HHS Public Access

Author manuscript

Virus Res. Author manuscript; available in PMC 2020 May 01.

Published in final edited form as:

Virus Res. 2019 May ; 265: 74–79. doi:10.1016/j.virusres.2019.03.007.

Stronger together: multi-genome transmission of measles virus

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Abstract

Measles virus (MeV) is an immunosuppressive, extremely contagious RNA virus that remains a leading cause of death among children. MeV is dual-tropic: it replicates first in lymphatic tissue, causing immunosuppression, and then in epithelial cells of the upper airways, accounting for extremely efficient contagion. Efficient contagion is counter-intuitive because the enveloped MeV particles are large and relatively unstable. However, MeV particles can contain multiple genomes, which can code for proteins with different functional characteristics. These proteins can cooperate to promote virus spread in tissue culture, prompting to question of whether multi-genome MeV transmission may promote efficient MeV spread also *in vivo*. Consistent with this hypothesis, in well-differentiated primary human airway epithelia large genome populations spread rapidly through intercellular pores. In another line of research, it was shown that distinct lymphocytic-adapted and epithelial-adapted genome populations exist; cyclical adaptation studies indicate that suboptimal variants in one environment may constitute a low frequency reservoir for adaptation to the other environment. Altogether, these observations suggest that, in humans, MeV spread relies on *en bloc* genome transmission, and that genomic diversity is instrumental for rapid MeV dissemination within hosts.

Keywords

cyclical adaptation; epithelial spread; measles virus; quasispecies; tissue adaptation; virus transmission

1. INTRODUCTION

Measles remains a leading cause of death among children because it suppresses immune function, facilitating secondary infections (Griffin, 2013). Although eradication is possible, and the WHO has established measles elimination goals in all six regions, measles virus

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(MeV) still caused about 110,000 deaths worldwide in 2017 alone (*WHO Key Facts, Nov. 2018*, https://www.who.int/news-room/fact-sheets/detail/measles). Vaccine hesitancy is favoring measles re-emergence in Europe and North America, now reporting costly epidemics (Dabbagh et al., 2017; Hotez, 2016). However, several aspects of MeV multiplication and spread, within and between hosts, are incompletely understood.

MeV is a negative strand RNA virus of the genus *Morbillivirus* in the family *Paramyxoviridae* (Lamb and Parks, 2013), which includes deadly emerging viruses like Hendra virus, Nipah virus, and prevalent human pathogens like mumps virus and the parainfluenza viruses. The particles of all these viruses have similar pleomorphic morphologies and may contain more than one genome (Rager et al., 2002) (Fig. 1, top). All the morbillivirus genomes are organized into six contiguous, non-overlapping transcription units separated by three non-transcribed nucleotides and coding for eight viral proteins in the order (positive strand): 5'-N-P/V/C-M-F-H-L-3' (Fig. 1, bottom).

Interactions of the viral glycoproteins with cellular receptors determine the tropism of MeV and the other morbilliviruses. Since these viruses sequentially utilize two receptors to enter different tissue niches, they are considered dual-tropic (Takeda et al., 2011). After contagion the signaling lymphocytic activation molecule (SLAM) mediates virus entry (Tatsuo et al., 2000): SLAM-expressing alveolar macrophages and dendritic cells ferry the infection through the respiratory epithelium and spread it to lymphoid tissue (Ferreira et al., 2009; Ludlow et al., 2015) (Fig. 2a). The extensive spread of MeV and other morbilliviruses in immune tissues accounts for immunosuppression (Leonard et al., 2010; von Messling et al., 2006).

In the second phase of infection, immune cells deliver morbilliviruses to upper airways epithelial cells that express nectin-4 (Frenzke et al., 2013; Sawatsky et al., 2012; Singh et al., 2016). Nectin-4, a component of the adherens junction, is expressed preferentially in the trachea, and morbillivirus replication at this location accounts for extremely efficient contagion (Muehlebach et al., 2011; Sawatsky et al., 2012).

MeV is the most contagious human respiratory virus (Mateo et al., 2014; Monto, 1999). Its basic reproductive number is often cited as 12–18, which means that each person with measles would, on average, infect 12–18 other people in a totally susceptible population (Guerra et al., 2017). In specific cases individual patients can infect more than 200 new patients (Christensen et al., 1953). Thus, even if the enveloped MeV particles are large and relatively unstable, they are very effective in transmitting infection.

We review here data indicating that MeV particles contain multiple genomes and that MeV spread in well-differentiated primary human airway epithelia relies on the transmission of genome populations. Moreover, adaptation studies indicate that distict quasispecies are selected in different cellular environments. Thus, *en bloc* genome transmission and genome diversity can be instrumental for MeV spread and pathogenesis.

2. GENOME DIVERSITY OF MeV AND OTHER RNA VIRUSES

MeV genomes, as those of all other RNA viruses, are quasispecies, mutant distributions (also termed mutant swarms or mutant clouds) that are generated upon the replication of RNA viruses in infected cells and organisms (Andino and Domingo, 2015). Genetic diversity allows virus populations to rapidly adapt in dynamic environments, and to develop resistance to antibodies and antiviral drugs (Lauring and Andino, 2010). Next generation sequencing, which greatly expands the capacity to examine the composition of viral quasispecies in infected cells and host organisms, is beginning to reveal the mechanisms driving mutant spectra adaptation during viral infections, including those of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (Ansari et al., 2017; Cuevas et al., 2015). These two viruses cause organ- or tissue-specific diseases: HCV causes liver disease, and HIV immunosuppression. Secondary infections of other organs occur but have minor effects on pathogenesis, and are irrelevant for spread to the next host. By contrast, MeV and the other morbilliviruses have to replicate sequentially in two different but equally relevant tissue niches before spreading to the next host. The sequential, dual-tropic nature of the MeV infectious cycle (Takeda et al., 2011) promotes it to an ideal system to characterize potential tissue adaptation of RNA virus genome populations.

Genomic diversity of RNA virus populations is due mainly to the intrinsic error rate of the viral polymerase (Borderia et al., 2016). The mutation rate of the MeV polymerase was estimated to 0.9×10^{-4} per base per replication (Schrag et al., 1999), or lower (Zhang et al., 2013). Our early analyses of MeV infections of permissive cell lines estimated a few positions per genome intra-population variability (Cattaneo et al., 1988). We also documented higher levels of MeV genome diversity in brains of patients who died with subacute sclerosing panencephalitis (SSPE), a rare but always lethal disease that can develop 5–10 years after acute MeV infection (Bellini et al., 2005).

Remarkably, a significant fraction of the changes in MeV RNA from the brains of SSPE patients were accounted by large clusters of biased transitions (U-to-C or A-to-G, depending on the polarity of the strand analyzed), an unprecedented finding when these studies were published (Cattaneo et al., 1988). Shortly thereafter, it was proposed that that the newly discovered cellular enzyme adenosine deaminase acting on RNA 1 (ADAR1) (Bass and Weintraub, 1988; Wagner et al., 1989), which converts adenosine residues to inosine in double-stranded RNA, may cause these A-to-I hypermutation events (Bass et al., 1989). Subsequently, similar A-to-I editing events were detected in several other RNA virus genomes (Cattaneo, 1994), including the Chinese MeV vaccine strain Shanghai-191 (Rota et al., 1994).

The advent of massive parallel genome-wide sequencing then revealed that many cellular RNAs are frequently edited (Li et al., 2009), not only in mammals but also across all orders of metazoans (Nishikura, 2016; Yablonovitch et al., 2017). Sequencing studies also demonstrated that extensive ADAR1 editing occurs frequently in defective-interfering MeV RNA genomes, and also in standard MeV genomes (Pfaller et al., 2015; Pfaller et al., 2014; Suspene et al., 2011). Very recently, it was formally demonstrated that ADAR1 accounts for MeV genome editing (Pfaller et al., 2018) and provides a mechanism that shields

immunostimulatory defective-interfering RNAs from recognition by the innate immune system.

Another family of cellular enzymes edits viral and cellular nucleic acids: the APOBEC cytidine deaminase enzymes introduce C-to-U or C-toT changes, respectively, in RNA or DNA templates (Chen et al., 2017; Harris and Dudley, 2015). The APOBEC family member 3G restricts HIV and other *Retroviridae* through this mutagenic process (Harris et al., 2003; Zhang et al., 2003). Interestingly, MeV and two other negative strand RNA viruses are also sensitive to APOBEC3G inhibition, but specific C-to-U hypermutation events were not detected (Fehrholz et al., 2012).

Altogether, these observations indicate that both the intrinsic error rate of the MeV polymerase, and sometimes A-to-I mutations introduced by ADAR1, can contribute to MeV quasispecies diversity, while APOBEC3G may not.

3. THE MeV INFECTIOUS UNIT: MORE THAN ONE GENOME

A fundamental concept in virus research is that genomes are packaged into particles for spread in the extracellular milieu. In most viruses, individual genomes are packaged. However, the pleomorphic particles of MeV and the other members of the *Paramyxoviridae* family often incorporate more than one genome, as deduced initially from particle sedimentation and ultraviolet inactivation studies (Dahlberg and Simon, 1969; Hosaka et al., 1966).

A second characteristic of MeV biology consistent with the complex nature of the MeV infectious “unit” is the cell-associated nature of infections. In cultivated cells, MeV particles accumulate below the plasma membrane (Bohn et al., 1983) and the ratio of intracellular to secreted infectivity is about 10:1 (Cathomen et al., 1998). In monolayers of most immortalized cell lines, MeV infections spread mainly through fusion of an infected cell with recipient cells expressing an appropriate receptor (Navaratnarajah et al., 2009). Receptors on recipient cells trigger the viral membrane fusion apparatus expressed on the surface of infected cells to form fusion pores; pore expansion then results in coalescence of plasma membranes, and formation of large multinucleated syncytia. This mechanism promotes the spread of progressively larger genome populations, without selection for individual genomes. From this vantage point, the MeV infectious “unit” appears to be highly complex and diverse.

Genetic analyses of MeV infections have provided independent evidence of multi-genome MeV transmission (Rager et al., 2002; Shirogane et al., 2012). In a study originally aimed at re-targeting MeV entry, Rager et al. unexpectedly characterized a stable MeV mutant, which spread depends on the simultaneous packaging of two types of genomes, one of which arose fortuitously. Each genome is defective for one function, namely either cell attachment or polymerase, but the two genomes complement each other. Importantly, the infection kinetics of this two-genomes mutant indicated that packaging of multiple genomes does not negatively affect growth. It was also shown that polyploid particles are produced in standard infections at no expense to infectivity (Rager et al., 2002).

Another example of multi-genome MeV transmission (Shirogane et al., 2012) is discussed in more detail in another contribution to this special issue of Virus Research. In brief, Shirogane et al. observed that a non-fusogenic recombinant MeV can evolve into mutant viruses which regain the ability to induce membrane fusion. Unexpectedly, the spread of one of these mutant MeV was dependent on the simultaneous packaging of two types of genomes, one encoding the wild-type F protein, the other a mutant version with a single amino-acid substitution. Neither the wild-type, nor the mutant F protein mediated membrane fusion by themselves, but together they exhibited enhanced fusion activity through hetero-oligomer formation (Shirogane et al., 2012). Thus, biochemical studies of viral particles, analyses of MeV cell biology, and genetic studies are all consistent with the hypothesis that, at least in stable cell lines, the “unit” that spreads MeV infection can be constituted by more than one genome.

4. MULTI-GENOME MeV TRANSMISSION IN PRIMARY HUMAN AIRWAY EPITHELIA (HAE) CULTURES

The observation that different MeV genome types can cooperate to allow virus spread in tissue culture prompted the question as to whether multi-genome transmission may promote MeV spread also in natural hosts. Animal studies of MeV pathogenesis are demanding because only certain primate species are good models (de Swart, 2009; Delpeut et al., 2017; McChesney et al., 1997). Nevertheless, it is clear that MeV infections remain largely cell-associated during spread in lymphatic tissue and peripheral blood mononuclear cells (PBMC) of humans and other primates. In PBMC of experimentally infected monkeys, or of infected humans, no free MeV is detected, and titers are measured by overlaying leukocytes onto SLAM-expressing cells (McChesney et al., 1997; van Binnendijk et al., 1994).

To begin addressing the question of multiple genome transmission in a relevant model tissue system, we have relied on well-differentiated primary HAE cultures. Previously, MeV infections have been studied extensively in monolayers of immortalized cell lines, where large multinucleated syncytia are formed after extensive cell fusion (Navaratnarajah et al., 2009).

In HAE cultures, formation of large syncytia is not observed. Instead, infectious centers form while tissue structures, including plasma membranes, remain intact and trans-epithelial resistance is maintained, indicating functional tissue integrity (Singh et al., 2015; Sinn et al., 2002). Infectious center formation without visible cell fusion is consistent with the existence of intercellular fusion pores, which may not expand much but are wide enough to allow the transfer of viral ribonucleocapsids, or partially assembled viral particles (Cifuentes-Munoz et al., 2018). Remarkably, presumably because of viral utilization of preserved apical cytoskeletal structures, infections spread to naïve cells within 2–4 hours, which is faster than the spread of other respiratory viruses in the same HAE culture system (Singh et al., 2015). Analogous intercellular pores may account for rapid epithelial spread of MeV genome populations during infections of natural hosts, implying that the intra-host infectious “unit” is highly complex and diverse.

5. CYCLICAL ADAPTATION OF MeV GENOMES TO LYMPHOID AND EPITHELIAL CELLS

Inter-host adaptation of RNA viruses, for example of arboviruses to arthropods and vertebrates (Coffey et al., 2008; Weaver et al., 1999), or of influenza viruses to birds and mammals (Kuiken et al., 2006; Manz et al., 2013) are well characterized, but insights about genetic diversity and adaptation of viruses that replicate sequentially in two tissue niches of the same host are rare. The switch of MeV tropism from lymphatic tissues to epithelial cells provides an ideal experimental system to address the question of whether, and how, intra-host evolution occurs. To gain insights into the extent of MeV genome variability after growth in different tissues, we adapted MeV either to lymphoid (Granta-519) or epithelial (H358) human cells. We also passaged it consecutively in both cell lines (Donohue et al., 2019) (Fig. 3A). Since MeV passaged in these cell lines had different replication kinetics, we sought to investigate the underlying genetic mechanisms by performing deep-sequencing analyses.

As shown in Fig. 3B, lymphoid adaptation reproducibly resulted in accumulation of variants mapping within an 11-nucleotide sequence located in the middle of the phosphoprotein (P) gene. This sequence mediates polymerase slippage and addition of a pseudo-templated guanosine to the P mRNA (Cattaneo et al., 1989). This form of co-transcriptional RNA editing leads to the expression of an interferon antagonist, named V, in place of a polymerase cofactor, named P. Indeed, we found that lymphoid-adapted MeV produces minimal amounts of edited transcripts, and of V protein. In contrast, epithelial-adapted MeV produced similar levels of edited and non-edited transcripts, and of V and P proteins (Donohue et al., 2019). Importantly, adaptation is reversible: in epithelial cells V-competent MeV genomes rapidly out-compete the V-deficient variants (Fig. 3C). Thus, suboptimal variants in one environment may constitute a low frequency reservoir for adaptation to the other environment.

The mechanisms of this process, which was named cyclical genome re-equilibration, were further characterized by rescuing four recombinant MeV carrying individual editing site-proximal mutations. Three mutations interfered with RNA editing, resulting in almost exclusive P protein expression. The fourth preserved RNA editing and a standard P-to-V protein expression ratio (Donohue et al., 2019). However, it altered a histidine involved in Zn^{2+} binding, inactivating V function (Ramachandran and Horvath, 2010). Positive selection of this mutant suggests that inactivation of V protein function, rather than enhanced P protein expression, is key for adaptation to lymphoid cells. Thus, the lymphoid environment favors replication of V-deficient MeV, while the epithelial environment has the opposite effect, resulting in rapid and thorough cyclical quasispecies re-equilibration.

Adaptation by V-protein inactivation was characterized in two different lymphocytic cell lines (Donohue et al., 2019), but it remains to be established whether it occurs during infections of natural hosts. Analyses of the expression of the V and P proteins, or of P/V transcripts, in lymphoid cells from experimentally infected primates are not yet available, but G-insertion efficiency is reduced to 5–20% in MeV mRNA extracted from brain autopsy

material of SSPE patients (Millar et al., 2016). This indicates that editing efficiency can vary *in vivo*, and could reflect quasispecies adaptation to the neural environment.

6. CONCLUSIONS AND PERSPECTIVES

MeV genomes, as those of all other RNA viruses, are quasispecies, or mutant distributions. MeV genomic diversity is due mainly to the intrinsic error rate of its polymerase, and also in part to editing by the cellular RNA-dependent adenosine deaminase ADAR1. Studies of viral particles, analyses of MeV cell biology, and genetic studies are all consistent with the hypothesis that, at least in stable cell lines, the “unit” that spreads MeV infection is constituted by more than one genome. Studies of MeV spread in well-differentiated HAE cultures suggest that also in this *ex vivo* system, MeV infections spread as a highly complex genome population.

We think that, even within natural hosts, MeV spread may rely on *en bloc* genome transmission, and that genomic diversity is instrumental for rapid dissemination and virulence. Interestingly, we have observed that the original epithelial-adapted genome sequence is conserved at low frequency in lymphoid cells, and is rapidly re-selected in epithelial cells. Thus, the compounded selective pressures for sequential adaptation to two different cell types within a host may account, at least in part, for the genetic stability of the dual-tropic morbilliviruses. Similar cyclical quasispecies re-equilibration processes may occur during natural infections with other dual-tropic RNA viruses. These include noroviruses, which infect epithelial and non-epithelial cell types (Wobus, 2018), and HIV, which infects T-cells and macrophages.

FUNDING:

This work was supported by National Institutes of Health grants R01 AI125747 and R01 AI128037 to RC.

Abbreviations:

ADAR1	adenosine deaminase acting on RNA 1
HAE	human airway epithelia
HCV	hepatitis C virus
HIV	human immunodeficiency virus
MeV	measles virus
PBMC	peripheral blood mononuclear cells
SLAM	signaling lymphocytic activation molecule
SSPE	subacute sclerosing panencephalitis

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Highlights:

- Measles virus is dual tropic: it replicates in lymphoid and epithelial cells
- Measles virus quasispecies rapidly adapt to lymphoid and epithelial cells
- In primary human epithelia measles virus spread relies on *en bloc* transmission
- Genomic diversity appears instrumental for rapid virus dissemination within hosts

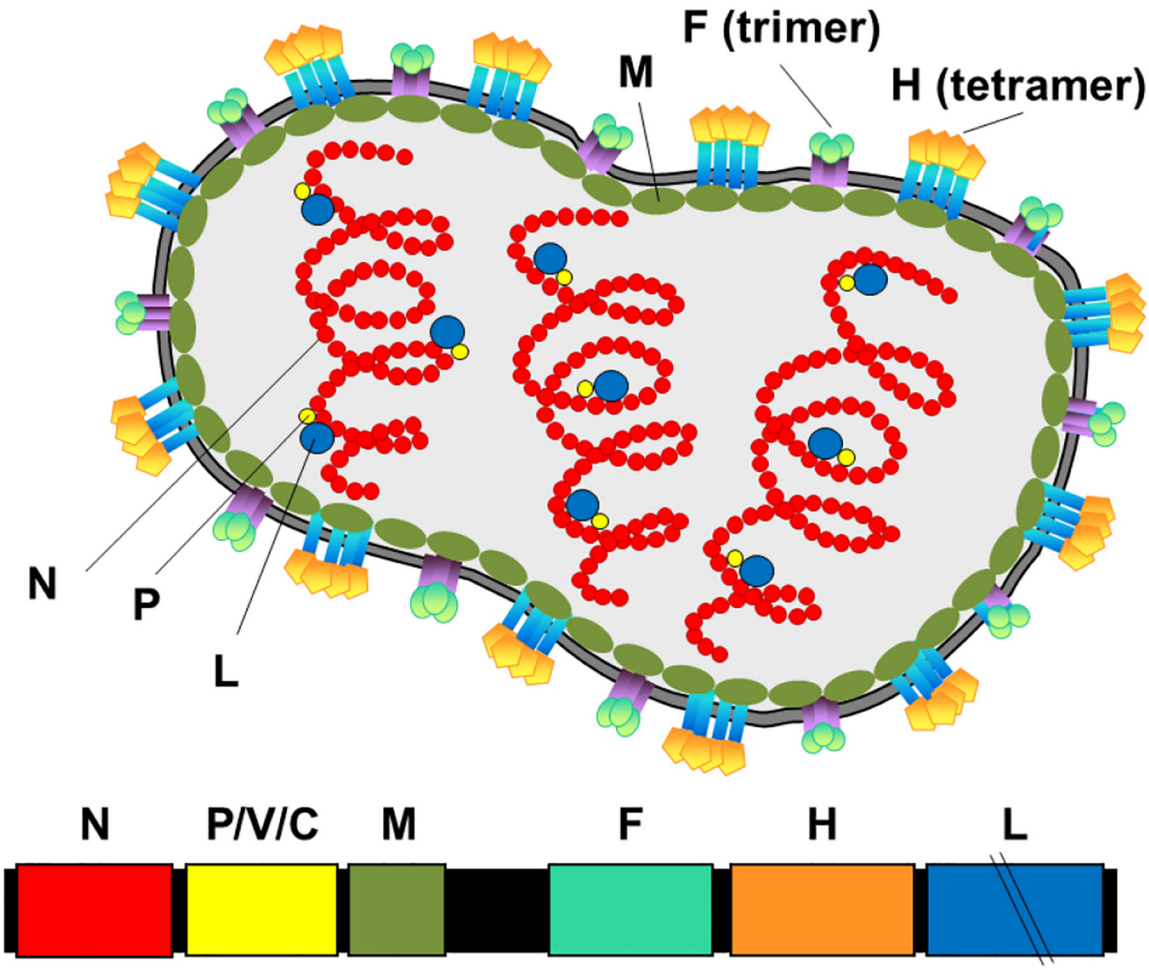

Figure 1. Diagram of a MeV particle (top) and of the viral RNA genome (bottom).

The particle is drawn with its six main components: the nucleocapsid (N) that covers the genomic RNA and interacts with the phosphoprotein (P), and polymerase (large, L), forming the ribonucleocapsid complex; the fusion (F) and hemagglutinin (H) proteins forming the membrane fusion apparatus; and the matrix (M) protein controlling particle assembly as well as transcription and membrane fusion. Particles contain multiple encapsidated genomes, of which three are drawn schematically. On the genome (shown here as positive strand, bottom), the coding regions of the proteins are color-coded, non-coding regions are black. The P gene codes also for the V and C proteins (see Fig. 3 for details).

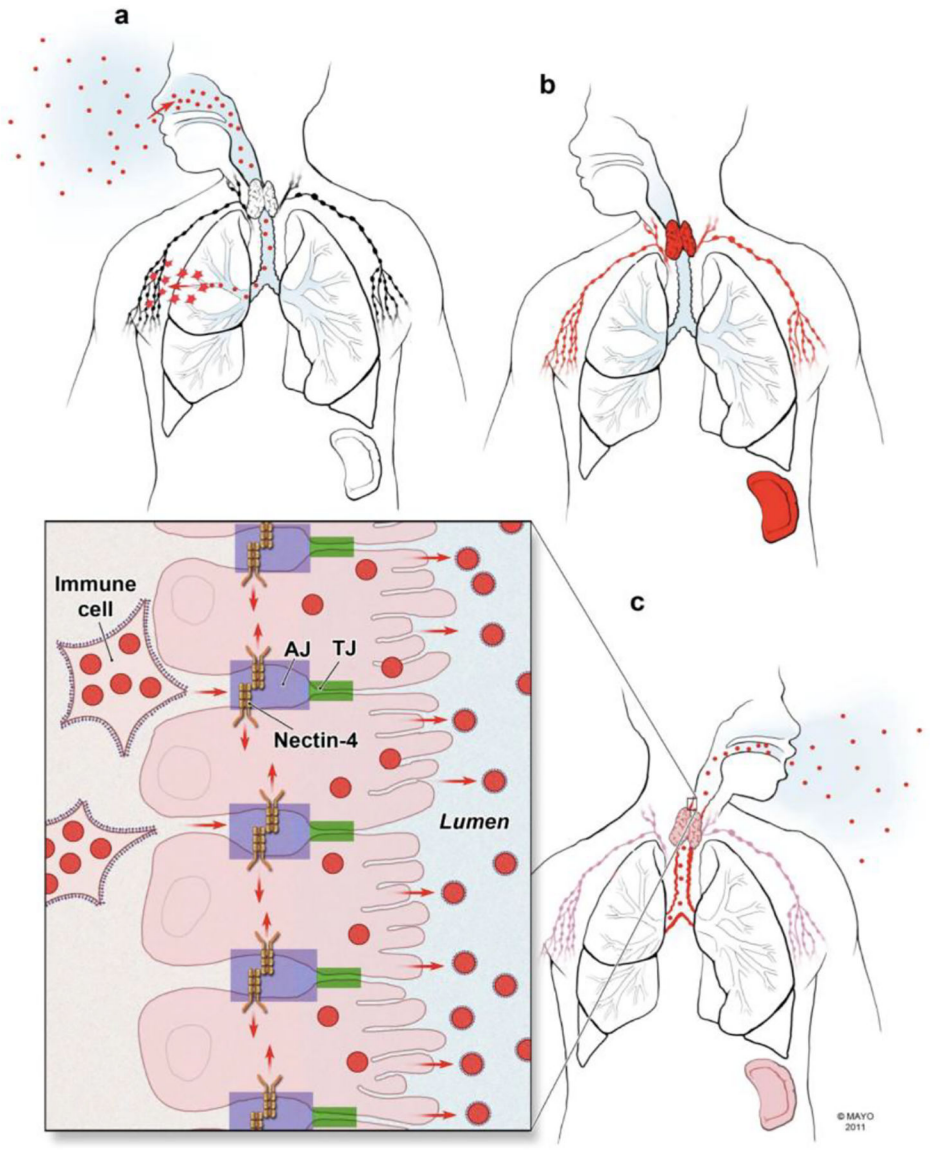

Figure 2. The MeV infectious cycle (Muehlebach et al., 2011).

The lymphatic phase of infection is shown in (**a** and **b**); the epithelial phase is shown in (**c** and **inset**). Infectious viral particles are shown as red spheres; viral glycoproteins as small spikes on extracellular viruses and infected immune cells (inset). Infected cells and organs are red at the peak of infection, or pink after the peak. **a**, MeV enters the airways and infects macrophages and dendritic cells, which ferry the infection to the regional lymph nodes. **b**, MeV infects the local lymph nodes and the infection spreads rapidly to the primary lymphatic organs (red). **c**, infection spreads to epithelia. **Inset**: MeV enters the airway epithelium carried from an infected immune cell that expresses the viral glycoproteins on its plasma membrane. The viral hemagglutinin binds to nectin-4 (orange) in the adherens junction (AJ, blue rectangle), which is located basolateral to the tight junction (TJ, green rectangle). Infection spreads laterally via AJ (red arrows). Epithelial cells express viral glycoproteins in their membranes (not shown).

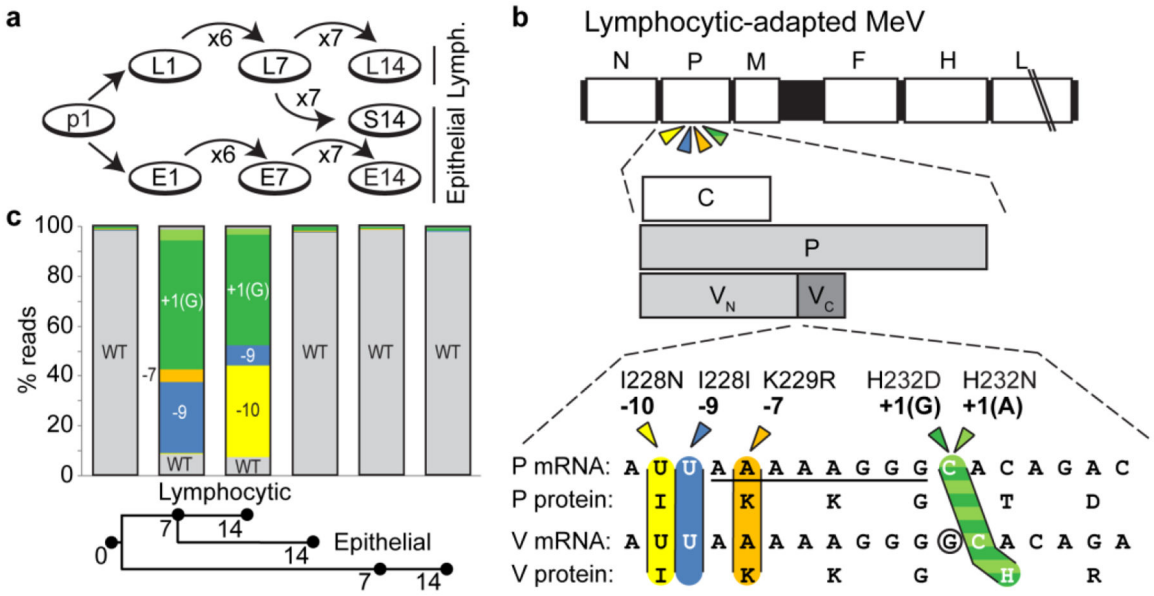

Figure 3. Cyclical quasispecies re-equilibration.

(**A**) Strategy for adaptation of MeV to two cell types. The original inoculum (p1) was passaged 14 times on lymphoid cells (L1 to L14) or epithelial cells (E1 to E14). L7 was also passaged on epithelial cells seven times to generate S14. (**B**) P gene variants selected by adaptation to lymphoid cells. (**Top**) The MeV genome. Coding regions are shown as white boxes, non-coding regions are in black. Four nearby P gene variant positions are shown by colored arrows. (**Center**) Schematic of P gene coding regions. The P protein is translated from the first AUG start codon. The C protein is translated from the second AUG, on a different reading frame. V is generated from transcripts with an additional G inserted after the AAAAAGGG sequence AUG start codon (see bottom). V shares the first 231 amino acids with P (V_N), but has a different C-terminal domain (V_C). (**Bottom**) Sequence and position of the lymphoid variants. Variants are indicated by colored arrowheads, and their positions relative to the G insertion site (circled) are shown above the arrowheads. Amino acid sequences are shown below the nucleotide sequences. (**C**) Analysis of editing site-proximal variants across passage history. The y-axis shows the percentage reads with the indicated alleles. Alleles are colored as in panel B: wild type (WT, grey), −10 (yellow), −9 (blue), −7 (orange), +1(G) (dark green), and +1(A) (light green). The passage numbers analyzed are indicated on the horizontal axis, and the passage history is drawn schematically on the bottom.