans. SLAM, on the other hand, is expressed on various types of cells of the immune system and it is involved in lymphocyte activation. Initially, SLAM was identified as a human T cell activation receptor (Cocks et al., 1995). Generally it has been accepted that laboratory adapted and vaccine strains use both CD46 and SLAM as receptors, whereas wild-type MV strains appear to use only SLAM. Lymphoid organs are major targets of MV and this correlates well with the distribution of SLAM (Yanagi et al., 2006). However, MV spreads through the respiratory route and propagates in lung epithelial cells and other cells that are SLAM negative. It has been suggested, that wild-type MV strains have the ability to bind also to CD46 at a lower affinity (Manchester et al., 2000, Massé et al., 2002), but recently reports with increasing evidence have indicated the possibility of a non-SLAM, non-CD46 receptor (Andres et al., 2003, Hashimoto et al., 2002, Takeda et al., 2007, Takeuchi et al., 2003, Yanagi et al., 2002).

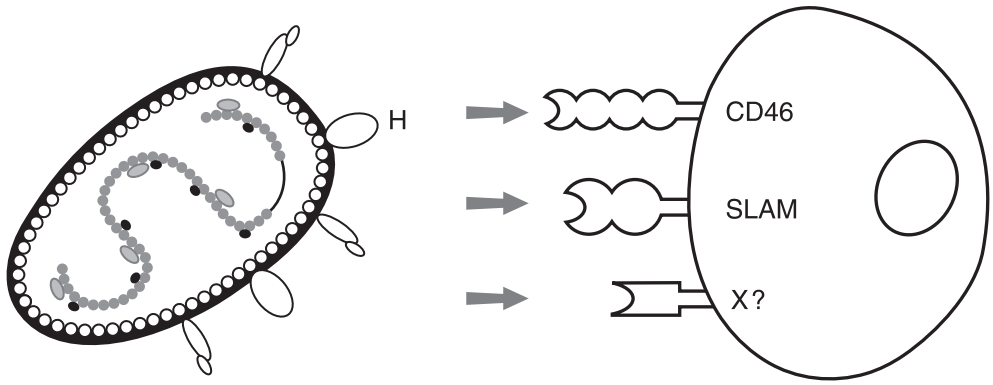

Figure 4. Receptors of MV. Laboratory adapted and vaccine MV strains use both CD46 and SLAM as receptors whereas wild-type MV strains apparently use only SLAM and possibly a yet unidentified molecule (X) as receptors.

2.2. Virus interplay with cellular structures

2.2.1. Cell cytoskeleton

The cytoskeleton is a dynamic structure composed of three primary types of fibers, the microfilaments, microtubules, and intermediate filaments. It gives the cell stability and physically it is associated with molecules involved in chemical signaling events by providing the major trafficking pathways in the cell. However, it also plays an important role in a number of biological processes, including cell movement, morphogenesis, endocytosis, and cytokinesis (Alberts et al., 2002). Practically all viruses use the cytoskeleton at some point in their life cycle; during attachment, internalization, endocytosis, nuclear targeting, transcription, replication, transport of progeny particles, assembly, or release (Radtke et al., 2006).

The individual subunits of actin are termed globular actin (G-actin). As these assemble they form the filamentous actin (F-actin) polymers and two parallel F-actin strands twisted around each other give rise to the microfilaments. The polymerized microfilaments are highly dynamic, constantly being assembled and disassembled, and reorganized as the cell changes shape, divides or attaches to a surface. Polymerization is regulated by several proteins that can bind to either the fast growing, barbed end or to the pointed, slower growing end, thereby inhibiting addition or loss of subunits at the ends. The microfilaments interact with the cell membrane usually by linkage between specific groups of actin binding proteins (Pollard & Borisy, 2003, Winder & Ayscough, 2005).

Microtubules consist of highly ordered structures of polymerized α- and β-tubulin heterodimers forming hollow cylinders. They have a highly dynamic, fast growing plus end and a minus end, which is usually attached to a microtubule-organizing center. The microtubules are components of the cytoplasmic cytoskeleton contributing to cell shape and motility and providing anchoring for protein complexes. They are also found in the mitotic spindle playing a major role during mitosis (Alberts et al., 2002).

Different pharmacological agents can be used to manipulate the cytoskeleton network. By the use of cytoskeletal inhibitors it has become apparent that different viruses use different elements of the cytoskeleton for entry, transportation, replication and virus release (Cudmore et al., 1997, Sodeik, 2000). Latrunculins are actin-modulating drugs (Spector et al., 1983) that have become important tools in cytoskeleton research. Latrunculin-A (lat-A) is highly specific in sequestering monomeric actin (Ayscough et al., 1997). By binding to and performing a complex with G-actin (Coue et al., 1987) it effectively depletes G-actin stores used to make F-actin and thereby it inhibits polymerization and

promotes depolymerization of F-actin. Previously cytochalasins were commonly used to study actin organization. The cytochalasins, however, act via multiple mechanisms to disrupt the actin filaments and their effects are therefore more complex to interpret (Cooper, 1987, Sampath & Pollard, 1991, Spector et al., 1999). Jasplakinolide (jaspla) is a cell-permeable cyclic peptide with actin as a specific functional target. It polymerizes actin by binding to F-actin and stabilizing filaments (Bubb et al., 1994). Nocodazole is a common agent for disrupting the microtubulus structure. It binds to β-tubulin and prevents interchain bonds. In Table I commonly used cytoskeleton modulators are listed.

Table I. Commonly used cytoskeletal modulators and their mode of action.

Cytoskeletal target	Modulator	Mode of action
Actin	Latrunculin-A	Binds to monomeric (G) actin, disrupts polymerization
	Cytochalasins	Bind to filamentous (F) actin, disrupt polymerization
	Jasplakinolide	Binds to and stabilizes F actin
Microtubulus	Nocodazole	Binds to β-tubulin, prevents interchain bonds
	Paclitaxel	Binds to N-terminus of β-tubulin, promotes formation of highly stable microtubules

2.2.2. Nuclear matrix

The cell nucleus is a highly organized, dynamic structure containing the genetic information and it is the site of many important processes such as DNA replication and RNA synthesis. It is surrounded by the nuclear envelope, which is a double membrane that keeps the nuclear content separated from the cytoplasm. The envelope is perforated with nuclear pores to allow and regulate the passage of molecules between the cytoplasm and the nucleus. The inner surface of the membrane has a protein lining called the nuclear lamina (Fig. 5). Nuclear matrix is the dynamic, proteinaceous framework of the nucleus similar to the cytoskeleton in the cytoplasm, and it is defined as the nonchromatin structure of the nucleus. The four main structural components are the nuclear lamina, nuclear pores, nucleoli and the interior filament network. The peripheral nuclear lamina has been described as a support- and shape-giving component of the nucleus (Schirmer et al., 2001, Stuurman et al., 1998) but the role of the interior protein network is not as clear. In addition to providing general support to the nuclear structures, the nuclear matrix also plays a role in several important cellular processes. It has been suggested to be involved in organizing the chromosomes,

localize the genes, and regulate gene expression and DNA replication (Alberts et al., 2002). The nuclear lamina is almost completely composed of interconnected fibrillar proteins called lamins (Aebi et al., 1986). Lamins are members of the intermediate filament protein family possessing a nuclear localization motif, which distinguishes them from other intermediate filaments (Stuurman et al., 1998). Lamins mainly underlie the inner nuclear membrane supporting the nuclear architecture and they are in close contact with several inner nuclear membrane proteins. The lamina interacts directly with chromatins and together with the integral membrane proteins it provides the structural connections between DNA and the nuclear envelope. The cytoskeleton is also associated with the nuclear matrix and may influence directly or indirectly nuclear matrix-DNA interactions.

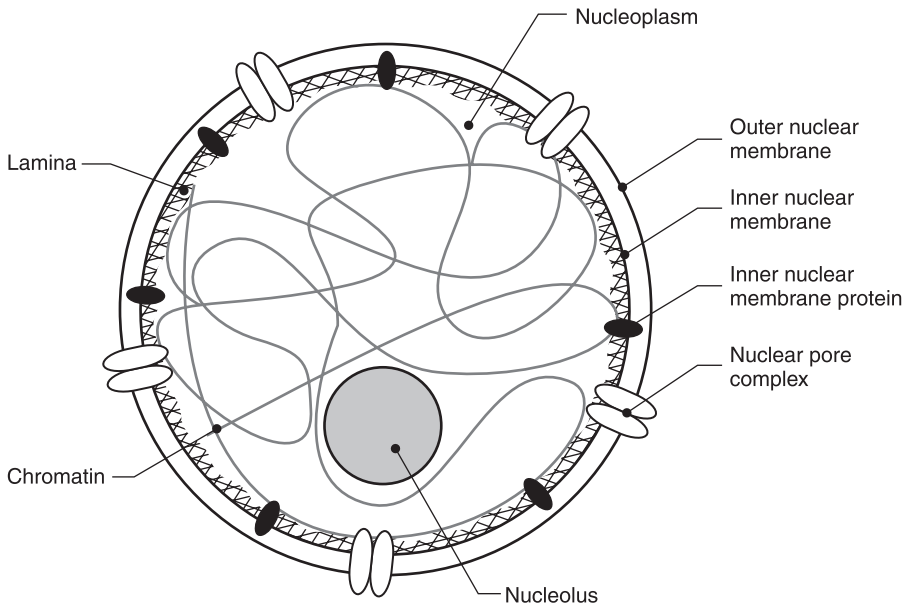

Figure 5. Schematic presentation of the cell nucleus. The major components are the outer and inner membranes, nuclear pores and inner nuclear membrane proteins, lamina, chromatin, and the nucleolus.

Two nuclear matrix components targeted and degraded during apoptosis are Nuclear Mitotic Apparatus protein (NuMA) and nuclear lamins. NuMA plays a structural role during interphase cells and redistributes to the spindle pole regions in mitosis (Kallajoki et al., 1991, Lydersen & Pettijohn, 1980). Several studies show that NuMa is specifically degraded during apoptosis (Greidinger et al., 1996, Gueth-Hallonet et al., 1997, Hirata et al., 1998, Hsu & Yeh, 1996, Taimen & Kallajoki, 2003, Weaver et al., 1996) and also during necrotic cell death (Bortul et al., 2001). The majority of cellular changes during

apoptosis are mediated by cystein proteases with aspartic acid specificity known as caspases (Nicholson & Thornberry, 1997). Lamins were among the first caspase target proteins to be identified and their cleavage has been suggested to be of importance in the apoptotic breakdown of the nuclear structure (Lazebnik et al., 1995, Rao et al., 1996, Takahashi et al., 1996). Caspase-6 has been shown to cleave lamins A and C (Ruchaud et al., 2002), whereas caspase-3 seems to be involved in lamin B cleavage (Slee et al., 2001).

Associations between virus infections and nuclear envelope alterations have recently been published. Most reports concern DNA viruses replicating in the nucleus, examples being cytomegalovirus-induced phosphorylation of lamins (Muranyi et al., 2002), herpes simplex virus type 1-induced disruption of lamina (Scott & O'Hare, 2001), and human immunodeficiency virus-induced herniations of the nuclear envelope (de Noronha et al., 2001). It has also been reported that some RNA viruses replicating in the cytoplasm also may interfere with host cell nuclear functions. Nuclear import of reovirus σ1s protein induced herniations in the nuclear envelope redistributing nuclear pore complexes and lamins, and the authors suggested that this could induce cell cycle arrest during reovirus infection (Hoyt et al., 2004).

2.3. Measles virus infection in cells of the immune system

MV infects cells of the immune system and has been shown to suppress immune functions both in vivo and in vitro. This was first described when von Pirquet (1908) discovered suppressed skin test responses to tuberculin for several weeks after recovery from measles. In vitro, the lymphoproliferative responses to mitogens are suppressed (Sullivan et al., 1975). MV is known to infect all subsets of human peripheral blood mononuclear cells (PBMC), monocytes being the primary cell type (Esolen et al., 1993), and a high proportion of PBMC of patients with SSPE has been reported to contain viral RNA (Fournier et al., 1985). Replication occurs in unstimulated lymphocytes but is highly restricted (Hyypiä et al., 1985). In the absence of mitogenic stimuli monocytes seem to represent the major infected population (Salonen et al., 1988), but the infection in monocytes is not productive, not even upon activation of the cells (Karp et al., 1996, Vainionpää et al., 1991). Helin and coworkers (1999) have shown, that MV replication is dependent on the differentiation stage of myelomonocytic cells, being productive only in immature bone marrow progenitor cells and restricted in mature monocytes/macrophages. Phytohemagglutinin (PHA) is a commonly used mitogen that triggers resting lymphocytes into metabolic activity. Binding of PHA to its receptor is known to activate a group of biochemical reactions associated with inositol phospholipids breakdown, Ca^{2+} mobilization and protein kinase C activation and to cause cell

proliferation. PHA stimulation, as well as, ionophore A23187 and TPA treatments, activate the productive MV infection cycle in PBMC (Hyypiä et al., 1985, Vainionpää et al., 1991), indicating that effective replication requires components that are present only in activated cells. Activation of the cell cycle has been believed to be the main reason for productive MV infection, however, mitogenic stimuli also affect the cytoskeleton structure in lymphocytes, which is to be taken into consideration. In unstimulated lymphocytes, as much as 70% of actin is present in the monomeric, globular form (Cassimeris et al., 1990, Rao, 1984). This is dramatically changed upon stimulation with PHA, which causes a concentration dependent conversion of globular actin to filamentous actin (Rao, 1984). Many paramyxoviruses require cytoskeletal elements for different stages of their life cycle and they were indeed among the first viruses to be shown to contain actin in the virion (Lamb et al., 1976, Mountcastle & Choppin, 1977, Tyrrell & Norrby, 1978).

2.3.1. Innate immunity

MV infection leads to a strong, temporary immunosuppression during the acute phase of infection, affecting both the innate and adaptive immune systems. Activation of the innate immune system is the first response upon a virus infection, consisting of a complex system of defenses including immediate chemokine and IFN responses, followed by initiation of the adaptive response (Sen, 2001). The innate immune system detects invading pathogens through interactions of host cell pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs, Takeda et al., 2003). This triggers an intracellular signaling cascade activating the production of proinflammatory cytokines such as IFNs, interleukins (ILs), and tumor necrosis factor-α (TNF-α). IFNs are a family of virus-induced cytokines that represent an important first line of defense. They are divided into two types, namely type I IFNs or IFN-α/β super family induced by virus infection, and type II IFN (IFNγ) induced by mitogenic or antigenic stimuli (Samuel, 2001). Type I IFNs induce an antiviral state in the cell, maturation of dendritic cells, enhance cytotoxic T cell responses and stimulate production of chemokines, which recruit inflammatory cells to the site of infection.

Type I and II IFNs bind to different receptors on the cell surface and trigger related signal transduction pathways resulting in the activation of IFN-stimulated genes, whose products inhibit various stages of virus replication. The many IFN-α species have a broad effecting range since they usually inhibit more than one step of the viral replication cycle depending on virus family and host cell types (Samuel, 2001). Virus infection induces the transcription of several cellular genes either directly through the activation of cellular transcription factors such as interferon regulatory factor-3

(IRF-3) or indirectly through prior induction of IFN-α/β. IRF-3 is expressed widely in all cell types and is directly activated by double stranded RNA (dsRNA) or virus infection, whereafter it can bind and activate the IFN-α and IFN-β promoters and IFN-α/β-responsive genes (Nguyen et al., 1997, Schafer et al., 1998, Yoneyama et al., 1998). MV has been shown to activate signaling pathways involving IRF-3 (tenOever et al., 2002) and also nuclear factor-κB (NF-κB, Helin et al., 2001). IRF-7 is another important inducer of IFN-α gene transcription in the response to virus infections and most probably plays a role in the regulation of IFN-β synthesis (Marie et al., 1998, Wathelet et al., 1998). Several studies have suggested that MV induces both IFN-α and IFN-β production (Dhib-Jalbut & Cowan, 1993, Feldman et al., 1994, Schneider-Schaulies et al., 1993), however, it has been shown that wild-type strains induced notably lower production in human PBMC or a marmoset B-cell line as compared to laboratory adapted strains (Naniche et al., 2000). In plasmacytoid dendritic cells the normally efficient production of IFNs was shown to be abolished by MV in a manner of MV-induced disruption of the toll-like receptor 7/9-mediated signaling (Schlender et al., 2005). Not only could the virus avoid an immediate IFN alert triggered by itself but also one triggered by unrelated pathogens.

Toll-like receptors (TLRs) are a family of structurally similar transmembrane proteins, expressed on different cell types, which detect conserved microbial components. Their role in viral recognition has become more evident as a growing range of viruses has been studied (Mogensen & Paludan, 2005, Takeda et al., 2003). Single stranded RNA (ssRNA) is recognized by TLR7 and TLR8 (Diebold et al., 2004, Heil et al., 2004, Lund et al., 2004) whereas TLR3 is a receptor for dsRNA (Alexopoulou et al., 2001, Matsumoto et al., 2002), and it has been suggested that TLR3 might be involved in MV recognition (Tanabe et al., 2003). However, two additional recognition receptors of dsRNA, namely melanoma differentiation-associated gene 5 (mda-5) and retinoic acid inducible gene I (RIG-I) have recently been described (Andrejeva et al., 2004, Yoneyama et al., 2004) and found to have an important role in the recognition of RNA viruses (Kato et al., 2006). These are genes with similar properties in dsRNA-induced IFN-β production. Both are IFN-inducible host cell proteins with a helicase domain and a caspase recruitment domain (CARD). CARDs apparently transmit signals leading to activation of IRF-3 and NF-κB upon binding of dsRNA (Andrejeva et al., 2004, Yoneyama et al., 2004), thereby promoting the production of IFN-β and IFN response genes such as chemokines CCL5 and CXCL10. IRF-3 is essential for the innate response and viruses from different families utilize alternative mechanisms to regulate its antiviral activities. The exact role of mda-5 and RIG-I in stimulating the IFN-β promoter is yet to be determined. The V proteins of several paramyxoviruses

have been shown to bind mda-5 and thereby reduce the amount of IFN production in infected cells but no direct binding to or inhibition of RIG-I has been detected (Andrejeva et al., 2004, Childs et al., 2007).

2.3.2. Adaptive immune response

During the incubation period MV replication is controlled by nonspecific, innate antiviral host responses. The onset of the typical rash is coincident with the appearance of MV-specific adaptive immune responses and antibody to the viral proteins can be detected (Bech, 1959, Bouche et al., 2002, Graves et al., 1984). The most rapidly produced and most abundant antibody is against the N protein. Antibodies against N protein and H protein increase notably during the second week and also antibodies against M protein and F protein can be detected but only in low amounts (Graves et al., 1984). Antibodies to MV protect from reinfection and maternal antibodies protect infants from MV infection. Clearance of the virus takes place during the time of the rash, but immune suppression and immune activation continue for weeks after apparent recovery.

During the first week after the rash, spontaneous proliferation of PBMC occurs, reflecting an activation of the immune system where all immunologically important cell types participate (Ward et al., 1990). T lymphocyte-mediated immune responses are key factors in recovery from measles. Abundant evidence suggests that CD8+ T cells are activated during infection and CD8 T cell memory is established by infection (Jaye et al., 1998, Nanan et al., 1995, van Binnendijk et al., 1990). Also CD4+ T cells are activated in response to the infection. Both IFN-γ and IL-2, which characterize type 1 CD4+ T cells, IFN-γ also CD8+ T cells, are clearly elevated in plasma of acutely infected patients (Griffin & Ward, 1993, Griffin et al., 1990). MV infection also upregulates major histocompatibility complex class I and II expression, effectively presenting MV proteins to T cells (Gerlier et al., 1994, Herberts et al., 2001, Leopardi et al., 1993).

2.3.3. Mechanisms of immunosuppression

The high mortality rate associated with measles is mainly due to secondary infections during the immunosuppression that accompanies MV infection. It presents a unique state of prolonged depression of the immune response in the absence of detectable virus and can last up to 6 months after acute measles. The immunosuppression is most likely a multifactor process during which immune responses to new antigens

are impaired and thereby increasing susceptibility to other infections. Despite its clinical relevance, the mechanisms underlying MV-induced immunosuppression are still not understood. Several mechanisms have been suggested to lead to lymphopenia after measles and the number of lymphocytes may reduce even to 10 % of normal values (Okada et al., 2000). Lymphopenia alone may not, however, contribute to immunosuppression to a large extent since it is a common feature of many acute viral infections including those not associated with immunosuppression. MV-induced apoptosis (Esolen et al., 1995) and cell cycle arrest (Engelking et al., 1999, McChesney et al., 1987, 1988, Naniche et al., 1999) are also strongly linked to prolonged immune suppression.

A MV-induced arrest of the cell cycle progression in G_1 phase in lymphocytes has been described (McChesney et al., 1987, 1988). Mitogen stimulation activates MV replication but this suppresses lymphocyte proliferation and differentiation causing cell cycle arrest. Proliferation can also be arrested in uninfected cells by direct contact (Schlender et al., 1996), probably mediated by F protein expressed on the surface of infected cells (Weidmann et al., 2000). In epithelial cells it has been shown the MV-induced arrest is followed by apoptosis (Valentin et al., 1999), which is a cause of cell death of MV-infected cells (Esolen et al., 1995). Interestingly, results show that MV induces apoptosis also indirectly in cells that are not infected and do not express MV antigen (Fugier-Vivier et al., 1997, Okada et al., 2000, Vuorinen et al., 2003).

Because several MV proteins have been suggested to interfere with immune responses this can be of importance in the induction of immunosuppression (Kerdiles et al., 2006). Interaction of the H protein with CD46 has been suggested to impair the capacity of dendritic cells and monocytes to produce IL-12, which is a proinflammatory cytokine important for the initiation of the cellular immune response (Fugier-Vivier et al., 1997, Karp, 1999, Marie et al., 2001) and has been shown to be suppressed in measles patients (Atabani et al., 2001). As SLAM was identified to be a receptor for MV-induced it was also suggested to play a critical role in immunosuppression. It is expressed on different subsets of immune cells, which are potential targets of MV-immunosuppressive action and a recent report states that SLAM-mediated MV-dendritic cell interaction inhibits IL-12 synthesis through TLR4 activation (Hahm et al., 2007). The N protein, in addition to complexing the viral RNA and the P protein, is released into the extracellular compartment after apoptosis and/or secondary necrosis. It has been shown to interact with two inhibitory cell-surface receptors: the type II IgG Fc receptor (FcγRII, Ravanel et al., 1997), thereby suppressing the inflammatory immune response in vivo in a murine delayed-type hypersensitivity model (Marie

et al., 2001), and a yet uncharacterized nucleoprotein receptor (Laine et al., 2003). This nucleoprotein receptor inhibits both spontaneous and activated cell proliferation suggesting that, in addition to H protein, also N protein is involved in inducing immunosuppression, possibly by inducing cell cycle arrest and apoptosis (Laine et al., 2003, 2005).

3. AIMS OF THE STUDY

The hypothesis of the first part of this study was that the lack of a filamentous actin structure in PBMC could partly explain the highly restricted MV replication in unstimulated cells. This was approached by studying infection both by disrupting the well-organized cytoskeleton in Vero cells, which normally support MV replication, and further by inducing filamentous actin in PBMC. In the second part we continued studies on structural elements, now focusing on structural changes of the cellular nucleus during MV infection. MV is known to induce apoptosis but so far it has not been studied whether or not nuclear matrix proteins are affected as a result of MV-induced apoptosis. Furthermore, MV-induced innate immune responses were studied. We set out to analyze which factors of this early defense system were triggered by MV infection.

The specific aims of the study were:

1. To investigate the role of the cytoskeleton in productive MV replication.

2. To study nuclear changes, especially the fate of proteins of the nuclear matrix, which occur during MV-induced apoptosis.

3. To determine MV-induced gene expression of the innate immune system in lung epithelial and endothelial cells.

4. MATERIALS AND METHODS

4.1. Virus (I-IV)

A wild-type laboratory adapted strain of measles virus (Halonen strain, Vainionpää et al., 1978) with a high infectivity titer (>1 x 10^7 plaque forming units (PFU)/ml) was used throughout the study. The inoculum virus was propagated in Vero cells (American Type Culture Collection, ATCC, CCL-81). Cells were infected at a multiplicity of infection (m.o.i.) of 0.01-5 by adding the inoculum virus to medium, which was applied to the cells and adsorption was performed at 37°C. Uninfected control cells were treated with supernatant fluid of uninfected Vero cells. The inoculum virus of human rhinovirus (HRV) 1B and HRV 9 (ATCC) had a titer of 10^8 $TCID_{50}$/ml (50% tissue culture infective dose, III). Control cells and cells infected with MV were incubated at 37°C, whereas control cells and cells infected with HRV 1B or HRV 9 were incubated at 33°C, with 5% CO_2.

4.2. Cells (I-IV)

Vero cells of monkey kidney origin (ATCC, CCL-81) were cultured in Basal Eagle's Medium (MP Biomedicals) supplemented with 0.2% bovine serum albumine, 5% tryptose phosphate broth, and gentamycin (I, II). PBMC were isolated by density centrifugation from heparin-blood drawn from adult volunteers using Ficoll-Paque (Amersham Pharmacia Biotech). Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS, Bioclear, Wilshire, UK) and gentamycin and maintained at 37°C in 5% CO_2 (II). HeLa SS6 cells (ATCC) were cultured in DMEM (Gibco) supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, and 100µg/ml streptomycin (III). The human lung adenocarcinoma cell line A549 (ATCC, CCC-185) was cultured in Ham's F-12 medium (MP Biomedicals) supplemented with 1% FCS and gentamycin and human umbilical vein endothelial cells (HUVEC, ATCC, CRL-1730) were cultured in RPMI 1640 medium supplemented with 10% FCS and gentamycin (IV).

4.3. Antibodies (I-IV)

Table II. Primary and secondary antibodies used in the study.

Primary antibody	**Reference**	**Publ.**
Rabbit polyclonal MV antiserum	Dept of Virology, University of Turku*	I-III
Mouse monoclonal MV-N antiserum	″	I, III
Mouse monoclonal MV-H antiserum	″	II
Rabbit polyclonal HRV 1B antiserum	″	III
Mouse monoclonal anti-β-tubulin ab	Sigma, St. Louis, MO, USA	I
Mouse monoclonal anti-ezrin ab	NeoMarkers, Fremont, CA, USA	I
Mouse monoclonal anti-moesin ab	″	I
Mouse monoclonal NuMA ab (aa 255-267, SPN-3 clone)	Kallajoki et al., 1991, 1993	III
Mouse monoclonal lamin A/C ab	Novocastra Laboratories, Newcastle upon Tyne, UK	III
Goat polyclonal lamin B ab	Santa Cruz Biotech, CA, USA	III
Mouse monoclonal PARP-1 ab	Sigma, St. Louis, MO, USA	III
Rabbit polyclonal ab detecting cleaved p85 fragment of PARP-1	Promega, Madison, WI, USA	III
Rabbit monoclonal ab detecting cleaved caspase-3 (Asp 175)	Cell Signaling Technology, Beverly, MA, USA	III
Rabbit polyclonal caspase-8 ab (Ab-4)	NeoMarkers, Fremont, CA, USA	III
Rabbit polyclonal caspase-3 ab	BD Pharmingen, San Diego, CA, USA	III
Mouse monoclonal caspase-7 ab	″	III
Rabbit anti-RIG-I ab	Imaizumi et al., 2002	IV
Rabbit anti-mda-5 ab	Lin et al., 2006	IV
Secondary antibody		
Alexa Flour 488-conjugated phalloidin	Molecular Probes, Eugene, OR, USA	I, II
Alexa Flour 568-conjugated anti-rabbit ab	″	I, II
Alexa Flour 488-conjugated anti-mouse ab	″	I
FITC-conjugated goat anti-mouse IgG	Cappel Laboratories, Couchranville, PA, USA	II, III
TRITC-conjugated goat anti-rabbit IgG	Zymed Laboratories, San Fransisco, CA, USA	III
HRP-conjugated goat anti-mouse IgG	Dako, Klostrup, Denmark	I
HRP conjugated goat anti-rabbit IgG	″	I-IV
HRP-conjugated sheep anti-mouse IgG	Amersham, Buckinghamshire, UK	III
HRP-conjugated donkey anti-rabbit IgG	″	III
HRP-conjugated rabbit anti-goat IgG	Zymed Laboratories, San Fransisco, CA, USA	III

*Purified measles virus, H- and N-proteins (Lund & Salmi, 1981, Salmi & Lund, 1984, Vainionpää et al., 1978) and purified HRV 1B (Smyth et al., 1993) were used for preparation of antisera. Unimmunized sera were used as negative controls.

4.4. Reagents (I-III)

Latrunculin-A (lat-A, Molecular Probes) and nocodazole (noc, Sigma) were used to disrupt actin filaments and microtubules, respectively. Jasplakinolide (jaspla, Molecular Probes) was used to assemble F-actin. All drugs were dissolved in dimethylsulfoxide (DMSO, final concentration >0.1%) and different dilutions in culture medium were assessed for optimizing further working concentrations; 1 μM lat-A and 5 μM noc (I) and 0.5 μM jaspla (II), respectively. PHA (Difco Laboratories) was used at a concentration of 25 μg/ml (II). 100 μM benzyloxylcarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK, Bachem, Bubendorf, Switzerland) dissolved in chloroform-methanol was used for inhibiting caspase activity and blocking apoptotic cell death (III).

4.5. Immunoperoxidase staining (I, III)

Immunoperoxidase staining of Vero cells (I) and HeLa cells (III) was performed as described previously (Waris et al., 1990). Briefly, cells were grown on 12-well plates and grown until confluence. 1 h p.i. cells were washed extensively with phosphate buffered saline (PBS) to remove inoculum virus and thereafter 1 μM lat-A or 5 μM noc was added to appropriate samples (I). The effect of removal of the drug was studied by washing the samples 48 h p.i. and continuing incubation for another 48 h with fresh medium with or without addition of drugs. In cells where caspase activity was studied there was addition of 100 μM z-VAD-FMK 30 min before infection (III). 1 h p.i. cells were washed thoroughly and fresh medium containing 100 μM z-VAD-FMK was added. Cultures were incubated and samples collected at 24 h intervals up to 96 h (I) or 72 h (III). Supernatants were collected for virus infectivity assay and cells were fixed with cold 75% acetone for 15 min at +4ºC. Cells were stained with polyclonal MV (I, III) or HRV 1B (III) antiserum in 5% nonfat milk in PBS as primary antibody and horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG as secondary antibody. To visualize the infected cells, a substrate solution containing 3-amino-9-ethylcarbazole dissolved in dimethylformamide, acetate buffer and H_2O_2 was added and infected cells were detected by light microscopy.

4.6. Assays for virus titers (I-III)

The amount of infectious virus was determined by incubation of dilution series of supernatants from treated or untreated MV-infected cells on monolayers of Vero cells on 12-well plates. After a 60 min adsorption period cells were covered with 0.5% CMC (blanose cellulose gum type 7HF, Hercules Incorporated, Wilmington, DE) and after 6-8 days cells were fixed and stained with crystal violet. Virus titers were determined by

standard plaque counting (Vainionpää et al., 1978). Virus titers were also determined by $TCID_{50}$ method where serial dilutions of supernatants were titered in Vero cells (MV) or HeLa cells (HRV 1B) to assess the dilution of virus, which was not able to cause a cytopathic effect in cells and thereby quantify the amount of infectious virus (III).

4.7. Immunofluorescence microscopy (I-III)

For immunofluorescent staining cells were grown on 12 mm coverslips (I, III). PBMC were fixed onto microscopic slides using a Shandon-Elliot cytocentrifuge to collect 2 x 10^6 cells per slide. At the time intervals indicated in the results section, cells were washed once with PBS, fixed in 3% paraformaldehyde (I), 4% formalin (II) or 3.7% formaldehyde (III) in PBS for 15 min at room temperature (RT), permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT and finally washed with PBS. To reduce background samples were pretreated shortly with 5% bovine serum albumin (BSA, Sigma) or 5% normal goat serum (Dako) in PBS, washed and incubated with primary antibody diluted in 1-2% BSA in PBS for 1-2 h in a humid chamber at RT. Samples were washed with PBS and incubated with secondary antibody for 1 h at RT. After washing samples were stained for DNA with Hoechst 33258 (1µg/ml in 25% ethanol/75% PBS) for 5-15 min, washed and embedded in Mowiol 4.88 (Hoechst AG, Frankfurt, Germany). Samples were analyzed using a Leica DM L microscope and Leica IM50 Image Manager (I), a confocal laser-scanning microscope (Leica TCS NT, II) or an Olympus BX50 fluorescence microscope and AnalySIS software (Soft Imaging Systems, III).

4.8. TUNEL assay (III)

TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labelling) assays were performed using the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Briefly, cells grown on coverslips were fixed in 3.7% formaldehyde in PBS for 15 min RT and permeabilized with 0.1% TritonX-100 in PBS for 15 min. Samples were pre-equilibrated with equilibrating buffer for 10 min at RT and then incubated with equilibrating buffer containing nucleotide mix and TdT enzyme for 1 h at 37°C. The reaction was terminated by immersing the samples in 2x SSC (1x SSC; 0.15 M NaCl, 0.015 M trisodium citrate) for 15 min at RT. Samples were washed three times in PBS, stained with desired antibodies and for DNA with Hoechst 33258, and analyzed as described above.

4.9. Immunoblotting (I-IV)

Total cell extracts were prepared by lysis in ice-cold RIPA buffer (50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.4% Na-deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin). Protein concentrations were determined according to the Bradford-method using the Bio-Rad Protein assay kit. Equal amounts of protein (10-50 µg/lane) in Laemmli-sample buffer were separated by 12% SDS-PAGE and electrophoretically transferred to a PVDF-membrane (I, II, IV). In paper III cells were scraped into the medium, washed with PBS, and pelleted at 12 000 x g for 5 min. Cell numbers were counted with hemocytometer and cell pellets were suspended directly into hot SDS-PAGE electophoresis sample buffer at a concentration equivalent to 10^7 cells/ml and sonicated for 5 s. Samples were loaded onto 5% polyacrylamide gels for NuMA, 10% gels for lamins, PARP-1, and viral proteins, and on 10%, 12%, or 14% gels for caspases. Proteins were transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Protein transfer was controlled by incubating the filters with Ponceau staining 20 minutes and rinsing them with distilled water. 5% nonfat milk in PBS (I, II, IV) or 4% BSA (III) in 0.2% Tween 20 in TBS (Tris-buffered saline; 20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, IV) was used for over night blocking. The filters were washed with PBS-0.5% Tween 20 (I, II, IV) or TBS-0.2% Tween 20 (III) and incubated with primary antibody diluted in 5% nonfat milk (I, II, IV) or 1% BSA in TBS-0.2% Tween 20 (III) for 1-3 h at 37°C or RT or over night at 4°C. After thorough washing, filters were incubated with HRP-conjugated secondary antibody diluted in 5% nonfat milk (I, II, IV) or 1% BSA in TBS-0.2% Tween 20 (III) for 1 h at RT. Again, thorough washing was performed and proteins were visualized using the enhanced luminescence system (ECL Western blotting detection system, Amersham Biosciences). When incubated with another primary antibody, filters were washed and incubated with stripping buffer (2% SDS, 100 mM ß-mercaptoethanol, 63 mM Tris, pH 6.8) at 50ºC for 30-60 min before pre-incubation in blocking buffer.

4.10. RNA isolation and Northern blot analysis (IV)

Total cellular RNA was extracted using the Qiagen RNeasy Kit according to the manufacturer's instructions. Samples containing equal amounts (10 µg) of RNA were size-fractionated on 1% formaldehyde-agarose gels, transferred onto Hybond-N membranes (Pharmacia Amersham Biotech, Buckinghamshire, UK) and hybridized with the indicated probes. Probes for IFN-β (Ronni et al., 1997), IL-28 and IL-29 (Sirén et al., 2005), CCL5 and CXCL10 (Matikainen et al., 2000), and TLR3 (Miettinen et al., 2001) have been previously described. Probes for mda-5 and RIG-I were cloned from

total cellular RNA obtained from Sendai virus-infected macrophages by RT-PCR using oligonucleotides GCTCACAGTGGATCCGGAGTTATCGAA and CACCATCATGGA TCCCCAAGCCTGGCC (mda-5) and TGTTTCCAGGGATCCCAGCAATGA and AC TTCACATGGATCCCCCAGTCATGGC (RIG-I). Ethidium bromide staining of rRNA bands was performed to ensure equal loading. Probes were labeled with [α-^{32}P] dATP using a random primed DNA labeling kit (Boehringer Mannheim, Germany). Membranes were hybridized (Ultrahyb; Ambion, Austin, TX) and washed in 1x SSC/0.1% and exposed to Kodak AR X-Omat films (Eastman Kodak, Rochester, NY) at –70ºC using an intensifying screen.

4.11. Transfections (IV)

To study the possible involvement of mda-5, RIG-I and TLR3 in MV-induced antiviral gene expression we adopted the reporter gene assay based strategy in A549 epithelial cells. Plasmids containing the sequences mda-5 or RIG-I have previously been described (Imaizumi et al., 2002, Kang et al., 2002). TLR3 plasmid was purchased from Invitrogen. For luciferase assays, A549 cells were transfected with indicated expression plasmid, Renilla luciferase plasmid and firefly luciferase reporter under the control of IFN-β promoter using FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. The firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega) and Victor multilabel reader (Wallac). The results were normalized and presented as firefly/Renilla luciferase activity ratios.

5. RESULTS AND DISCUSSION

5.1. Role of cytoskeleton factors in measles virus replication (I, II)

Cytoskeletal components are known to be essential in various steps of the replications cycle for a number of viruses (Cudmore et al., 1997). The focus in our work was on the connection of MV replication with actin filaments. Our hypothesis was that the arrangement of the actin structure in PBMC plays a crucial role in productive MV replication. To approach this issue, MV infection in Vero cells, which have a distinct actin cytoskeleton, and in PBMC, which mainly contains globular actin and lacks a structured filament network, were studied. In order to determine how rearrangement of the cytoskeletal network affected MV, we treated Vero cells (I) with the cytoskeletal inhibitors latrunculin-A and nocodazole and PBMC (II) with the actin filament stabilizer jasplakinolide.

5.1.1. Reorganization of actin filaments or microtubule structures affects production of infectious measles virus

The replication cycle of MV is known to be 16-18 h and we followed infection up to 96 h. We studied events after entry of MV and therefore the inhibitors were added 1 h p.i. not to affect adsorption of the virus. Disruption of actin filaments and microtubules by lat-A and noc, respectively, inhibited production of new infectious virus particles. When performing immunoperoxidase staining (I, Fig. 1) we could count about the same numbers of infected cells in the untreated and treated cell cultures 24 h p.i. and we concluded that these were cells infected by inoculum virus. At 48 h, and even more so at 72 h (data not shown) and 96 h p.i., the difference between the control cells and treated cells became evident. In the untreated cell culture, we could at 48 h p.i. detect multinucleated cells fused to form large syncytia, typical for MV infection, as well as single infected cells, representing cells infected by newly synthesized virus, and 96 h p.i. all cells were infected. In the lat-A or noc treated cell cultures only large syncytia were detected at these time points, suggesting that the virus was able to spread from cell to cell by fusion but release of infectious virus was not taking place. Interestingly, similar experiments with respiratory syncytial virus (RSV) showed that RSV did not form syncytia after actin filament disruption (Kallewaard et al., 2005). To ensure that the reduction of newly infected cells seen in immunoperoxidase staining was indeed due to the absence of release of new infective virus and not to restricted attachment or entry in the treated cell cultures, a plaque titration assay was performed. Supernatants from the different cell cultures were inoculated onto confluent Vero cells and plaque

forming units could be counted 6-8 days later. The number of PFU/ml was dramatically less, approximately 1/10, in the treated cell cultures (I, Fig. 2A). These results support previous studies where cytochalasins, which are different in their modes of actions, have been used to disrupt actin filaments causing almost complete inhibition of virus release (Stallcup et al., 1983).

The reversible effect of the two inhibitors was demonstrated by washing treated cell cultures 48 h p.i., and continuing incubation for another 48 h with fresh medium without inhibitors. In the immunoperoxidase staining the number of positive foci increased (I, Fig. 1 48h + 48h) and the number of PFU/ml tripled (I, Fig. 2B). This demonstrated not only the reversibility of the drugs and that the cells were viable after the treatments, but also that the release of infectious virus started upon removal of the drugs and that new replication cycles in non-neighboring cells were initiated when the cytoskeletal structures reformed.

Effects of jaspla and/or PHA treatments on productive MV infection PBMCs are summarized in Table 1 (II). Interestingly, and supporting our hypothesis, jaspla-induced release of virus particles (clarified supernatants produced 130 PFU/ml) and increased the total amount of infected cells in the treated cell culture (15% MV positive cells), without causing stimulation of cell proliferation when examined by thymidine incorporation. This suggests that actin polymerization to the filamentous form has a great impact on MV replication and that the restricted replication can be turned into an active one without stimulating the cells. In addition, jaspla inhibited the effects of PHA when cells were treated with both drugs. As expected, PHA stimulated cell proliferation and activated productive MV replication (2400 PFU/ml, 32% infected cells). In cell cultures treated with both drugs effects were similar to those of only jaspla treated cells (120 PFU/ml, 17% infected cells) and no cell proliferation was detected. In untreated cells virus release was nonexisting (10 PFU/ml can represent remains of inoculum virus) and the proportion of infected cell was low (3%).

5.1.2. Intracellular localization of measles virus proteins

To study the intracellular localization of the virus proteins, cells were prepared for immunofluorescence analysis. In lat-A treated cell cultures actin was mainly in monomer form when detected with phalloidin (I, Fig. 3B) but no clear-cut change in the appearance of the virus proteins could be noticed when detected with polyclonal anti-MV serum (I, Fig. 3A and 3B). Similarly, noc treatment disrupted the microtubulus network without a dramatic effect on the distribution of virus proteins (I, Fig. 3C and 3D). This indicated that

although actin or microtubulus structures were disrupted and release of new infectious virus was highly inhibited, there was still restricted replication occurring. Although the drugs are highly specific for actin or microtubules, the loss of one structure can influence another. Cultured cells often round up and even detach from the surface when lacking cytoskeletal frameworks. To verify the specificity of the inhibitors, we performed staining of the microtubules in lat-A treated cells and of actin filaments in noc treated cells (I, Fig. 4). The untreated structures were intact in the respective cell cultures. This confirmed that the effects on virus production were not due to general breakdown of the cytoskeletal proteins. The immunofluorescence study was also performed with confocal microscopy but no apparent colocalization between actin or tubulin and viral proteins was detected (data not shown).

In PBMC a dramatic change in the intracellular distribution of MV proteins was detected after jaspla treatment (II, Fig. 1). In contrast to untreated, infected PBMC where only small spots of MV proteins were observed, the virus proteins were widely distributed in jaspla treated cells, more similar to PHA treated cells. We were not able to visualize the effect of jaspla on the actin structure due to previously described competitive binding of jaspla and phalloidin (Bubb et al., 1994, Kurashima et al., 1999) and attempts to do so with an anti-actin antibody also failed. Jaspla has, however, previously been shown to effectively modify and polymerize the actin cytoskeleton, specifically also in T-lymphocytes (Posey & Bierer, 1999). Like in Vero cells we could not detect any colocalization between MV proteins and actin in PBMC.

The indication of restricted MV replication in inhibitor treated Vero cells gained support by immunoblotting. MV-N protein was detected in all three cell cultures 48 h p.i. and increasingly 72 h p.i. (I, Fig. 6). This demonstrated that virus protein synthesis was active although virus release was strongly inhibited. In PBMC no virus proteins were detected in untreated cells but in jaspla treated cells a slight increase was seen (II, Fig. 2). In PHA treated cells effective protein synthesis occurred as expected, but interestingly the effect of PHA was again inhibited by jaspla in cells treated with both drugs. This correlated with the results from the plaque titration assay and flow cytometry. This phenomenon was most likely due to the fact that jaspla is cell-permeable and rapid in reorganizing the actin structure (Bubb et al., 1994, Patterson et al., 1999) thereby preventing the receptor-mediated, more slow actions of PHA (Rao, 1984) at the cell periphery. Also to be considered is that jaspla-induced actin polymerization inhibits depolymerization and prevents the dynamic conversion from F- to G-actin. This loss of the reversible conversion can add to the loss of PHA effect.

5.1.3. Possible connections with other cytoplasmic proteins

No direct colocalization between MV proteins and actin or tubulin has been shown, yet these structures are compulsory for efficient MV production. This strongly suggests the need for one or more actin-binding proteins, as has been suggested also for other viruses. Interesting proteins in this context are the ezrin/radixin/moesin (ERM) proteins. These play an important role in the interactions of the cytoskeleton with transmembrane proteins, as they regulate the organization of the actin cytoskeleton through their role as crosslinkers between actin filaments and membrane proteins (Sato et al., 1992). We studied the possible connection between ezrin or moesin with MV proteins but could not detect colocalization by immunofluorescent microscopy (I, Fig. 5). By performing Western blotting we could, however, detect changes in the amount of ezrin due to MV infection (I, Fig. 6). Likewise, the levels of tubulin and less obvious the levels of actin diminished as the infection proceeded. This was surprising, as we could not see any dramatic changes in the actin or tubulin structures in our microscopic studies (I, Fig. 3A and 3C), which supports previous studies (Duprex et al., 2000). Sagara and coworkers (1995) have detected ERM proteins in the virions of rabies virus, vesicular stomatitis virus (VSV), Newcastle disease virus and influenza A virus and they could show a close interaction between the ERM proteins and rabies virus G protein suggesting a role for the actin-ERM complex in virion formation by the budding process. For RSV it was shown that, in addition to actin, a cellular transcription factor identified as profilin is needed for optimal replication (Burke et al., 2000). Profilin acts as an actin monomer binding protein, that regulates the normal distribution of F-actin structures. Filamentous actin is not required for RSV transcription but appears to be critical for viral budding (Burke et al., 1998) and a role for profilin in the post-replicative stages to promote actin polymerization and thereby facilitating RSV budding was suggested. Very recent data show, however, that there is a direct interaction between actin and mature RSV particles (Jeffree et al., 2007).

As for several other viruses the host cell cytoskeletal structures are evidently necessary for effective productive MV infection. This area has been immensely studied but still there is a lack in our understanding of the details of this dependency. The link between these cellular proteins and MV proteins is so far undefined. It has been shown that the M protein of Sendai virus and Newcastle disease virus specifically interacts with actin *in vitro* (Giuffre et al., 1982), but no such activity has been shown for any other paramyxovirus. It was recently shown that the MV-M protein forms a complex with nucleocapsid structure to be transported to the plasma membrane (Runkler et al., 2007)

and it is likely that cytoskeleton structures are needed for this since budding does not occur without intact actin filaments.

Paramyxoviruses seem to utilize the cytoskeletal components in different manners. Ribonucleoprotein of human parainfluenza virus type 3 has been shown to colocalize with actin filaments and these, but not microtubules, seem to be directly involved in RNA synthesis (Gupta et al., 1998). For RSV it has recently been shown that, whereas actin plays a prominent role in virus release, tubulin is more dominant in the formation of cell-associated virus (Kallewaard et al., 2005). The interaction between MV proteins and cytoskeletal proteins is less clear. Tubulin has been suggested to function as a subunit of the viral RNA polymerase of Sendai virus and MV, and actin is known to associate with MV nucleocapsids (Bohn et al., 1986, Moyer et al., 1986, 1990, Stallcup et al., 1983), however, no colocalization between these has been detected. Early reports (Fagraeus et al., 1981, Fagraeus et al., 1978) suggest that MV infection disrupts actin structures whereas others state the opposite (Bedows et al., 1983, Duprex et al., 2000). Duprex and coworkers (2000) could not detect any disruption of the actin, tubulin or the intermediate filament vimentin structures due to MV infection, only the glial-fibrillary-acidic protein filament (GFPA) network was affected in an astrocytoma cell line. In syncytia the filaments were integrated into larger but similarly organized networks.

Our understanding of the regulation of MV replication in PBMC is still limited. The highly restricted replication in resting lymphocytes and effective release of virus upon stimulation, have indicated that parameters present only in activated cells are required. However, our results suggest that in addition to the active cell stage itself, attention should be paid also to the cytoskeleton components and their arrangement. The commonly used mitogen PHA does not only activate cell proliferation but it also causes a conversion of globular actin to filamentous form and jaspla treatments show that stabilization of F-actin alone can activate productive MV replication.

5.2. Effects of measles virus on nuclear proteins (III)

We have investigated the changes of nuclear matrix during MV infection and for comparison also during human rhinovirus (HRV) infection, which replication strategy differs from MV. Rhinoviruses form the largest genus of the family *Picornaviridae*. This is a family of small nonenveloped RNA viruses with a single positive-stranded genome and replication and assembly takes place in the cytoplasm of the host cell. In this study, NuMA and lamins A/C and B were used as markers for internal nuclear matrix and peripheral nuclear lamina, respectively. Preliminary experiments additionally included

human adenovirus and herpes simplex virus type-1 and the fate of NuMA, lamins A/C and B, and PARP-1 was analyzed by immunoblotting. The effects were less distinct with these viruses and to narrow study they were left out from further analysis.

5.2.1. Virus infections resulted in chromatin condensation and redistribution of NuMA and lamins

To study the morphological changes of nuclear matrix in virus infected cells, HeLa cells were infected with MV or HRV 1B and immunofluorescence analysis was performed. In the MV-infected cell culture (III, Fig. 2), the typical syncytia made it difficult to characterize changes. In the nuclei in the center of syncytia, NuMa was excluded from the condensed chromatin and the staining for lamins A and C had lost the round shape and was wrinkled and indistinct. This was, however, not seen until late time points, 48-72 h p.i. Changes of lamin B were nearly identical to lamin A/C (data not shown). For HRV 1B changes in cell structure were noticed at earlier time points, 12 h p.i. (III, Fig. 1) when a large amount of viral proteins were detected in the cytoplasm. Although some cells had started to shrink and detach, there were no detectable changes in the distribution of NuMa, lamin A/C or DNA. 24 h p.i. (data not shown) some cells showed partial chromatin condensation and NuMa started to separate from the condensed chromatin, whereas lamins A and C had lost the round shape and formed a wrinkled staining pattern instead. Cells with apoptotic bodies were detected 48 h p.i. and in these cells both NuMa and lamin A/C seemed to encircle the nuclear fragment.

We then wanted to study whether chromatin condensation and cytoplasmic blebbing seen in both virus infections were due to caspase activation and apoptotic cell death. A number of viruses are known to induce apoptosis and the relevance of this phenomenon for viral pathogenesis is being intensively studied. Apoptotic cell death has also been detected in MV-infected cells (Esolen et al., 1995) and this can be one possible mechanism of the MV-induced immunosuppression. We performed staining with antibodies detecting either cleaved caspase-3 or the 85 kDa cleavage product of poly(ADP-ribose) polymerase-1 (PARP-1), which is considered a characteristic of apoptotic cell death (Germain et al., 1999, Tewari et al., 1995). Both MV- and HRV 1B-infected cells, in which NuMA was redistributed and chromatin was condensed, stained positive for the antibodies, whereas cells with normal nuclear morphology did not (III, Fig. 3A). Again the changes in MV-infected cells were not seen until late in infection when large syncytia had formed. When performing TUNEL assay, both MV- and HRV 1B-infected cells showed apoptotic bodies with TUNEL-positive chromatin clumps (III, Fig. 3B), whereas only a few single positive cells were detected in uninfected control cells (data not shown).

Western blot analysis was performed to study possible processing of NuMA, lamins, PARP-1, and pro-caspases in cells infected with MV, HRV 1B or HRV 9 (III, Fig. 4). HRV 9 was included to test whether proteolytic changes were shared with other rhinoviruses. Full length NuMA was gradually cleaved into 190- and 180-kDa fragments in all infected cells and in addition a ~160-kDa fragment was detected in the HRV-infected cells. The 70-kDa lamin A and 60-kDa lamin C were cleaved into ~68- and ~50-kDa in MV-infected cells, whereas the heavier fragment was of ~62-kDa in HRV-infected cells suggesting a different cleavage site in these cells. Following all infections lamin B was cleaved into a ~45-kDa fragment, for HRV 9 levels were however close to that of control cells. PARP-1 was cleaved into a ~85-kDa fragment but interestingly also partial cleavage into ~50-kDa and minor ~60-kDa fragments was noted in HRV-infected cells but were completely lacking in MV-infected cells. Similar pattern of PARP-1 cleavage into smaller "atypical" fragments has been described during necrosis (Casiano et al., 1998, Gobeil et al., 2001). As NuMa, lamins, and PARP-1 were cleaved due to accumulation of viral proteins, we could detect simultaneous cleavage of pro-caspase-8, -3, and -7 presumably due to their activation. Some differences were noted between the infections. Pro-caspase-8 was cleaved into ~42/44-kDa fragments in all cells but in HRV 1B-infected cells additionally a ~25-kDa fragment could be detected. Pro-caspase-7 was cleaved to a ~31-kDa fragment in MV-infected cells and differently to ~31/33-kDa fragments in HRV-infected cells. Pro-caspase-3 was cleaved more markedly in MV or HRV 1B-infected cells that in HRV 9-infected cells.

MV infection induced features of typical apoptotic cell death, which is in agreement with previously data stating that MV induces apoptosis (Auwaerter et al., 1996, Esolen et al., 1995, Fugier-Vivier et al., 1997, McQuaid et al., 1997, Vuorinen et al., 2003). The finding that NuMA and lamins are specific target proteins has not, however, been described before. Other viruses in the family picornaviridae have been reported to induce apoptosis in host cells (Agol et al., 1998, Carthy et al., 1998, Jelachich & Lipton, 1996, Tolskaya et al., 1995, Tsunoda et al., 1997), but the connection between human rhinoviruses and apoptosis has so far been unclear. The activation of caspases, the chromatin condensation and fragmentation, and the specific cleavage of several caspase target proteins in HRV-infected cells demonstrate that the cell death induced by rhinoviruses is, at least partially, apoptotic.

5.2.2. Caspase inhibitor prevents NuMa, lamin and PARP-1 cleavage and measles virus-induced apoptosis

The influence of caspase activity on the morphology of infected cells and on cleavage of cellular proteins was studied by repeating the experiments in the presence of the

caspase inhibitor z-VAD-FMK. A marked impact on MV-infected cells was detected (III, Fig. 5B). No cellular shrinkage or chromatin condensation was observed and instead larger syncytia with intact nuclei, based on normal distribution of NuMA, lamin A/C and DNA, were formed. In HRV 1B-infected cells (III, Fig. 5A) a delay in cell death was observed. Cells shrank, detached partially, and alterations in the chromatin structure were detected but no apoptotic bodies were formed. Based on phase contrast viewing together with the altered chromatin structure we concluded that most of the infected cells died in the presence of z-VAD-FMK. These results indicated, that inhibition of caspase activity could rescue MV-infected cells but not HRV 1B-infected cells from cell death. Inhibition of caspase activity in z-VAD-FMK treated cell was verified by immunostaining of cleaved caspase-3 or p86 fragment of PARP-1, which was negative in both infected cell cultures (data not shown).

Immunoperoxidase staining confirmed the morphological observations. In MV-infected cells the morphological difference between z-VAD-FMK treated and untreated cell cultures was pronounced (III, Fig. 6). In the treated cell culture infection seemed to spread only by fusion to neighboring cells forming multinucleated giant cells, although we could not detect any reduction of released virus particles (data not shown). Evidently MV replication is effective in the absence of caspase activation, but the initiation of new infection cycles in non-neighboring cells is disturbed by the pan-caspase inhibitor. A slight accumulation of infected cells in z-VAD-FMK treated HRV 1B-infected cell cultures was detected (III, Fig. 6) but no significant difference in the amount of new virus particles determined by virus infectivity assay (data not shown) was observed.

The presence of z-VAD-FMK in both infected cell cultures inhibited the cleavage of NuMA, lamin B, partially lamin A, and PARP-1 as detected by western blotting (III, Fig. 7). The inhibitor did not significantly affect the production of virus proteins showing that z-VAD-FMK inhibits several late features of virus-induced cell death including cleavage of cellular proteins and fragmentation of DNA without significantly affecting the progress of viral infection itself.

Since we could notice activation of caspases, morphological changes such as cellular shrinkage and partial chromatin condensation, and specific cleavage of several caspase target proteins in the infected cells, we conclude that both infections induced apoptosis. MV-infected cells completely lacked all features of cell death when treated with z-VAD-FMK. This, together with the typical apoptotic cleavage pattern of NuMa, lamin B and PARP-1 (Nicholson & Thornberry, 1997), supports previous data of MV-induced apoptosis in host cells. In HRV 1B-infected cells, however, z-VAD-FMK did not prevent

the major morphological changes mentioned above or cell death. The cells were TUNEL negative and our results suggest that in addition to caspase-mediated apoptosis also noncaspase-mediated apoptosis or necrosis are involved in HRV-induced cell death. Our study shows that NuMa and lamins are target proteins during programmed cell deaths induced by viral infections. This seems to be a late effect due to activity of caspases or other proteases rather than a result of virus replication and blockade of cellular protein synthesis.

Although apoptosis due to MV infection has been extensively studied, it is still not completely understood. It has been shown that in MV-infected dendritic cell-T cell cocultures, apoptosis of dendritic cells is mediated through the Fas/APO-1/CD95 receptor, whereas T cell apoptosis cannot be inhibited by blocking the Fas pathway (Servet-Delprat et al., 2000). The cytotoxic activity of MV-infected dendritic cells has been shown to be TRAIL (TNF-related apoptosis inducing ligand)-mediated (Vidalain et al., 2000), which triggers the apoptotic cell death machinery and was therefore suggested to be responsible for the T cell apoptosis in the coculture. Dendritic cells are potent antigen-presenting cells that initiate the immune response, but their functions are compromised by MV infection, and this is most likely an important factor in immunosuppression.

5.3. Mechanisms of the innate immune system induced by measles virus in lung epithelial cells (IV)

The purpose of this project was to study the regulation of antiviral cytokines in response to MV infection. Both epithelial and vascular endothelial cells are highly susceptible to MV infection and the spread of infection occurs via endothelial cells followed by systemic infection. In this work we used two cell lines, i.e. A549 lung epithelial cells and human umbilical vein endothelial cells (HUVECs), to study MV-induced cytokine and chemokine response. A failure to activate innate immune responses could contribute to the outcome of MV-induced immunosuppression affecting the result of MV infection.

5.3.1. Measles virus-induced gene expression in human epithelial and endothelial cells

To study cytokine expression induced by MV infection, total cellular RNA was isolated from uninfected and MV-infected cells. Analysis by Northern blotting showed an increasing expression of IFN-β, IL-28, IL-29, CCL5, and CXCL10 mRNA in MV-infected A549 cells and HUVECs (IV, Fig. 1). This suggested that cytokines derived

from these cells have important roles in activation of innate immune response during MV infection. In accordance with our results, Tanabe and coworkers have shown that laboratory-adapted but not wild-type strains of MV upregulate IL-28 and IL-29 gene expression in A549 cells (Tanabe et al., 2003). Like IFN-α and IFN-β, the two novel type I IFN-related cytokines IL-28 (IFN-λ2/3) and IL-29 (IFN-λ1) have antiviral activity (Kotenko et al., 2003, Sheppard et al., 2003, Österlund et al., 2005). IFN-α/β and TNF-α are key regulatory cytokines in the innate immune response. They activate the principal effector cells, i.e. the dendritic cells, natural killer cells and macrophages and inhibit viral replication. Similar to IFNα/β, IL-28 and IL-29 are produced in response to virus infection and activate the JAK-STAT signaling pathway. The signal transducer and activator of transcription (STAT) family of proteins are latent cytoplasmic transcription factors that become tyrosine phosphorylated by the Janus family of tyrosine kinase (JAK) enzymes in response to cytokine stimulation and different members of these families have distinct functions in cytokine signaling. MV activates transcription factors in epithelial cells indirectly via IFN-α/β leading to STAT activation but also directly by activating NF-κB (Helin et al., 2001). IL-28 and IL-29 are likely to be involved in controlling the spread of MV infection and activation of innate immune response.

Mda-5, RIG-I, and TLR3 are receptors for dsRNA and binding results in activation of IFN-β gene expression (Alexopoulou et al., 2001, Andrejeva et al., 2004, Yoneyama et al., 2004). Mda-5, RIG-I and TLR3 mRNA expression was clearly activated by MV infection in both A549 and HUVEC cells (IV, Fig. 2) and this was confirmed by analyzing protein expression of mda-5 and RIG-I (IV, Fig. 3). Our results also show that mRNA expression of these receptors clearly precedes that of IFNs and chemokines suggesting that enhanced expression of mda-5, RIG-I, and TLR3 is required for proper cytokine response in MV-infected cells.

TLR3 gene expression is upregulated by IFN-α in human endothelial and epithelial cells (Miettinen et al., 2001, Tissari et al., 2005) and similarly, mda-5 and RIG-I are IFN-α target genes (Andrejeva et al., 2004, Kang et al., 2004, Kang et al., 2002, Yoneyama et al., 2004). TLR2 has been shown to be involved in MV-induced IL-6 expression in human monocytic cells (Bieback et al., 2002), and therefore TLR2 was included in our experiments to study whether IFN-α and IFN-β regulate the expression of mda-5 and RIG-I in A549 cells and HUVEC cells. TLR2 was expressed in high levels in INF-α-stimulated human primary macrophages used as a positive control, but remained negative throughout the rest of the experiment (IV, Fig. 4A). IFN-α or IFN-β treatment, however, strongly upregulated mda-5 and RIG-I mRNA expression (IV, Fig. 4A) and protein expression (IV, Fig. 4B) in both cell types. This is in accordance with previous

reports showing that mda-5, RIG-I, and TLR3 are upregulated by IFN-α (Andrejeva et al., 2004, Kang et al., 2004, Kang et al., 2002, Miettinen et al., 2001, Tissari et al., 2005, Yoneyama et al., 2004). We have previously shown that A549 cells produce biologically active form of type I IFNs in response to MV infection (Helin et al., 2001) and by using neutralizing antibodies against type I IFNs we could prove that MV-induced expression of mda-5, RIG-I, and TLR3 is mediated by IFN-α or IFN-β (IV, Fig. 5). Our results suggest that at early phases of MV infection low levels of IFN-α and/or IFN-β are produced resulting in strong upregulation of mda-5, RIG-I, and TLR3 expression. At late phases of infection high amounts of dsRNA binding molecules are available and IFNs and chemokines are efficiently produced in response to MV-derived dsRNA. Currently, the cellular receptors responsible for low initial type I IFN production in MV-infected endothelial and epithelial cells are unknown.

5.3.2. Mda-5 is involved in measles virus-induced expression of IFN-β

To study the role of mda-5, RIG-I, and TLR3 in MV-induced expression of cytokines, we measured IFN-β mRNA production using a reporter gene assay, specifically the dual luciferase assay (Promega). This assay enabled us to monitor the efficiency of transfection and to normalize the results, which are presented as firefly/Renilla luciferase activity ratio. TLR3 transfection had no effect on basal or MV-induced IFN-β promoter activity (data not shown). However, forced expression of mda-5 or RIG-I resulted in activation IFN-β promoter (IV, Fig. 6). Furthermore, IFN-β promoter was highly activated in mda-5-transfected and MV-infected A549 cells, whereas the virus did not enhance IFN-β reporter gene activity in cells transfected with a plasmid expressing the RIG-I protein. The results suggest that mda-5 functions in the activation of IFN-β mRNA synthesis in response to MV infection in A549 epithelial cells.

The TLR7- and TLR9-dependent IFN-inducing pathways in plasmacytoid dendritic cells have been shown to be effectively inhibited by MV (Schlender et al., 2005) possibly partly explaining the MV associated immunosuppression. Recently, it was shown that ssRNA from influenza A virus, VSV and HIV type 1 are recognized by TLR7 and TLR8 leading to INF-α production in plasmacytoid dendritic cells (Diebold et al., 2004, Heil et al., 2004, Lund et al., 2004). It is therefore likely that genomic ssRNA of MV activates TLR7 and/or TLR8 pathway in dendritic cells resulting in IFN-α production. TLR7 and TLR8 are, however, not expressed in human epithelial or endothelial cells (Tissari et al., 2005) and can therefore not be involved in MV-induced IFN response in these cells.

The role of mda-5 and RIG-I in IFN induction is presently being extensively studied. The V protein of several paramyxoviruses has been shown to bind to and inhibit the function of mda-5, but not RIG-I, thereby reducing the production of IFN (Andrejeva et al., 2004, Childs et al., 2007). The IFN-β induction was, however, not reduced to zero suggesting that the target for V proteins is not the only route to IFN-β induction. This V protein-mda-5 interaction can though in part contribute to immunosuppression during MV infection. Another study has shown that functional RIG-I is required for recognition of MV infection (Plumet et al., 2007). The authors proposed that RIG-I recognizes MV transcription and triggers an IFN response when encountering a free 5´-triphosphate ended leader RNA resulting from a mislocated transcription activity. Both mda-5 and RIG-I are IFN-inducible genes and thus the IFN-dependent upregulation of these genes could be prevented in MV infection by blocking IFN signaling. Hence, the production of IFN can be severely limited.

6. CONCLUSIONS

The first part of this thesis aimed to elucidate the role of cytoskeleton proteins and their conformation in the productive MV infection. By using cytoskeletal modulators we were able to confirm the necessity of intact actin filaments and microtubules in MV replication. Of great interest was the observation that filamentous actin formation alone, without stimulation of the cell cycle, could induce active MV infection in PBMC.

Secondly, we pursued studies in cell structures by investigating MV-induced changes of nuclear components using NuMa and lamins A/C and B as markers for internal nuclear matrix and peripheral nuclear lamina, respectively. We could determine morphological changes correlating with typical features of apoptosis. Furthermore, by inhibiting the caspase activation by the pan-caspase inhibitor z-VAD-FMK MV-infected cells were rescued from cell death showing that caspases were responsible for MV-induced apoptosis.

We also investigated the effect of MV-infection on the innate immune system. Our results suggest that at early phases of infection low levels of IFN-α and/or IFN-β are produced resulting in a strong upregulation of mda-5, RIG-I, and TLR3 expression. We also suggest that mda-5 is involved in MV-induced expression of antiviral cytokines in human endothelial cells.

Our results support cumulating data of a strong interplay between MV proteins and the cytoskeletal components of the host cell. A suitable cellular environment enhances active MV infection, which triggers immunological mediators of the innate immune system, which further affect cell cycle events.

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