Pathogenic measles viruses cannot evolve to bypass vaccine-induced neutralizing antibodies

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1 **<u>Abstract</u>**

2 After centuries of pestilence and decades of global vaccination, measles serotypes capable of

3 evading vaccine-induced immunity have not emerged. Here, by systematically building

4 mutations into the H-glycoprotein of an attenuated measles strain and assaying for serum

5 neutralization, we show that virus evolution is severely constrained by the existence of numerous

6 codominant H-glycoprotein antigenic sites, some critical for binding to pathogenicity receptors

7 SLAMF1 and Nectin-4. We further demonstrate the existence in serum of protective

8 neutralizing antibodies targeting codominant F-glycoprotein epitopes. Calculations suggest that

9 evolution of pathogenic measles viruses capable of escaping serum neutralization in vaccinated

10 individuals is a near-zero probability scenario.

11 Measles (MeV) is a highly transmissible airborne pathogen that spreads systemically and causes

12 transient immune suppression (*1, 2*). Childhood infection is associated with significant mortality

13 and MeV elimination remains a high priority for the World Health Organization (*3*). MeV cell

14 entry via the immune cell receptor SLAMF1 (CD150) drives MeV immunopathogenesis (*4*),

15 whereas a second epithelial cell receptor, NECTIN-4 (PVRL*4*) is exploited for virus

16 transmission (*5*). Receptor attachment and virus entry are mediated by the concerted action of the

17 hemagglutinin (H) and fusion (F) surface glycoproteins (*2*). The MeV polymerase has a high

18 mutation rate and a correspondingly high frequency of monoclonal nAb-escape mutants but has

19 nevertheless remained monotypic (*6, 7*). Vaccination with a lab-adapted isolate of the genotype

20 A strain MeV, isolated from the throat of David Edmonston in 1954, still confers full protection

21 against all currently circulating genotypes. The evolutionary stability of MeV remains poorly

22 understood but could be due to the inability of its surface glycoproteins to tolerate sequence

23 modification (*8-10*), the multiplicity of B cell epitopes displayed on its surface and/or the low

24 likelihood of mutational escape from combined B and T cell mediated antiviral defences.

25

26 Here, to elucidate the serotypic constraints on MeV evolution, we introduced mutated

27 hemagglutinin glycoproteins into the Moraten MeV vaccine whose attenuated phenotype is

28 highly stable with no recorded cases of reversion to pathogenicity or person to person

29 transmission (*11*). Mechanistically, vaccine attenuation is multifactorial, arising from the

30 acquisition of CD46 tropism (*12*), inactivating mutations in V and C immune combat proteins,

31 mutations in the L (polymerase) protein (*13*), and mutations in noncoding sequences (*14*).

32

33   To facilitate the detailed analysis of MeV-H neutralization we assembled a comprehensive set of

34   30 published anti-MeV-H monoclonal antibodies known to neutralize virus infectivity. For those

35   antibodies with unknown target epitopes we propagated the virus in the presence of the antibody,

36   selected escape mutants and sequenced them for epitope identification. Subsequently, we

37   introduced increasing numbers and varying combinations of epitope escape mutations into an

38   antigenically advanced and relatively malleable MeV-H protein obtained from the H1 genotype

39   of MeV (*15*) which, to facilitate the evaluation of mutants with disrupted SLAMF1 and

40   NECTIN-4 receptor binding sites, had been engineered to bind to CD46 by introducing

41   mutations N481Y, H495R, and S546G (**Fig. S1**). We thereby generated a CD46-tropic MeV

42   (MeV-H Δ8) in which all 7 previously reported antigenic sites (*15*), plus an 8th site (IIc)

43   identified in the course of the current study were successfully disrupted **(Fig. S2 and Fig. S3).**

44   Neutralization assays confirmed that viruses encoding the MeV-H Δ8 showed a decreased

45   susceptibility to neutralization by all 30 mAbs **(Fig. 1A and Fig. S4)**.

46

47   Interim analysis of the neutralization of mutated viruses by anti-MeV H antisera had revealed

48   that disruption of 4 or fewer antigenic sites did not abrogate polyclonal antibody neutralization

49   (*15-17*). However, in MeV-H Δ8 we observed an 8-fold reduction in the neutralization titer of

50   mouse anti-H antiserum (**Fig. 1B**). We therefore back-mutated the disrupted antigenic sites to

51   create a panel of Δ7 viruses, each one uniquely presenting a single intact antigenic site.

52   Interestingly, with the exception of the Δ7 viruses retaining antigenic sites Φ, IIa or IIb, all the

53   other four Δ7 viruses were serologically indistinguishable from the parental virus, **(Fig. 1B).**

54   These results were replicated using sera from MeV-H-immunized rabbits (**Fig. S5**). Thus,

55   simultaneous disruption of at least five antigenic sites is required to manifest resistance to

56   neutralization by MeV-H antisera.

57

58   We next sought to determine whether polyclonal sera from measles vaccinees paralleled the

59   above reactivity of mouse and rabbit antisera. Serum samples from Dutch individuals were tested

60   before and after depletion of MeV-F reactive antibodies (**Fig. S6**). Whereas MeV-F–depleted

61   human serum retained its neutralization potency against viruses displaying MeV-H A, the Δ8

62   viruses were less readily neutralized (7-fold reduced susceptibility after averaging individual

63   titers) **(Figure 1C).**

64

65   Since antigenic site III is known to overlap with the SLAMF1 and NECTIN4 receptor-

66   interacting surfaces of the MeV-H (*18*), the elimination of B cell epitopes in this region was

67   expected to negatively impact binding to these critical pathogenicity determining receptors. We

68   therefore examined the receptor tropisms of the Δ8 variant and of the Δ7 variants exhibiting

69   reduced susceptibility to serum neutralization (**Fig. S7**). None of these viruses was able to

70   mediate infection via SLAMF1, indicating that the emergence of a pathogenic measles strain

71   with reduced susceptibility to serum neutralization would require the virus to reconfigure the

72   receptor interacting residues in antigenic site III in such a way as to destroy the dominant B cell

73   epitopes it shares with existing MV vaccine strains, while retaining NECTIN4 and SLAMF1

74   receptor binding affinities.

75

76   Having demonstrated that the multiply mutated MeV-HΔ8 protein could not interact with

77   pathogenicity-determining MeV receptors, we next sought to eliminate the confounding effect of

78   neutralizing antibodies recognizing the MeV-F glycoprotein (**Fig. 2**). To achieve this, we

79   substituted the MeV-F gene with the corresponding gene from canine distemper virus (CDV).

80   Mutations were introduced into the CDV-F and MeV-H coding sequences to restore and

81   optimize the fusogenicity of this heterologous F/H pairing (**Fig. S8**). This virus, hereafter termed

82   MR (Moraten Resurfaced) was subjected to Sanger sequencing and protein composition analysis

83   to confirm its identity (**Fig. S9**). Propagation of the MeV MR virus on Vero cells was

84   significantly slowed versus the comparable virus incorporating an unmodified MeV-H genotype

85   A protein (**Fig. S10A**). Propagation of MeV-HΔ8 with the parental MeV-F glycoprotein was

86   similarly impaired, indicating that mutation of multiple surface residues in MeV-H compromised

87   protein folding and/or function (**Fig. S10B**).

88

89   We next tested whether MeV MR was resistant to human serum from vaccinated Dutch (n=13,

90   cohort 1), Minnesotan (n=6, cohort 2) and Hispanic individuals (n=4, cohort 3) using an

91   improved luciferase-based infection neutralization assay (**Fig. S11** and **Fig. 2A**). ND_{50} values of

92   the tested serum samples gave an overall geometric mean neutralization titer 4 $\log_2$-fold lower

93   MeV-MR versus MeV-A (**Fig. 2B**), suggesting that resistance to neutralization of the MeV MR

94   is fully manifest only at or below a MeV-A ND_{50} titer of 1591 mIU/mL (Fig. 2C).

95

96   Interestingly, measles-immune serum does retain some level of neutralizing activity against the

97   MR virus, suggesting that it may also contain protective antibodies directed against subdominant

98   epitopes in the H glycoprotein. To test this, we inoculated the MR or A viruses into

99   immunocompetent HuCD46Ge-IFNar[KO] mice, harvested sera 4 weeks later and tested for the

100   presence of IgG antibodies directed against the nucleocapsid (MeV-N) or against the MeV-H

101   proteins of pathogenic MeVs (**Fig. S12**). Interestingly, the data confirm that antisera raised

102   against MeV MR do weakly crossreact with wild type MeV-H, indicating that subdominant B

103   cell epitopes may play a significant role in MeV defense. Conversely, antibodies raised against

104   the A virus were able to crossreact weakly with subdominant epitopes in the MeV-H Δ8 protein.

105

106   Since MeV-MR is partially resistant to neutralization by measles-immune human sera, it was

107   important to confirm that, like MeV-Δ8, it lacks the ability to use the pathogenicity-determining

108   receptors SLAMF1 and NECTIN4, and enters cells exclusively via CD46. This was confirmed

109   using CHO cells expressing CD46, NECTIN4, or SLAMF1 where, unlike MeV-A, MeV-MR

110   infected only cells expressing CD46 (**Fig. 4A** and **Fig. S13**). This selective tropism is

111   particularly interesting because previous reports claimed that NECTIN4 tropism could not be

112   eliminated independently of CD46 tropism (*19, 20*) (**Fig. S13**). We therefore measured the

113   densities of CD46 and NECTIN4 receptors on our respective CHO cell transfectants and found

114   them to be equivalent (**Fig. S1B**). Cotransfecting plasmids encoding MeV-F and MeV-H Δ8

115   confirmed that intercellular fusion occurred only in CD46-positive and not NECTIN4-positive

116   CHO cells (**Fig. 4B**) and was similar with CD46 of nonhuman primate origin (**Fig. S15**).

117

118   Further mechanistic studies into the discrimination of CD46 over NECTIN-4 showed that MeV-

119   H Δ8 bound more strongly to CD46 than to NECTIN4, and negligibly to SLAMF1. This

120   contrasted with the binding pattern for MeV-H A **(Fig. 4C)** and suggested that MeV-H Δ8

121   discriminates between CD46 and NECTIN4 via differences in its binding affinities to each of

122   these receptors. We identified no second-site mutations in known contact residues to explain this

123   unexpected segregation of CD46 and NECTIN4 tropisms and therefore postulate that the

124   phenotype may be partially attributable to specific noncontact residues in the MeV-H protein of

125   genotype H1.

126   Measles-immune human serum is known to negate seroconversion in infants during the first year

127   of life and negates the therapeutic effect of systemically administered oncolytic MeV. Hence,

128   we sought to investigate the impact of passive immunization on the infectivity of the MR virus

129   versus the MeV vaccine. Whereas passive immunization with MeV antisera led to a decrease in

130   luciferase signal from MeV A, there was no reduction in the case of the MeV MR (Fig. S16).

131   Accordingly, systemically administered MeV MR in tumor-bearing mice could reach its target

132   (the tumor cells), in the presence of passive antibodies (Fig. S17).

133

134   In summary, by engineering the surface glycoproteins of a MeV vaccine, we have elucidated a

135   number of critical factors determining the remarkable antigenic stability of this monotypic virus.

136   First, MeV has numerous immunologically codominant antigenic sites on its H and F surface

137   glycoproteins. Second, antibodies to each of the seven known antigenic regions on the H

138   glycoprotein are capable of neutralizing virus infectivity. Third, MeVs retaining even a single

139   immunodominant antigenic site on the H glycoprotein remain fully susceptible to neutralization

140   by measles immune human serum. And fourth, the receptor binding surface of the MeV-H

141   glycoprotein is itself an immunodominant antigenic site. Hence MeVs cannot escape their

142   susceptibility to neutralization by measles-immune human serum unless they also lose their

143   tropism for the pathogenicity-determining receptors SLAMF1 and NECTIN4.

144

145   Given that a minimum of 5 immunodominant antigenic sites must be disrupted to impact the

146   susceptibility of MeV-H to serum neutralization and that the error rate of the MeV polymerase is

147   approximately 1×10^{-5} mutations per site per round of genome replication (*21*), we estimate the

148   probability of such a virus arising to be of the order of 1 in 10^{25} progeny virions (weighing

149   approximately 10,000,000 kilograms)(*22*). Further, the simultaneous disruption of fewer than

150   five major antigenic sites would confer no selective advantage on the virus, making stepwise

151   evolution an unrealistic pathway to achieve a neutralization-resistant phenotype. However, even

152   a virus insensitive to anti-H antibodies would still be efficiently neutralized by MeV-F-specific

153   antibodies in immune human sera and, even more critical, would lack the SLAMF1 and

154   NECTIN4 receptor tropisms required for pathogenicity and transmission. We therefore conclude

155   there is a near-zero probability for the accidental emergence of a pathogenic MeV capable of

156   evading vaccine-induced immunity.

178 **Supplementary Materials:**

179 Materials and Methods

180 Figures S1-S17

181 References

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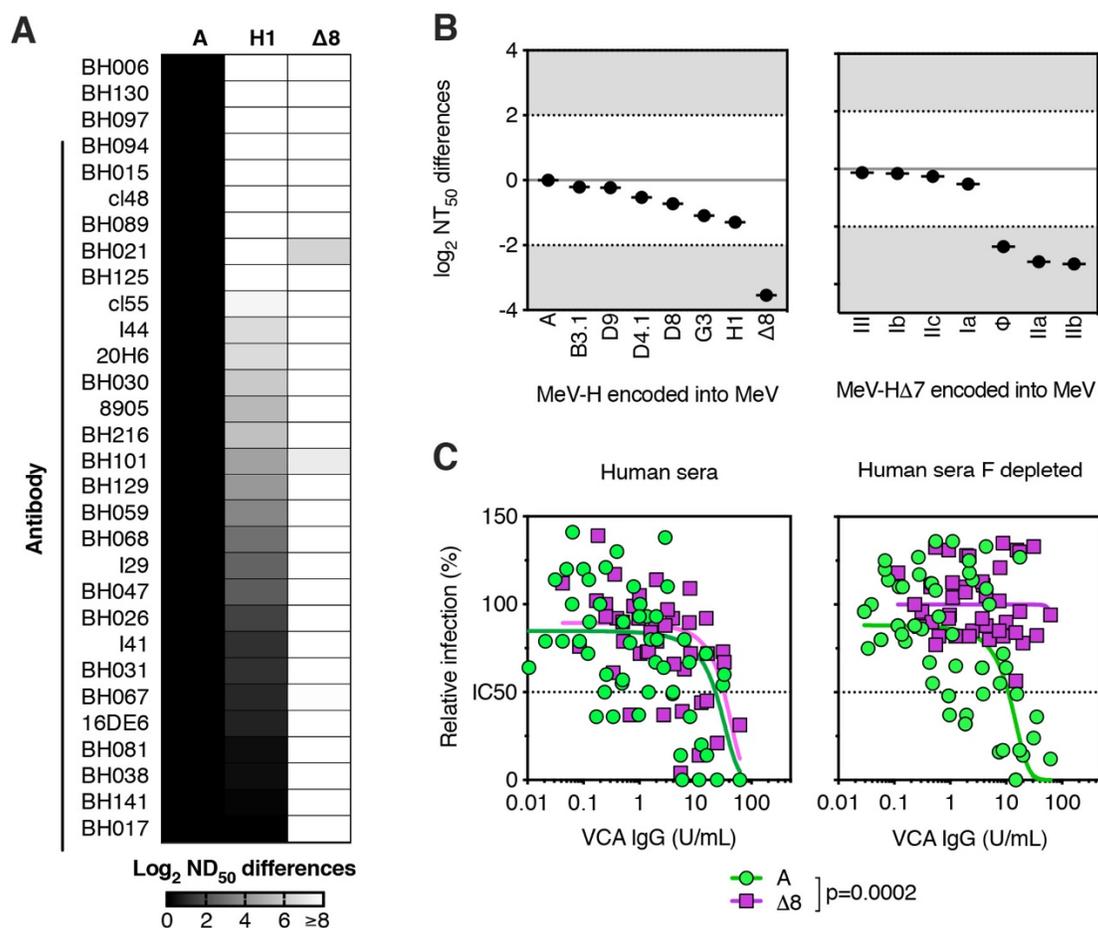
Figure 1. Rational Design of MeV-H Δ8. (A) Neutralization sensitivity of viruses encoding MeV-H A, H1, and Δ8 against a panel of 30 mAbs. Boxes are shaded according to $\log_2 ND_{50}$ reduction. (B, left panel) Neutralization sensitivity of viruses encoding genotype-specific MeV-H (left panel) or (B, right panel) MeV-H Δ 7 mutants (indicated is the antigenic site remaining intact) against mouse sera post-MeV A infection. A difference ≥2 $\log_2$ (grey-shaded region) is considered antigenically significant. (C) Neutralization sensitivity of MeV A or Δ8 against MeV-immune human sera after depletion of MeV-F-specific antibodies (Fig. S5). Epstein-Barr virus viral capsid antigen (VCA) IgG levels were used to accounting for dilution factors since depletion conditions should not affect their levels.
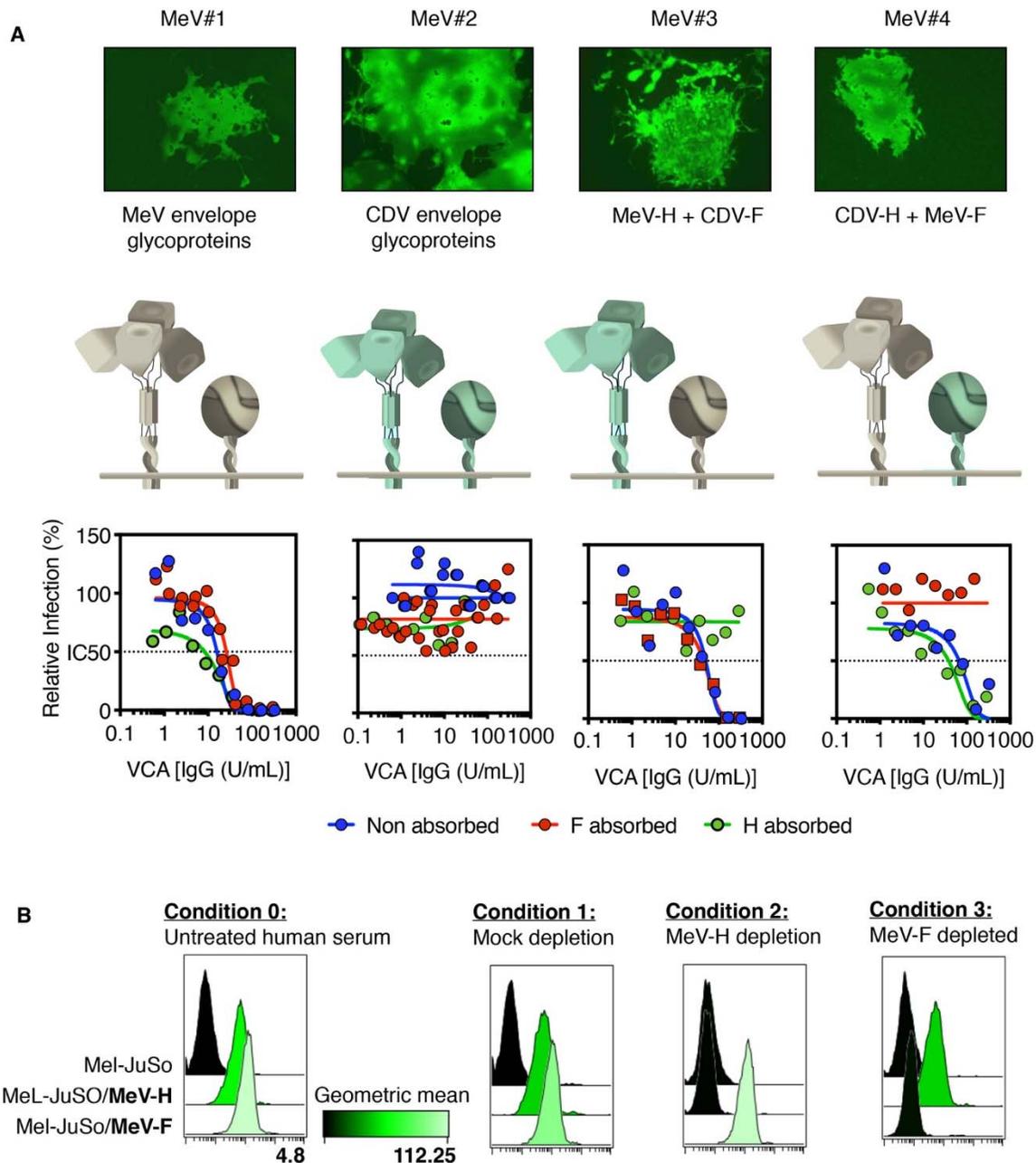
Fig. 2. Role of MeV-H and MeV-F in Virus Neutralization. (A) Virus neutralization assay of envelope-exchange viruses. Isogenic recombinant MeV encoding MeV envelope glycoproteins (virus 1), CDV (virus 2), or chimeric viruses (virus 3 and 4) were used to test neutralization sensitivity against pooled human AB sera depleted of antibodies against MeV-H or MeV-F. Representative syncytia are shown. (B) MeV glycoprotein specificity of pooled human AB sera. Conditions and IgG specific levels are described and determined as in Figure S5. The remaining MeV coat-specific antibodies were tested again to confirm successful depletion. Data are shown as histogram plots.
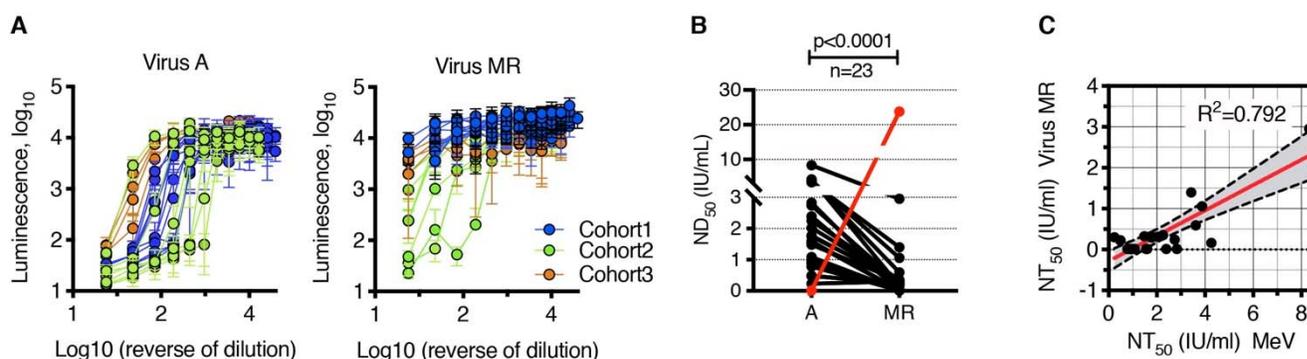
Fig. 3. Neutralization activity of human serum samples. (A) Individual neutralization curves. Samples belonging to different cohorts are colored-code. (B) ND_{50} values of MeV-immune human sera against the MeV A and MR. Each line represents an individual sample (N=23). The red line shows ferret serum anti-CDV, used as a control for neutralization. Statistical significance was inferred by a two-tailed paired t-test. (C) Correlation between ND_{50} for the vaccine virus and the MR virus. P<0.001, for both Pearson and Spearman correlation test. The red curve line represents the linear regression line, with dotted lines indicating the 95% CI for the regression analysis.
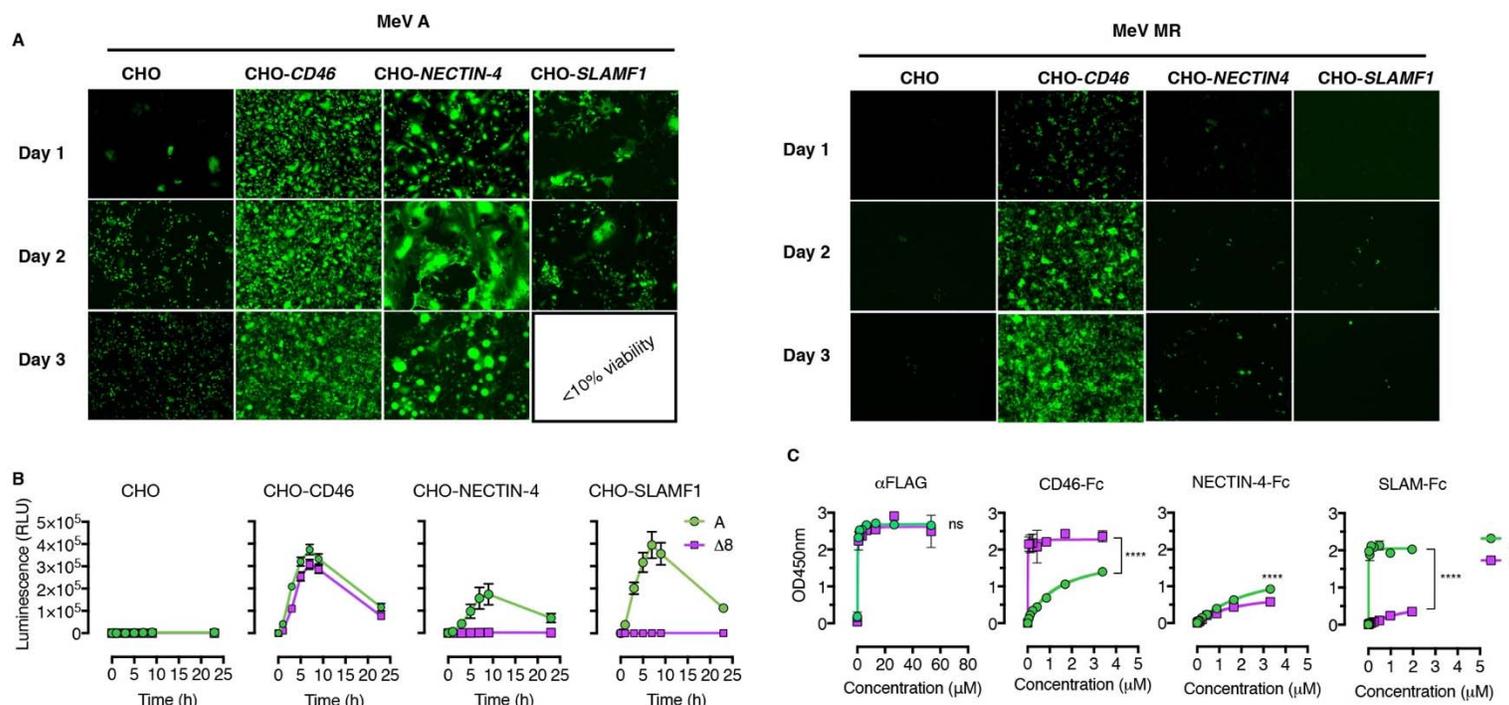
Fig 3. Receptor Usage. (A) CHO cells expressing different MeV receptors were infected at MOI of 1. Images were obtained 3 days after infection. Magnification ×40. (B) Kinetics fusion assay after coexpression of MeV-F with either MeV-H A or Δ8. Mean ± SD. (C) Binding of MeV receptors-Fc to MeV-H protein, monitored by OD. The FLAG epitope present in both MeV-H was used as a coating control. Data are presented as mean ± and were fitted to a 1-site mode of total binding ($R^2 \geq 0.99$). Statistical significance was determined using the Holm-Sidak multiple comparison test: ns, no significant; **** $P < .001$.