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Nebulized fusion inhibitory peptide protects cynomolgus macaques from measles virus infection

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1 **Nebulized fusion inhibitory peptide protects cynomolgus macaques from**
2 **measles virus infection**

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41

42 **Abstract:**

43 Measles is the most contagious airborne viral infection and the leading cause of child death among
44 vaccine-preventable diseases. We show here that aerosolized lipopeptide fusion inhibitors, derived
45 from heptad-repeat regions of the measles virus (MeV) fusion protein, block respiratory MeV
46 infection in a non-human primate model, the cynomolgus macaque. We used a custom-designed
47 mesh nebulizer to ensure efficient aerosol delivery of peptides to the respiratory tract and
48 demonstrated the absence of adverse effects and lung pathology in macaques. The nebulized
49 peptide efficiently prevented MeV infection, resulting in the complete absence of MeV RNA,
50 MeV-infected cells, and MeV-specific humoral responses in treated animals. This strategy
51 provides an additional shield which complements vaccination to fight against respiratory infection,
52 presenting a proof-of-concept for the aerosol delivery of fusion inhibitory peptides to protect
53 against measles and other airborne viruses, including SARS-CoV-2, in case of high-risk exposure,
54 that can be readily translated to human trials.

55

56 INTRODUCTION

57 Measles virus (MeV), a member of the *Paramyxoviridae* family of single-stranded negative sense
58 RNA viruses, is one of the most infectious microorganisms worldwide, with a primary
59 reproduction rate of 12-18¹. Despite the availability of a safe and effective vaccine, measles causes
60 3 to 4 million cases annually, claimed 207.500 lives in 2019, and remains a leading cause of
61 childhood death from vaccine-preventable diseases in many developing countries². Although
62 incidence has decreased considerably from 2000 to 2016 (from 145 to 18 per million), measles has
63 increased since 2017² and is expected to further increase in incidence as a result of the SARS-
64 CoV-2 pandemic and the intercurrent delays in childhood immunization programs and resultant
65 “immunity gaps” in the population³⁻⁶. In addition, in developed countries, imported outbreaks
66 pose a significant risk for immunocompromised people who rely on herd immunity and cannot
67 receive the current live vaccine⁷.

68 MeV is an airborne pathogen, transmitted by inhalation of respiratory droplets and smaller
69 aerosol. Initial infection targets susceptible cells in the respiratory tract^{8,9}. After an incubation
70 period of 7 to 10 days, the acute phase is characterized by fever, oculo-respiratory inflammation,
71 cough, and Koplick spots¹⁰. The characteristic erythematous skin rash occurs around 14 days after
72 infection¹¹ when MeV infects cells in the epidermis^{11,12}. MeV is amplified in regional lymphoid
73 tissues, followed by systemic infection when MeV-infected lymphocytes and dendritic cells (DCs)
74 migrate into the subepithelial cell layers and transmit MeV to the epithelial cells of various
75 tissues¹³. After amplification of MeV in the epithelia, progeny virus is released into the respiratory
76 tract, either free or as dislodged infectious centers consisting of infected cells¹⁴. Infection generally
77 resolves after three weeks, but is often followed by immune suppression which may last for several
78 months and is responsible for numerous complications associated with measles^{15,16}. Rare but
79 severe complications of measles involve central nervous system infection progressing to lethal
80 MeV encephalitis⁷.

81 MeV uses several receptors to infect target cells. The virus binds to CD150 (SLAMF1,
82 SLAM) to infect macrophages, DCs, and lymphocytes¹⁷, and the attachment is aided by the CD209
83 (DC-SIGN) co-receptor^{18,19}. Alternatively MeV can use nectin-4 as an attachment factor to infect
84 the basolateral side of the epithelial airway, a process that promotes viral dissemination^{20,21}. Recent
85 data suggest that entry may also occur from the apical side of the airway in a nectin-4-independent

86 manner^{8,9}. Finally, a role for CADM1 and 2 in neuroinvasion has been postulated²². Infection is
87 initiated following the attachment of the hemagglutinin (H) protein to one of MeV receptors; H
88 then activates the fusion protein (F), and the ensuing rearrangement of F promotes insertion of the
89 hydrophobic fusion peptide into the facing cellular membrane. A second folding event occurs
90 driven by interaction between N- and C- terminal heptad repeat (HR) regions (HRC and HRN
91 domains respectively) of F, completing virus-cell membrane fusion²³. Our previous work has
92 demonstrated that peptides derived from HRC region can interfere with the second folding event
93 required for virus-to-cell fusion during MeV infection^{24,25}. A dimerized version of a peptide
94 corresponding to the HRC region, conjugated to a cholesterol moiety (referred to as “HRC4”
95 peptide, Fig. 1D), inhibited the fusion process in cell culture and in organotypic brain cultures²⁴.
96 HRC4 peptide administered intranasally to cotton rats and to humanized transgenic mouse models
97 of lethal measles disease led to reduction of the viral titer in cotton rat lungs and a significant
98 increase in survival of mice ^{24,25}.

99 Advantages of inhaled protein therapeutics include the non-invasive needle-free drug
100 delivery route, and the ease of depositing drugs directly in the lungs while limiting systemic
101 toxicity²⁶. Since the approval of inhaled Dornase alfa for treating pulmonary disease in cystic
102 fibrosis, several peptides have been under clinical development for inhaled delivery²⁷. Nebulizers
103 can be used for high dose delivery with limited drug formulation development²⁶⁻²⁸. In the present
104 study, a mesh nebulizer was used to deliver MeV fusion inhibitory peptides to nonhuman primates
105 (NHPs) – cynomolgus macaques – a well-characterized model that recapitulates measles infection
106 in humans²⁹. The mesh nebulizer applied in this study uses a piezo-electric generator to push the
107 drug solution through a micro-perforated metal sieve, allowing a fast and silent drug delivery ²⁸.
108 Small diameters of the sieve pores generate aerosols smaller than 5 µm, enabling efficient
109 pulmonary drug delivery²⁷. Using this mesh nebulizer for respiratory administration of fusion
110 inhibitory peptide we effectively inhibit MeV infection in the macaque model. These results open
111 novel perspectives for antiviral prevention strategy against measles and possibly other airborne
112 viruses, including SARS-CoV-2.

113

114

115

116 RESULTS

117 Treatment with HRC4 lipopeptide does not promote selection of drug-resistant variants

118 Generation of escape variants is a concern with any antiviral³⁰, we initially tested for emergence
119 of peptide resistant MeV variants in cell culture. Recombinant MeV IC323-eGFP³¹ was grown on
120 Vero-hSLAM cells in the presence of either 1 μM HRC4 peptide or another fusion inhibitory
121 peptide (FIP) carbobenzoxy-(Z)-D-Phe-L-Phe-Gly peptide³², -[FIP-PEG₄]₂-Chol- which was
122 dimerized and coupled to cholesterol like HRC4³³. Viruses were sequenced after eight passages
123 (Fig. 1a). In the [FIP-PEG₄]₂-Chol-treated cells, two mutations in MeV HRC domain were
124 identified, in the same residues as described previously under the selective pressure of the
125 unconjugated FIP, V₄₅₉ and N₄₆₂³⁴ (Fig. 1a, b). However, no mutations were identified in the
126 HRC4-treated MeV. The FIP resistant MeV variants were susceptible to inhibition by HRC4
127 peptide, as determined using a quantitative fusion assay (Fig. 1c). Fusion between cells expressing
128 wt or variant MeV glycoproteins and cells expressing hSLAM was measured by β-galactosidase
129 complementation in the presence of 5 μM of FIP or HRC4. FIP inhibited membrane fusion
130 mediated by the wild-type F and F-V₄₅₉I but did not affect the fusion mediated by F-N₄₆₂S or F-
131 N₄₆₂S/V₄₅₉I mutated proteins. In contrast, HRC4 inhibited membrane fusion mediated by the wild-
132 type and mutant F proteins (Fig. 1c). These data demonstrated the absence of HRC4-resistant MeV
133 mutants following multiple viral passages and strengthen the selection of HRC4 lipopeptide (Fig.
134 1d) for further preclinical development of MeV fusion inhibitory peptide.

135

136 Analysis of the dose and schedule of HRC4 administration in murine model of MeV infection

137 We next evaluated the dose and schedule of HRC4 peptide by intranasal administration in the
138 humanized murine transgenic model of CD150xIFNα/βR KO mice, previously shown to be very
139 susceptible to MeV intranasal infection^{35,36}. Mice were treated with HRC4 peptide (1 or 0.1 mg/kg)
140 either twenty-four or six hours prior to intranasal infection with a lethal dose of MeV-IC323-eGFP
141 (10⁴ PFU). All mock-treated mice succumbed to the infection by day 12 post-infection (p.i.), while
142 the treatment with the HRC4 (1 mg/kg) six and twenty-four hours before infection led to a
143 significantly higher survival rate (p <0.0001, Mantel-Cox test) (Fig. S1).

144 Based on these results and previous work^{25,37}, we selected for further studies a 4 mg/kg
145 dose, given 3 times by nebulization, 24 h and 6 h before infection, and 24 h after infection, to

146 optimize the antiviral effect of HRC4 in primates which are highly susceptible to MeV, considering
147 the possible loss of peptide delivered through nebulization. This choice was also driven by a
148 recently published study of the therapeutic three-time nebulization of antiviral compound in
149 respiratory syncytial virus (RSV)-infected children³⁸.

150

151 **Characterization of an aerosol device for lipopeptide delivery into the lung alveoli**

152 MeV infection of the respiratory tract targets the lung alveoli⁸, we therefore engineered an inhaled
153 strategy and used a customized mesh nebulizer to deliver the HRC4 lipopeptide aerosol deep into
154 the respiratory tract, to block virus infection. The particle size measurement of aerosol generated
155 following the nebulization of either HRC4 peptide or saline solution (0.9% NaCl) using a prototype
156 mesh nebulizer with a 3 μm pore sieve and a prototype face mask (Fig. 2a) was assessed by laser
157 diffraction (Fig. 2b). The nebulizer devices delivered particles with an average size of 4 μm , in
158 terms of the Volume Mean Diameter (VMD), of both peptide and saline solution, at a flow rate of
159 0.32 - 0.46 ml/min (Fig. 2b, detailed in Table S1). Approximately 58% of particles were smaller
160 than 5 μm , which is the aerosol size that reaches the airways²⁷, where MeV infection initiates.

161 The inhibitory effects of HRC lipopeptide *in vitro* were evaluated before and after
162 nebulization to address the possibility that nebulization itself could inducing aggregation or
163 degradation of peptide with resultant loss of activity^{26,27} (Fig. 2c and d). Nebulization of HRC4
164 did not cause any loss of activity. Cytotoxicity of the nebulized HRC4 before and after nebulization
165 was evaluated *in vitro* using Vero-E6 cells (Fig. 2d). No measurable cytotoxicity was observed at
166 doses ranging from 0.5 nM to 4 μM , indicating that the HRC4 therapeutic index is higher than 500
167 (4 μM / 8nM)³⁹.

168

169 **Biodistribution and safety of nebulized HRC4 in cynomolgus macaques**

170 Delivery of aerosol by the customized nebulizer to the macaques was measured by scintigraphy
171 imaging of animals nebulized with ^{99m}Tc-DTPA-labeled in NaCl 0.9% solution, chosen since
172 HRC4 peptide solution and NaCl shared similar aerodynamic properties (Table S1). After
173 nebulization, 40% of the total aerosolized product reached the respiratory tract, with 11.4%
174 distributed into the lungs (Fig. 3a). The deposition of the HRC4 peptide in the respiratory tract was

175 further analyzed using anti-HRC4 antibodies for the immunofluorescent detection of the peptides
176 in lungs of macaques, sampled either immediately after nebulization (15 min) or 16 h and 24 h
177 later (Fig. 3b and S2). As expected from the scintigraphy imaging, analysis of all three lung regions
178 revealed the presence of HRC4 within the alveoli surface area, suggesting peptide distribution
179 throughout the lungs following the nebulization.

180 We further analyzed whether peptide could reach the blood circulation following the
181 nebulization. HRC4 was found in low concentration (below 1nM) in the serum of nebulized
182 animals, up to 96 h after a third nebulization (Fig. 3c), while it was absent in the urine. Low entry
183 of the peptide into the circulation did not lead to the active immunization of animals, as HRC4
184 specific antibodies were not found in the serum 28 days after nebulization (Fig. 3d). In addition,
185 no adverse effects (pyrexia, allergic reaction) were observed during the 28 days after peptide
186 nebulization. Histological analysis of lungs collected from nebulized animals did not reveal any
187 abnormalities (Fig. S3).

188 Biochemical parameters in the plasma and cellular composition of the blood were
189 evaluated immediately before treatment and 1, 2, 3, 6, and 28 days after nebulization of either
190 saline or HRC4 peptide in non-infected and MeV-infected NHP, to search for early and late toxic
191 effects of the aerosol delivery (Fig. 4). Analysis of the numerous hematological and biochemical
192 parameters in noninfected animals did not show significant variations between groups. In both
193 control and peptide group, few animals experienced transient increase of creatine kinase over
194 physiological values⁴⁰ (Fig. 4a) which can be linked to intramuscular anesthesia⁴¹. In addition,
195 flow cytometry monitoring of the composition of major PBMC populations in blood did not reveal
196 any significant changes in animals nebulized with peptides compared to values obtained before the
197 nebulization (Fig. S6).

198

199 **Modelling of the virus and peptide deposition on the lung surface area**

200 To estimate the coverage of the lung surface area of a NHP following the aerosolization of a
201 peptide present within the nebulised particles, in the relationship to the administrated viral
202 inoculum, we performed the mathematical modelling, schematically presented in the Fig. 5. The
203 estimation is based on the calculation of either virus or peptide dose per unit of lung surface as
204 described in the FDA guidance for inhalation product⁴². The calculation took into consideration

205 the number of droplets formed following the administration, the distribution of viral particles and
 206 the amount of peptide molecules covering the pulmonary area ⁴³. Calculation of the number of
 207 infectious viral particles (N_v) administrated in the macaque's lung took into account the method
 208 of viral administration which consists in delivering 10000 plaque-forming unit (PFU) of virus,
 209 (N_v) in a form of liquid (V_v), through the endo-tracheal tube, leading to the formation of the thin
 210 liquid layer in the lung conductive airways⁴⁴. Taking into account the worst-case scenario, ie a
 211 liquid thickness (T_v) of 7 μm⁴⁵ recovering the epithelia in the conductive airways, we calculated
 212 the surface (S_v) covered by the 5 ml liquid volume (2 ml inoculum and 3 ml washout):

213
$$S_v = V_v / T_v = \mathbf{7140 \text{ cm}^2}$$

214 Then, the calculation of the virus concentration (C_v) in the region of infected surface lung
 215 was performed using the following formula:

216
$$C_v = N_v / S_v = \mathbf{1.4 \text{ PFU/cm}^2}$$

217 Determination of the number of droplets administrated in the macaque lung was based on
 218 results presented in the Fig. 2A, showing that the deposition fraction in the lung (E) is around 10%,
 219 with the nebulizer charge V_p = 3ml, giving thus 0.3 ml in a form of deposited droplets in the lung.
 220 Knowing the mean diameter of droplets (D_p = 4 μm), we can calculate the number of deposited
 221 droplets (N_d) as follows:

222
$$\text{particles volume} = \frac{4 \pi}{3} \left(\frac{D_p}{2}\right)^3$$

223
$$N_d = \frac{E V_p}{\text{particles volume}} = \frac{6 E V_p}{\pi D_p^3} = \mathbf{9 \times 10^9 \text{ droplets}}$$

224 Based on the peptide concentration (400 nmol/ml) and the nebulizer charge (V_p), we can
 225 calculate the number of peptide (N_p) deposited in the lung as follow:

226
$$N_p = N_a C_m V_p E = \mathbf{7 \times 10^{16} \text{ peptide molecules}} \quad (N_a: \text{Avogadro number})$$

227 If we consider a homogenous deposition of the liquid in the lungs, we can calculate the
 228 concentration in terms of number of peptide molecules per surface of lung as the ratio of the
 229 number of peptide molecules (N_p) and the macaque's lung surface:

230
$$[R] = \frac{N_p}{\text{alveoli area} \times \text{total number of alveoli}} = \frac{7.22 \times 10^{16}}{(0.19 \text{mm}^2 \times (2 \times 57.8,106))} = \mathbf{3 \times 10^{11} \text{ peptide /cm}^2}$$

231 Consequently, in the lung of the macaque, a surface of 7140 cm² was covered with the
232 virus 1.4 PFU / cm². On the same lung surface, we deposited by nebulisation 3x10¹¹ peptide/cm².
233 This concentration of peptide was administrated homogeneously to the totality of the lung.
234 Interestingly, we have obtained a 2x10¹¹ ratio between the peptide and the virus per cm² in the
235 infected lung surface, being thus largely in favor of the peptide deposition (Fig. 5), and highly
236 encouraging for the further *in vivo* assay using MeV infected NHP.

237 Finally, when the estimation of the peptide and virus deposition in the *in vitro* tissue culture
238 was performed using a similar type of calculation, the large excess of the peptide to virus was also
239 obtained (5x10¹¹ peptides/cm², Fig. S4), in accord to the highly efficient inhibition of virus
240 infection seen *in vitro* (Fig. 2c and d).

241

242 **Nebulized HRC4 peptide protects cynomolgus macaques from MeV infection**

243 To assess antiviral efficacy of nebulized HRC4 peptide in NHPs, groups of 3 animals were infected
244 with 10⁴ PFU MeV IC323-eGFP by intra-tracheal inoculation, and either mock-treated with
245 nebulized saline solution (C1, C2, and C3) or treated with nebulized HRC4 peptide (P1, P2, and
246 P3) twenty-four and six hours before infection and twenty-four hours after infection (Fig. 6a). The
247 NHPs were housed in cages accommodating two animals per cage for ethical reasons, so that one
248 cage contained one peptide-treated and one saline-nebulized macaque. The animals were
249 monitored for 28 days for the appearance of clinical signs, including temperature, weight, and
250 behavior changes, none of which were observed during the experiment.

251 As in human infection, measles infection in NHPs induces a skin rash that can be followed
252 macroscopically during infection with eGFP-encoding MeV¹⁷. Observation of the fluorescent skin
253 rash is facilitated by utilizing a blue LED light, particularly in cynomolgus macaques where the
254 red skin rash is less apparent than in rhesus macaques⁴⁶. After infection with MeV-IC323-eGFP,
255 skin and oral mucosa were monitored under blue light every three days post-infection (p.i.). All
256 mock-treated animals, but no HRC4-treated animals, had a GFP-fluorescent rash (Fig. 6b-c). The
257 fluorescent rash appeared as early as day 6 p.i. (animal C3) and lasted until day 16 (C1 and C2) or
258 up to day 28 p.i. (C3), mainly located in the mouth (tongue, palate, gum, and chin) and skin
259 (preferentially on armpits, groin, and back) (Fig. 6b-c).

260 MeV infects and replicates in circulating immune cells^{13,15}. Infection of PBMCs was
261 detected by flow cytometry on day three p.i. in animals C1 and C3, on day 6 p.i. in animal C2 and
262 at a low level in animal P2 at day 9 p.i. (Fig. 6d, left panel). Consistent with eGFP expression,
263 viral RNA followed a similar kinetic trajectory (Fig. 6d, central panel). Viremia lasted 9 to 16 days
264 in mock-treated animals and 6 days in HRC4-treated animal P2. This NHP was housed in the same
265 cage with mock-treated C3, which exhibited a high level of MeV infection and most probably
266 transmitted it as a secondary infection to P2 after the end of the peptide treatment, confirming the
267 high contagiousness of MeV infection. The viremia of animal C3 peaked at day 6 p.i. while animals
268 C1 and C2 peaked at day nine p.i.. However, despite the delayed infection, animal P2 had less
269 viremia than mock-treated animals. Oral shedding of virus was monitored by viral genome
270 quantification in RNA extracts from throat swabs (Fig. 6d, right panel). Low levels of viral RNA
271 were found at early time points and likely represent leftovers of the initial inoculum, while viral
272 shedding peaked at day nine p.i. and lasted up to day 16 p.i. in saline-treated animals. Consistent
273 with the PBMC results, viral RNA was only detected late and transiently at day 13-16 p.i. in the
274 swabs from animal P2.

275

276 **HRC4 nebulization prevents MeV infection in peripheral immune cells**

277 Transient immunosuppression associated with leukopenia is a hallmark of MeV infection in
278 humans¹⁵ and is observed in MeV-infected NHPs. Hematological monitoring of MeV-infected
279 NHPs demonstrated a transient leukopenia in the saline-treated group and at later time points in
280 the infected animal P2, as observed in previous reports^{17,47}, with total white blood cell and
281 lymphocytes counts decreased on day 6 following MeV infection in saline-treated animals, but
282 remained stable in HRC4-treated macaques (Fig. 4B and S5). Leukopenia lasted longer in saline-
283 treated macaques (days 6-16 p.i.) compared to animal P2 (days 9-13 p.i.). In infected saline-treated
284 animals, leukopenia was associated with lymphopenia, which was not observed in the fully
285 protected macaques P1 and P3 (Fig. S5).

286 Flow cytometry studies revealed a transient decrease in B cells (CD20⁺) between day 9-13
287 p.i. in mock-treated MeV-infected animals (Fig. S6). The proportion of CD3⁺CD4⁺ and CD3⁺CD8⁺
288 T cells remained unchanged (Fig. S6) despite a decrease in absolute lymphocyte number (Fig. S6).
289 Evaluation of MeV-infected cell phenotype showed only a few CD14⁺ monocytes positive for GFP

290 between day 6 and 13 p.i, with CD4⁺ T lymphocytes and CD20⁺ B lymphocytes constituting the
291 main targets of the virus (Fig. 7a). Of the total cells infected, 40-60% were T cells and 20% were
292 B cells, with a peak of infection day 6 (C3) or day 9 (C1, C2 and P2) p.i.. The magnitude of
293 infection of CD3⁺CD8⁺ T cells and CD14⁺ monocytes was lower (Fig. 7a) and the majority of
294 infected cells among PBMCs were CD4⁺ lymphocytes (Fig. 7b). Interestingly, peptide-treated
295 animal P2, who likely was infected later by transmission from its non-treated cage-mate, had a
296 very low percentage of all infected cell populations, ranging between 5-30 times lower than saline-
297 treated animals, suggesting an anti-viral effect of HRC4 nebulization followed by secondary
298 infection from the co-housed actively infected C3 macaque (Fig. 7a).

299

300 **Humoral immune response in animals combatting MeV infection**

301 MeV infection induces life-long immunity to reinfection, characterized by the generation of a
302 MeV-specific lymphocyte response¹⁵. We evaluated peripheral blood B cell phenotype and serum
303 antibody responses in MeV-infected macaques using flow cytometry, to track the presence of
304 unswitched (CD20⁺ CD27⁺ CD38⁺ IgD⁺) memory B cells, secreting only IgM, and class-switched
305 (CD20⁺ CD27⁺ CD38⁺ IgD⁻) memory B cells, known to secrete IgG, IgA or IgE (Fig. 8a). Both B
306 cell populations increased from day 3-6 p.i. and day 9 p.i, respectively, in mock-treated animals
307 C2 and C3, although the response of C1 was much lower. The secondarily infected HRC4-treated
308 animal P2 displayed a similar but delayed increase in both class-unswitched and -switched B cell
309 populations. Notably, both P1 and P3 HRC4-treated animals were fully protected against MeV,
310 and B cell populations remained stable without any noticeable increase.

311 MeV-specificity of the B cell response was further confirmed by serological analysis (Fig.
312 8b and 8c). All saline-treated animals seroconverted after MeV infection, with a high MeV
313 antibody titer on day 28 p.i. The secondarily infected animal P2 had a slightly lower total antibody
314 titer (Fig. 8b). All seropositive animals secreted neutralizing antibodies with SN₅₀ values ranging
315 between 546 and 3465 (Fig. 8c). The absence of seroconversion of HRC4-treated animals P1 and
316 P3 correlated with the lack of viral replication and the distinct composition of lymphoid blood
317 compartment in those animals, underlining the efficient and robust protection provided by the
318 nebulized HRC4 lipopeptides.

319

320

321 **DISCUSSION**

322 Airborne infection is transmitted through small aerosolized particles suspended in the air and is
323 responsible for spreading many important infectious diseases of humans and animals. In this study,
324 we pioneered a nebulization approach to inhibit highly contagious MeV infection in the NHP
325 model with fusion inhibitory peptides. As measles continues to present a significant health problem
326 worldwide², there is a need for prevention modalities in addition to vaccination for those who
327 either cannot be vaccinated or do not respond appropriately to vaccination. In the current study,
328 we adopted an approach based on immunovirological and technological research, to develop a
329 drug and device that can be adapted to treat human patients. Fusion inhibitory HRC4 peptide
330 provided complete protection to MeV challenge after delivery by nebulization. This needle-free
331 therapy may find acceptance among people when compared to other routes of administration⁴⁸⁻⁵⁰.
332 The production of 4 µm aerosolized particles by the device used in this study supports its use for
333 targeting MeV and possibly other airborne viruses.

334 MeV infection of cynomolgus macaque mimics both pediatric respiratory infection
335 physiology and mild MeV infection in humans^{47,51-55}. Our results in this model have demonstrated
336 that aerosol peptide administration using the prototype mesh nebulizer device results in efficient
337 deposition of HRC4 peptide into lungs and persistence of detectable peptide twenty-four hours
338 after the nebulization. This application of HRC4 peptide aerosol represents a promising initial step
339 that supports its use in humans, where this device should work even better in the absence of the
340 anatomical constraints of primates⁵⁶. In addition, the excellent safety profile, absence of any
341 adverse reaction, and non-immunogenic character of the compound following nebulized
342 administration support the strategy for human use. Finally, in contrast to several other antiviral
343 compounds^{30,34,57}, repeated passage of MeV in the presence of HRC4 lipopeptide did not elicit
344 viral escape mutants (Fig.1), suggesting that frequent administration may not promote
345 development of drug resistant variants.

346 HRC4 peptide treatment abrogated the development of MeV infection in two out of three
347 animals, measured by the absence of fluorescent rash, PBMC infection, viremia, viral shedding,
348 and MeV-specific immune response. The third HRC4-treated animal (P2), housed with the mock-
349 treated highly-infected animal C3, developed a late paucisymptomatic infection without rash,
350 delayed and reduced viremia, low shedding, and late immune activation of B lymphocytes. Thus,

351 animal P2 might have been protected from MeV initial challenge by HRC4 aerosolization and
352 acquired a MeV infection from animal C3 once the activity of the nebulized HRC4 decreased. This
353 hypothesis agrees with the mode of action of HRC4 fusion inhibitor peptide, which is expected to
354 prevent initial infection through daily administration and provide protection that is estimated to
355 endure at least twenty-four hours. Although the approach presented in this work was not tested in
356 humans, we applied both *in vitro* and *in vivo* models and mathematical modeling of the peptide
357 and the virus deposition in the lungs to support future development and to predict how the drug
358 delivery system will behave in humans.

359 Animals that experienced MeV infection developed a transient leukopenia consisting
360 mainly of lymphopenia and moderate monocytosis. Those parameters were in accordance with
361 previous descriptions of MeV tropism and disease course in the cynomolgus macaque^{47,58}. The
362 recorded infection levels were within the range of those observed by De Vries *et al.*⁴⁷ and slightly
363 below those surveyed by De Swart *et al.*¹⁷. MeV-infected cells were preferentially CD3⁺CD4⁺ T
364 cells, followed by CD20⁺ B cells, CD3⁺CD8⁺ T cells, and subsequently CD14⁺ monocytes, as
365 previously described⁴⁷. One animal (C3) experienced a faster acute infection with a detectable
366 viremia on day 3 p.i. that peaked on day 6 p.i. lasted until day 16 p.i., while in other animals,
367 viremia peaked at day 9.

368 Seroconversion was observed in all animals that developed MeV infection, as evidenced
369 by the appearance of neutralizing antibodies and by the activation of B lymphocyte populations.
370 In animals C2 and C3, class-unswitched memory B cells increased at 3-6 days p.i., while class-
371 switched memory B cells started to appear day 9 p.i., consistent with primary IgM production
372 followed by a switch in immunoglobulin class leading to a secondary secretion of IgG, A or E.
373 Unexpectedly, animal C1 only displayed a minor and delayed modulation of its memory B cell
374 populations, while both total immunoglobulins and neutralizing antibodies were produced.
375 However, animal C1 demonstrated an unusual distribution of lymphocyte populations with B cell
376 counts, representing 35% of PBMCs compared to 6% on average for the other animals. In line
377 with this discrepancy, total Ig was delayed compared to the two other animals from the same group.
378 HRC4-treated animals P1 and P3 developed neither signs of seroconversion nor immune cell
379 activation, underlining the profound protection provided by HRC4 peptides. Despite displaying
380 limited and delayed B cell activation following the pauci-symptomatic infection, animal P2
381 seroconverted.

382 The last four years have witnessed a drastic increase in measles cases despite a highly
383 effective vaccine ³⁻⁶, suggesting the importance of developing an additional safe prophylactic
384 strategy to support global MeV eradication. The approach developed here, nebulization of fusion
385 inhibitory peptides, should be clinically applicable. A fusion inhibitory peptide inhibitor of HIV
386 entry (enfuvirtide) has been commercialized to treat HIV-infected patients by subcutaneous
387 administration⁵⁷, and an oral fusion inhibitor for respiratory syncytial virus (prestatovir) is in
388 clinical trials⁵⁹. Our efforts over the last decade have been directed to design such an entry inhibitor
389 approach for MeV^{25,37,60-62}. The results presented here show that nebulization of our entry inhibitor
390 peptides significantly reduces the clinical impact of MeV infection in NHP, providing a proof-of-
391 concept for antiviral prophylaxis to be developed for humans. This strategy holds potential for
392 protecting immunocompromised people who rely on herd immunity and cannot receive the current
393 live MeV vaccine, since nebulized peptide is capable to completely halt viral infection. Protection
394 against one of the most contagious aerosol-transmissible viral diseases¹ is a critical achievement,
395 suggesting the potential of the nebulization approach for airborne enveloped viruses with similar
396 entry pathways including SARS-CoV-2⁶³ or highly pathogenic Nipah virus⁶⁵. In the case of viral
397 evolution or the emergence of a new strain, the rapid development of a new antiviral based on a
398 modified peptide sequence is feasible. Efficacy of nebulization as an administration route suggests
399 that these antivirals are practical, possible, and within reach for use in the field where outbreaks
400 occur. In parallel to vaccines, when available, and protective equipment, i.e. masks, aerosolized
401 peptides may provide an additional shield to fight against extending outbreaks of airborne
402 transmissible viruses, notably in case of high risk exposure like indoor high density people
403 grouping (aircrafts, exhibitions, lectures ...). This antiviral strategy forms the basis for efficacious
404 and timely emergency response immediately following identification of a new airborne virus
405 which uses a similar fusion mechanism for viral entry ^{25,33,61,63}, now with the added benefit of a
406 suitable delivery device.

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413 **METHODS**

414 **Study design**

415 The primary objective of this study was to evaluate the biodistribution, safety, and antiviral
416 efficacy of nebulized MeV fusion inhibitory lipopeptide HRC4. The initial evaluation of the
417 peptide dose and the administration schedule was performed in CD150xIFN α / β R knock-out (KO)
418 mice, highly susceptible to the intranasal MeV infection ³⁵, using 52 mice (31 males and 21
419 females) separated into five groups. The study was completed using the NHP model of cynomolgus
420 macaque, well-characterized to reproduce MeV infection similar to what is seen in humans ²⁹. In
421 the setting of nebulization experiments with NHPs, the number of animals was minimized to 2
422 times two macaques for the study of biodistribution, pharmacokinetics, and toxicology, two
423 macaques to analyze scintigraphy gamma camera imaging of the aerosol delivery and to 2 groups
424 of 3 NHPs, nebulized with either HRC4 peptide or saline as a control, all 6 infected with MeV, for
425 the study of antiviral efficacy of the tested lipopeptide.

426

427 **Cells and virus**

428 Vero cells expressing human SLAM (Vero-hSLAM) were grown in DMEM glutamax (Thermo)
429 supplemented with 10% fetal bovine serum (FBS), glutamine and antibiotics (100 U/mL of
430 penicillin and 100 μ g/mL of streptomycin) in 5% CO₂ incubators at 37°C and were tested negative
431 for mycoplasma spp (MycoAlerte, Lonza LT07-318). Recombinant MeV-IC323 expressing the
432 gene encoding eGFP (MeV-IC323-eGFP) was generated using reverse genetics in 293-3-46 cells
433 as previously described ⁶⁶, using the plasmid encoding MeV IC323-eGFP kindly provided by Y.
434 Yanagi (Kyushu University, Fukuoka, Japan)³¹. Viral stocks were propagated and titrated on Vero-
435 hSLAM cells.

436 Vero hSLAM cells were infected with 100 PFU of MeV IC323-eGFP, then incubated for
437 2 h at 37°C and further treated with several concentrations of peptides to promote the emergence
438 of escape variants. Viruses were collected after five days and passaged similarly eight times. Viral
439 sequencing was performed using metagenomic next-generation sequencing as described
440 previously⁶⁷. Briefly, RNA was extracted from 50 μ L of culture harvest using the Quick-RNA
441 Viral Kit (Zymo) and treated with TURBO DNase (Thermo, Fisher). cDNA was generated from
442 the DNase-treated RNA using Superscript IV Reverse Transcriptase (Thermo, Fisher) and random
443 hexamers (IDT), followed by second-strand synthesis via Sequenase Version 2.0 DNA

444 Polymerase. The resulting double-stranded cDNA was then purified with the DNA Clean &
445 Concentrator Kit (Zymo). Libraries were constructed from 2µL of cDNA using Nextera XT kit
446 (Illumina) and sequenced on 1x192 bp Illumina MiSeq runs. Sequencing reads were adapter and
447 quality trimmed using Trimmomatic v0.38. Variants present at an allele frequency greater than
448 10% and greater than 10x depth were identified with LAVA ([https://github.com/greninger-](https://github.com/greninger-lab/lava)
449 [lab/lava](https://github.com/greninger-lab/lava)) using a previously sequenced MeV strain (NC_001498) as the reference genome. All
450 variants were manually confirmed by mapping sequencing reads to the same MeV reference strain
451 in Geneious v11.1.4. Sequencing reads are deposited in NCBI BioProject PRJNA828179.

452

453 **MeV infection of mice**

454 CD150xIFN α / β R KO mice^{35,36}, generated by crossing SLAM transgenic mice into an IFN
455 Receptor α / β deficient background, were bred at the institute's animal facility (PBES, ENS-Lyon,
456 France) as heterozygotes for SLAM transgenes. Three to 4 weeks old mice (males and females)
457 were infected i.n. with 10µl of MeV IC323 in both nares (10⁴ PFU/mouse) under isoflurane
458 anesthesia. CD150xIFN α / β R KO mice were given i.n. either 0.1 or 1 mg/kg of HRC4 peptide 24
459 h or 6 h before the infection. Control mice received the same number of administrations of the
460 diluent. All animals were observed and weighed daily for four weeks and those showing clinical
461 signs (neurological symptoms, ataxia, lethargy) were euthanized. The protocol was reviewed by
462 the Regional Ethical Committee CECCAPP and approved under the agreement reference APAFIS
463 N° 21141-2019042916294753v5.

464

465 **MeV infection of NHP**

466 Cynomolgus monkeys (*Macaca fascicularis*) were obtained from Bioprim® (Baziege, France).
467 The effect of HRC4 nebulization on MeV infection in NHPs was analyzed at the BSL2 primate
468 facility at the University of Tours, France. The experiment received approval from the French
469 ethical committee and was performed under the agreement reference MESRN N°29992-
470 2021022209579514. Six healthy female cynomolgus macaques, weighting 2.6-4 kg, aged 2-4
471 years, were housed in groups of 2 animals/cage. All animals were confirmed by serology to be
472 negative for MeV and canine distemper virus. Three macaques included in the control group (C1,
473 C2, C3) were nebulized with 0.9% of NaCl, while the others received HRC4 nebulization (P1, P2,
474 P3). The ethical obligation to house at least 2 NHPs together in the same cage resulted in two

475 animals from different groups, C3 and P2, being co-housed within the same cage, increasing the
476 risk of late MeV transmission between those two animals. The experiment started after 21 days of
477 acclimatization with the nebulization for a 10-15 min period, using a prototype mesh nebulizer,
478 with either 3ml of peptide (4mg/ml) or saline (0.9% NaCl), 24 h and 6 h before infection, and 24
479 h post-infection. Animals were infected under medetomidine/xylazine anesthesia by MeV IC323-
480 eGFP with 10^4 PFU in 2mL by the intra-tracheal route, and macaques were followed for 28 days
481 before euthanasia.

482 Blood samples for hematology analyses were collected at days -1, 0, 1, 2, 3, 6, 9, 13, 16
483 and 28. Blood samples for flow cytometry analyses were collected at days -1, 3, 6, 9, 13, 16 and
484 28. Throat swabs were collected at days 0, 1, 2, 3, 6, 9, 13, 16 and 28, using cotton swabs. Oxygen
485 saturation and heartbeat were monitored by a Radical-7® Pulse CO-Oximeter (Masimo) and
486 breathing was monitored by a Dräger Primus anesthesia machine. Hematological parameters were
487 measured on a Procyte DX (IDEXX) and biochemical parameters were evaluated on a Konelab 30
488 (ThermoFisher). Development of fluorescent rash was followed using a FastGene Blue/Green
489 LED Flashlight (Nippon genetics).

490

491 **Study of biodistribution and toxicology in NHP**

492 In the initial study, the pharmacokinetics and toxicology of HRC4 peptide nebulization were
493 analyzed in 2 two-year-old healthy female cynomolgus macaques, weighing 2.8 kg, at Cynbiose,
494 Marcy l'Etoile, France, accredited by AAALAC. The protocol was approved by the Ethics
495 Committee of VetAgro-Sup and approved under number 146 (MESR N° 2016072117544328).
496 Animals were initially acclimatized to their designated housing room for two weeks and gradually
497 trained during that period to remain calm when being held by the operators during manipulations
498 (blood sampling, monitoring of body temperature) using a reward-based training regimen. Before
499 nebulization, animals were anesthetized with ketamine (5 to 15mg/kg) and midazolam (0.5 to 1.3
500 mg/kg) by intramuscular injection and then placed on a baby chair. Aerosols were administered
501 through a face mask connected to the prototype mesh nebulizer (DTF-Medical, Saint-Etienne). For
502 the pharmacokinetic phase, the aerosolized peptide was administered via the same face mask as
503 above to anesthetized animals on day 0. Blood samples were collected for 4, 8, 24, 48, and 72 h
504 after nebulization. The animals had a washout period of 17 days, and blood samples were collected
505 on day 21, the day preceding the start of the toxicology phase. For the toxicology phase, the

506 nebulized peptide was administered via a face mask to anesthetized animals daily on three
507 consecutive days (22, 23, and 24). Urine and blood samples were collected on day 25, and animals
508 were euthanized for organ collection.

509 To evaluate immediate HRC4 biodistribution into lungs, one animal from the MeV-
510 infection experiment (C1) was nebulized with 3 ml (4 mg/ml) immediately after euthanasia under
511 mechanical ventilation (Dräger Primus anesthesia machine). For mid-term biodistribution, animals
512 were nebulized 24 h (C2) or 16 h (C3) before euthanasia.

513

514 **Scintigraphy Gamma camera imaging of the aerosol delivery into NHP**

515 A study of the biodistribution of the nebulized aerosol was performed at the University of Tours,
516 France. Following European recommendations, 2 five-year-old healthy female cynomolgus
517 macaques, 3–4 kg, were housed under conventional conditions in the animal facility. The
518 experimental protocol was conducted according to European regulations for animal
519 experimentation and approved under the agreement reference MESR N°
520 11682#2017100217166146. Animals were acclimatized to laboratory conditions and trained to
521 breathe an aerosol with a facemask spontaneously. Aerosol generated from 3 ml of 0.9% NaCl
522 mixed with 74 MBq of DTPA radiolabeled with technetium 99m ($^{99m}\text{Tc-DTPA}$) was administered
523 through a facemask connected to the prototype mesh nebulizers, as used in MeV infection
524 experiments (Fig. 2a). Deposition of aerosol was extrapolated based on the $^{99m}\text{Tc-DTPA}$ signal
525 measured at the end of the nebulization using a gamma camera (Orbiter 75 Ecam, Siemens
526 healthcare, Erlangen, Germany)⁶⁸. The nebulizer charge was measured by counting the
527 radioactivity in the syringe (that contained $^{99m}\text{Tc-DTPA}$) before and after loading the nebulizers.
528 Immediately after aerosol delivery, the animals were imaged using the gamma camera. The post-
529 anterior static scintigraphy acquisition was performed for 120s. The amount of $^{99m}\text{Tc-DTPA}$
530 deposited into airways and stomach and remaining in the nebulizer was determined from the
531 digitalized images taking into account the tissue attenuation coefficients, previously determined
532 by perfusion scintigraphy (intravenous injection of $^{99m}\text{Tc-macroaggregates}$ of albumin). The organ
533 body outline was specified using a specific Region Of Interest (ROI), and the lungs were delineated
534 using the perfusion scan ROI. The aerosol dose delivered to different organs of NHPs is reported
535 as a percentage of the nominal dose placed in the nebulizer for that given experiment, taking into
536 account the decay of technetium for all measurements.

537

538 **Peptide synthesis**

539 Unconjugated MeV HRC peptide and FIP (Carbobenzoxy-(Z)-D-Phe-L-Phe-Gly peptide) were
540 purchased from Shanghai Ruifu Chemical Co., Ltd. Bis-maleimide cholesterol was custom made
541 by Charnwood Molecular, Ltd. HRC4 and FIP-dimer cholesterol were conjugated and purified as
542 previously described³³. For the *in vivo* experiments in mice, HCR4 peptide was initially dissolved
543 in DMSO to 50 mg/ml and stored at -80°C. Peptides were then diluted in water to reach either 0.1
544 mg/kg or 1 mg/kg for intranasal administration. For nebulization of macaques, HRC4 peptide,
545 soluble in water, was dissolved in Milli-Q water filtered to obtain a final concentration of 4 mg/ml.
546 The pH of peptide solution was adjusted to 7 and stabilized using HEPES buffer. Peptide
547 preparations were kept at 4°C for four days or at -80°C for the long-term storage.

548

549 **Fusion assay**

550 HEK 293T cells transfected with SLAM-coding plasmid and the omega reporter subunit of β -gal
551 (“target cells”) were incubated with cells co-expressing viral glycoproteins (IC323 H and F) and
552 the alpha reporter subunit of β -gal (“effector cells”) in the absence or presence of inhibitory
553 peptides at the concentration of 5 μ M. In the absence of peptides, fusion between the target and
554 effector cells permits reconstitution of β -galactosidase activity, quantified using the luminescence-
555 based kit, Galacto-Star β -galactosidase reporter gene (ThermoFisher). Percent inhibition was
556 calculated as the ratio of the relative luminescence units in the presence of a specific concentration
557 of fusion inhibitory peptide and the relative luminescence units in the absence of inhibitor,
558 corrected for background luminescence.

559

560 **Cell Toxicity Assay**

561 Vero cells were incubated at 37°C in the presence or absence of the indicated peptides at indicated
562 concentrations up to 5 μ M HRC4 peptide as added into the media, and the cells were incubated a
563 37°C. According to the manufacturer's guidelines, the viability was determined after 24 h using
564 the Vybrant MTT (3-(4, 5-dimethylthiazolyl-2)-
565 5-diphenyltetrazolium bromide) cell proliferation assay kit. TritonX-100 (1%) was used as a
566 positive control. Absorbance was read at 540 nm using a Tecan M1000PRO microplate reader.

567

568 **Viral load quantification by RT-qPCR**

569 Viral RNA was extracted using Qiaamp Viral RNA Kit (Qiagen) for sera and swabs samples and
570 Nucleospin Kit (Macherey Nagel) for PBMCs. Viral load was evaluated by one-step RT-qPCR
571 (NEB Luna® Universal One-Step RT-qPCR kit) using MeV-N-specific primers (MeV-N FW:
572 GTG ATC AAA GTG AGA ATG AGC and MeV-N Rev: GCT GAC CTT CGA CTG TCC T)
573 and GAPDH primers if necessary (GAPDH FW: CACCCACTCCTCCACCTTTGAC, GAPDH
574 REV: GTCCACCACCCTGTTGCTGTAG). PCR amplification was recorded on a Step One plus
575 apparatus (Thermo). All samples were run in duplicates, and results were analyzed using the ABI
576 StepOne software v2.1 (Applied Biosystems).

577

578 **Laser diffraction measurement**

579 The aerodynamic performances of the aerosols generated by the prototype mesh nebulizer were
580 determined by laser diffraction using a Spraytec™ instrument (Malvern Instruments Ltd.,
581 Malvern, UK) and the Spraytec inhalation cell (Malvern Instruments Ltd., Malvern, UK)
582 connected to an aspiration carried out by a vacuum pump set to 30-50L/min⁶⁹. The prototype mesh
583 nebulizers (n=4) were loaded with 3 ml of either NaCl 0.9% or the HCR4 peptide (4 mg/ml,
584 dissolved as described above) and then connected to the inhalation cell. Nebulization duration was
585 notified at the end of the complete aerosolization of the loaded 3 ml. Diffraction data and volume
586 distribution were automatically registered by the Spraytec software. The volume mean diameter
587 VMD, in μm , the respirable fractions inferior to 5 μm ($\% < 5 \mu\text{m}$) and inferior to 2 μm ($\% < 2 \mu\text{m}$)
588 were calculated by the software.

589 The output of nebulizer was determined by the difference between the weight of the
590 nebulizer before and after nebulization and was expressed in percentage of the loaded volume. The
591 output rate of each nebulizer (in ml/min) was then determined as the ratio between the output and
592 the nebulization duration. At least, the residual volume corresponding to the volume of liquid
593 remaining in the reservoir at the end of the nebulization was also determined by weighting the
594 nebulizer before loading it and after nebulization.

595

596 **Enzyme-linked immunosorbent assay (ELISA)**

597 Determination of the HRC4 concentration in the serum and urine of macaques after the third
598 nebulization was determined by ELISA. Maxisorp 96 well plates (Nunc) were coated overnight

599 with purified rabbit anti-MeV-F HRC antibodies (Genescript) ($5 \mu\text{g/ml}$) in carbonate/bicarbonate
600 buffer pH 9.2 at $+4^\circ\text{C}$. Plates were washed twice using PBS followed by incubation with 3% BSA
601 in PBS (blocking buffer) for 60 min. Then, the blocking buffer was replaced with 2 dilutions of
602 each sample in 3% PBS-BSA in duplicate and incubated for 90min at room temperature (RT).
603 Wells were washed 3 times using PBS, and the peptide was detected using an HRP-conjugated
604 rabbit custom-made anti-MeV F HRC antibody (1:1500) in blocking buffer for 2h at RT. Detection
605 of HRP activity was measured by using the TMB substrate (Thermo) and reading absorbance at
606 405 and 620nm on Multiskan FC reader (Thermo). The standard curves were established for each
607 peptide, using the same ELISA conditions as for the test samples and the detection limit was
608 determined to be 0.04nM.

609 Sera of MeV-infected NHPs were tested for the presence of anti-MeV antibodies by
610 ELISA. Briefly, MeV nucleoprotein, produced as described previously⁷⁰ was coated onto 96-well
611 ELISA plates overnight ($1 \mu\text{g/well}$). Plates were blocked using a mix of PBS 1X-Milk 5% for 30
612 min at room temperature. Serial dilutions (1/50 and then 1/3 serial dilutions until 1/2952450) were
613 done in PBS 1X-Tween 0.05%-Milk and incubated 2h at RT. Secondary antibody goat anti-
614 monkey IgG-A-M conjugated to horseradish peroxidase (HRP), (Sigma-Aldrich) was incubated
615 for 1h at 37° and plates were revealed using TMB substrate solution (Thermo). Optical density
616 was measured at 450 and 620nm using an ELISA reader (Thermo) and the absorbance difference
617 between 450nm and 650nm was determined and corrected for blank readings. The serum sample
618 is considered positive when its value is higher than three times the average value obtained with the
619 negative sera of the same dilution and the results were expressed as reciprocal value of the last
620 serum dilution giving the measurable values.

621

622 **Sero-neutralization**

623 Neutralizing Ab titers were determined using plaque reduction number test. Serial dilutions of sera
624 (1:3) in DMEM medium containing 2% FCS were mixed with 50 PFU of MeV IC323-eGFP,
625 incubated 30 min at 37°C and layered on Vero SLAM cells in 6 well-plates for 90 min. The inocula
626 were replaced by DMEM 3% FBS / CMC 0.6% and plates were incubated for 3 days at 37°C .
627 Plaques were counted after crystal violet staining, and relative neutralization titers were defined as
628 the reciprocal dilutions of sera samples that completely inhibited the cytopathic effect of MeV.

629 Data were analyzed by Prism 8 software to calculate SN₅₀ values (non-linear regression, [inhibitor]
630 vs. response, variable slope fitting).

631

632 **Immunofluorescence and histochemistry**

633 To assess the bioavailability of the HRC4 peptide on lungs, lung slices of paraffin embedded
634 organs of 5µm thickness were stained and imaged by confocal microscopy as described previously
635 ²⁴. Briefly, after being blocked and permeabilized in 0.1% TritonX100, 5% BSA solution, slices
636 were sequentially incubated with a rabbit anti-HRC4 (Genscript) overnight at 4°C and with a
637 secondary goat anti rabbit alexa-555 (Thermo) and DAPI for 1H at room temperature. Slides were
638 imaged using a Zeiss LSM800 confocal microscope).

639 For hematoxylin-eosin staining, formalin-fixed tissues were processed and embedded in
640 paraffin and tissue sections were then deparaffinized, rehydrated, rinsed, and placed in PBS before
641 harrys hematoxylin staining (Diapath, diluted 1/3), washed with PBS and stained with eosin 1%
642 (Sigma-Aldrich). Slide were washed with water dehydrated and mounted with DPX mounting
643 medium (Sigma-Aldrich).

644

645 **Flow cytometry analysis**

646 Whole blood was collected on EDTA, then transferred into BD vacutainer CPT tubes (after
647 removal of anticoagulant solution from CPT tubes) and spun at 2500g for 20 min. The PBMCs
648 were collected and one-tenth were used to isolate RNA. The remaining cells were surface stained
649 on ice using three different panels, including Panel A: CD150 BV-421 (clone A12, BD), CD8 AF
650 647 (clone RPA-T8), CD20 APC-H7 (clone 2H7, BD), CD3 V500 (clone SP34-2, BD), CD14 Pe-
651 cy7 (clone M5E2, BD); Panel B: CD150 BV421, CD3 V500, CCR7 Pe-Cy7 (clone G043H7,
652 Biolegend), CD8 AF647, CD45RA APC-H7 (clone 5H9, BD); and Panel C: CD150 BV421, IgD
653 BV510 (clone IA6-2, BD), CD38 Pe-Cy7 (clone HB7, BD), CD27 AF647 (clone O323,
654 Biolegend), CD20 APC-H7. Cells were acquired on a MACSQuant®10 flow cytometer
655 (Miltenyi).

656

657 **Statistical analysis**

658 We used the 2-way Anova analysis and Mantel Cox test for statistical analyses of results of the
659 fusion test and animal survival and non-linear regression for the calculation of the serum

660 neutralization titer. We considered p-values of 0.05 or below (two-tailed tests) to be statistically
661 significant. Statistical analyses were performed using GraphPad Prism 8 software.

662

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675

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681 **Competing interests:**

682 S. Le Guellec is employed by DTF Medical (Saint Etienne, France) and L. Vecellio was employed
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684 **Data availability:** All data are available in the paper or the Supplementary materials.

685

686

687

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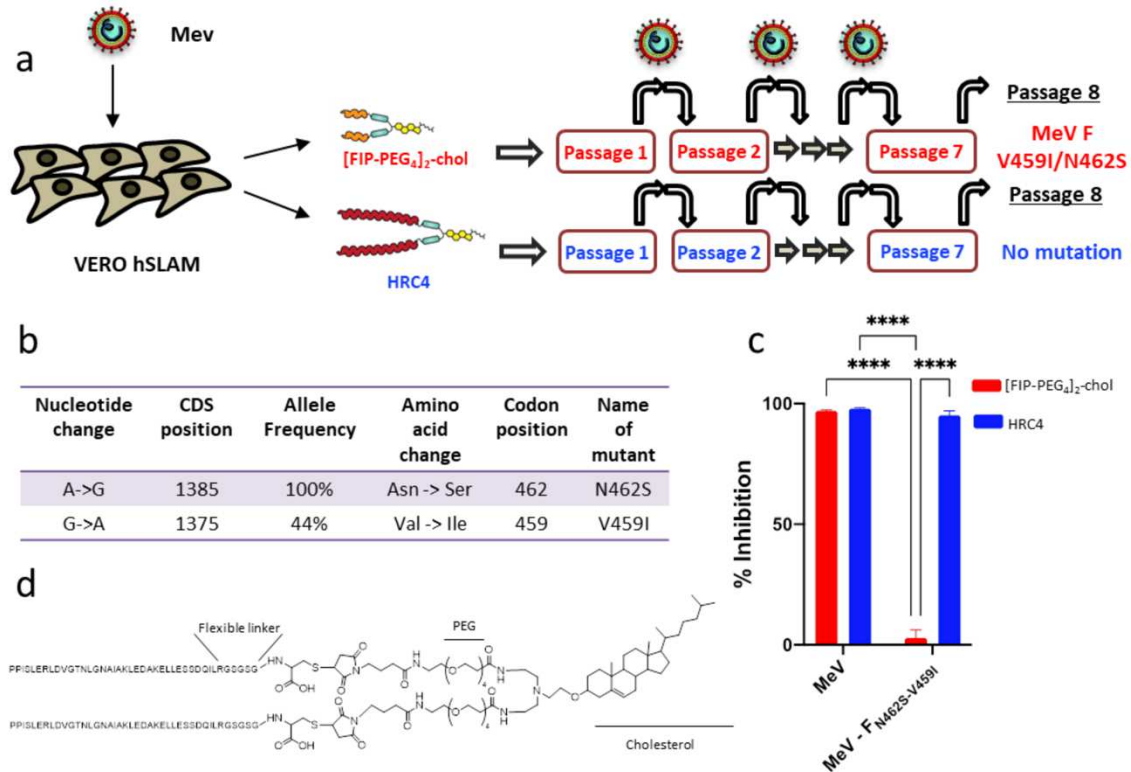
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Figures and figure legends

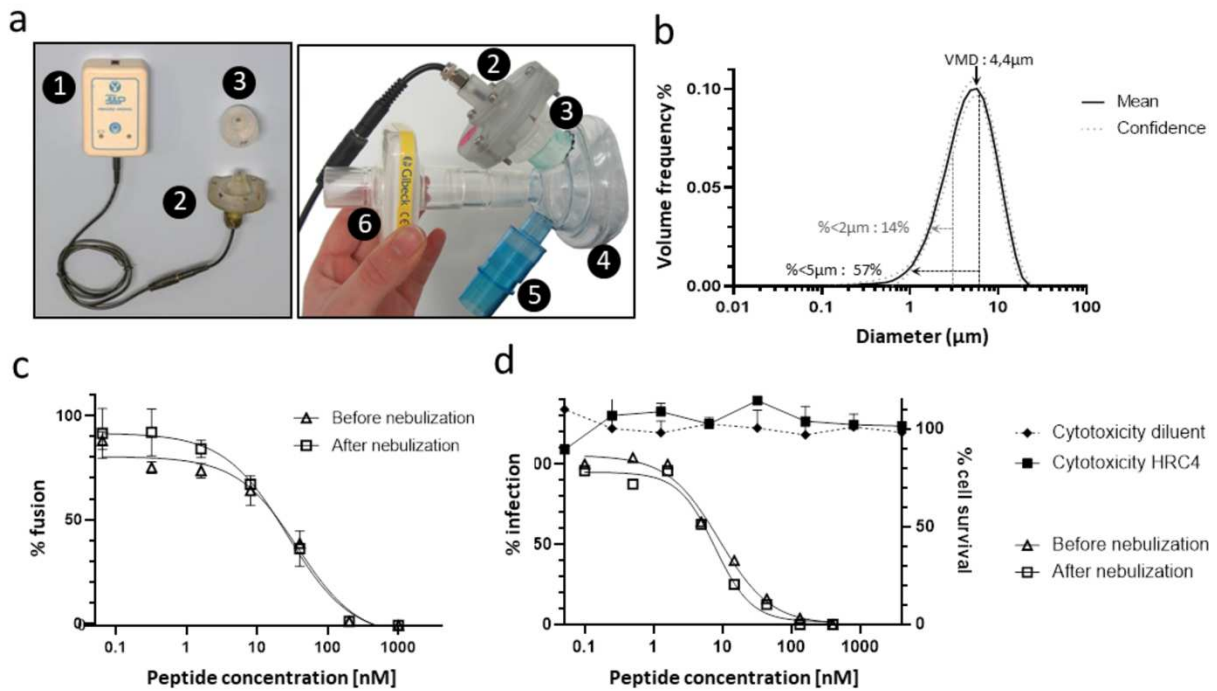


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860 **Fig. 1. HRC4 lipopeptide treatment does not generate MeV escape variants.** (a) Schematic
 861 presentation of viral passaging. MeV IC323-eGFP was serially passaged 8 times on the Vero
 862 SLAM cells in presence of either 1 μ M [FIP-PEG₄]₂-chol or HRC4 peptide, added to the culture
 863 after the infection. Virus was titrated after each passage and 100 PFU used for each subsequent
 864 infection. Sequencing of viral RNA after the 8th passage revealed two mutations in the F-HRC
 865 domain for the infection done in the presence of [FIP-PEG₄]₂-chol: V459I and N462S, while no
 866 mutations in the presence of HRC4 peptide were found. (b) The most frequent mutation events in
 867 F after MeV IC323 was serially passaged 8 times on the Vero SLAM cells in presence of [FIP-
 868 PEG₄]₂-chol. (c) Inhibition of cell-cell fusion mediated by MeV F bearing the indicated mutations
 869 by either 5 μ M [FIP-PEG₄]₂-chol or HRC4 peptide, using HEK-293T cells transfected with SLAM
 870 and the omega reporter subunit of β -gal, incubated with cells co-expressing viral glycoproteins
 871 (IC323 H and F) and the alpha reporter subunit of β -gal (**** p<0,001, Two-Way ANOVA
 872 analysis). (d) Schematic of the HRC4 lipopeptide, used in the further study.

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877 **Fig. 2. Utilization of the prototype mesh nebulizer with low Volume Medium Diameter**

878 **preserves functional activity of HRC4 lipopeptide.** (a) Composition of the prototype mesh

879 nebulizer used in experiments: 1. electronic controller; 2. piezo-electric vibrator; 3. reservoir

880 containing the mesh; 4. facemask; 5. one-way inspiratory valve; 6. absolute filter. (b) Graphical

881 representation of particle size distribution obtained from laser diffraction analysis of aerosolized

882 3ml of HRC4 lipopeptide. Plain line presents the mean values of four nebulizers used in the study

883 and dotted line standard deviation. Percentage of particles $< 5\mu\text{m}$ and $< 2\mu\text{m}$ present the fraction

884 of aerosol below the indicated size, corresponding to the aerosol penetrating into either lung in

885 general ($< 5\mu\text{m}$) or into alveolar regions of lungs ($< 2\mu\text{m}$). Volume Mean Diameter (VMD)

886 presents the mean size of generated aerosols. (c) Fusion inhibitory activity of HRC4, measured

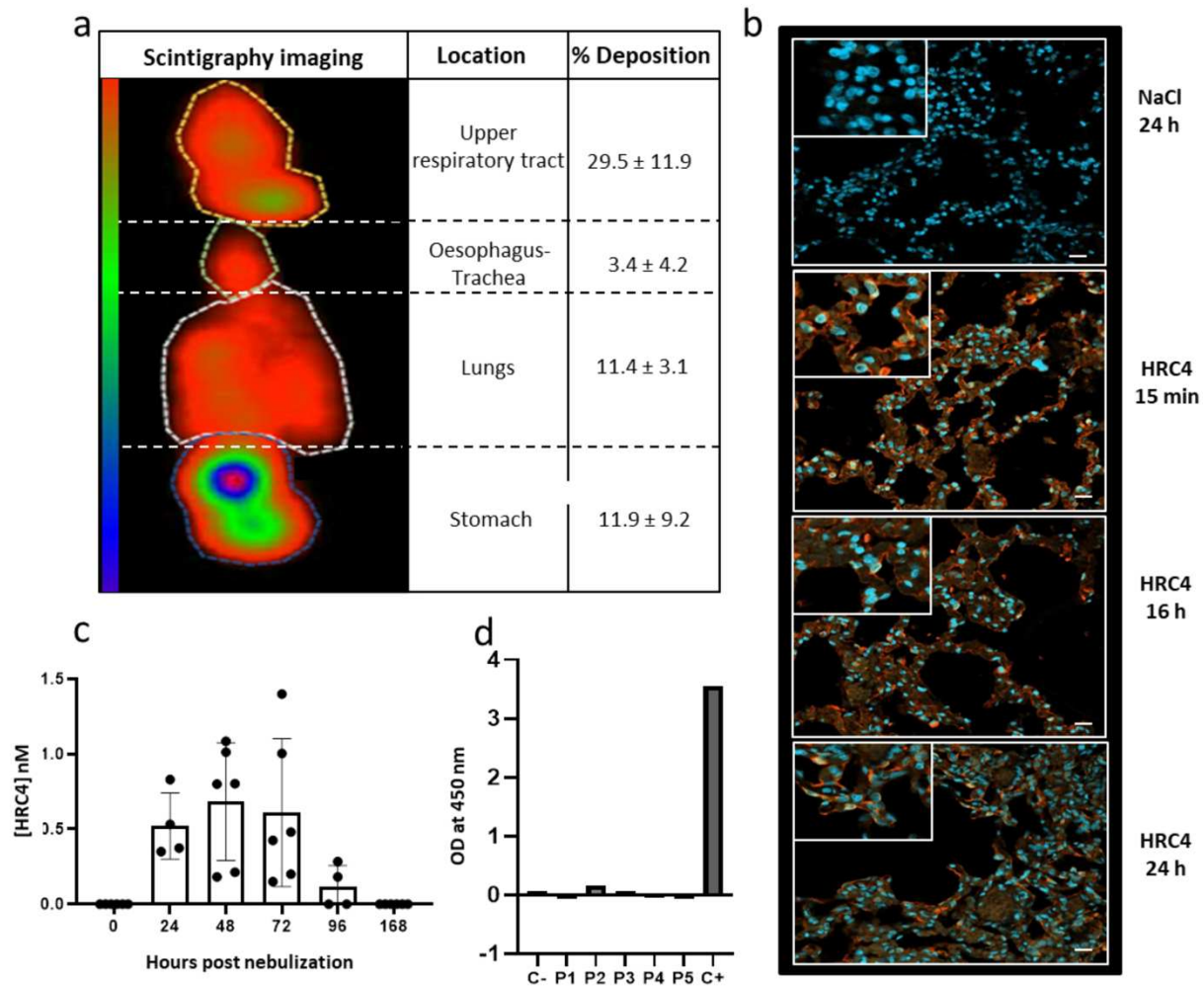
887 before or after peptide nebulization, using β -gal complementation assay. (d) Antiviral activity of

888 HRC4 measured prior and after nebulization, determined by IC_{50} measurement using plaque

889 reduction assay on Vero-hSLAM cells, and cytotoxicity assay, performed by assessing of cells

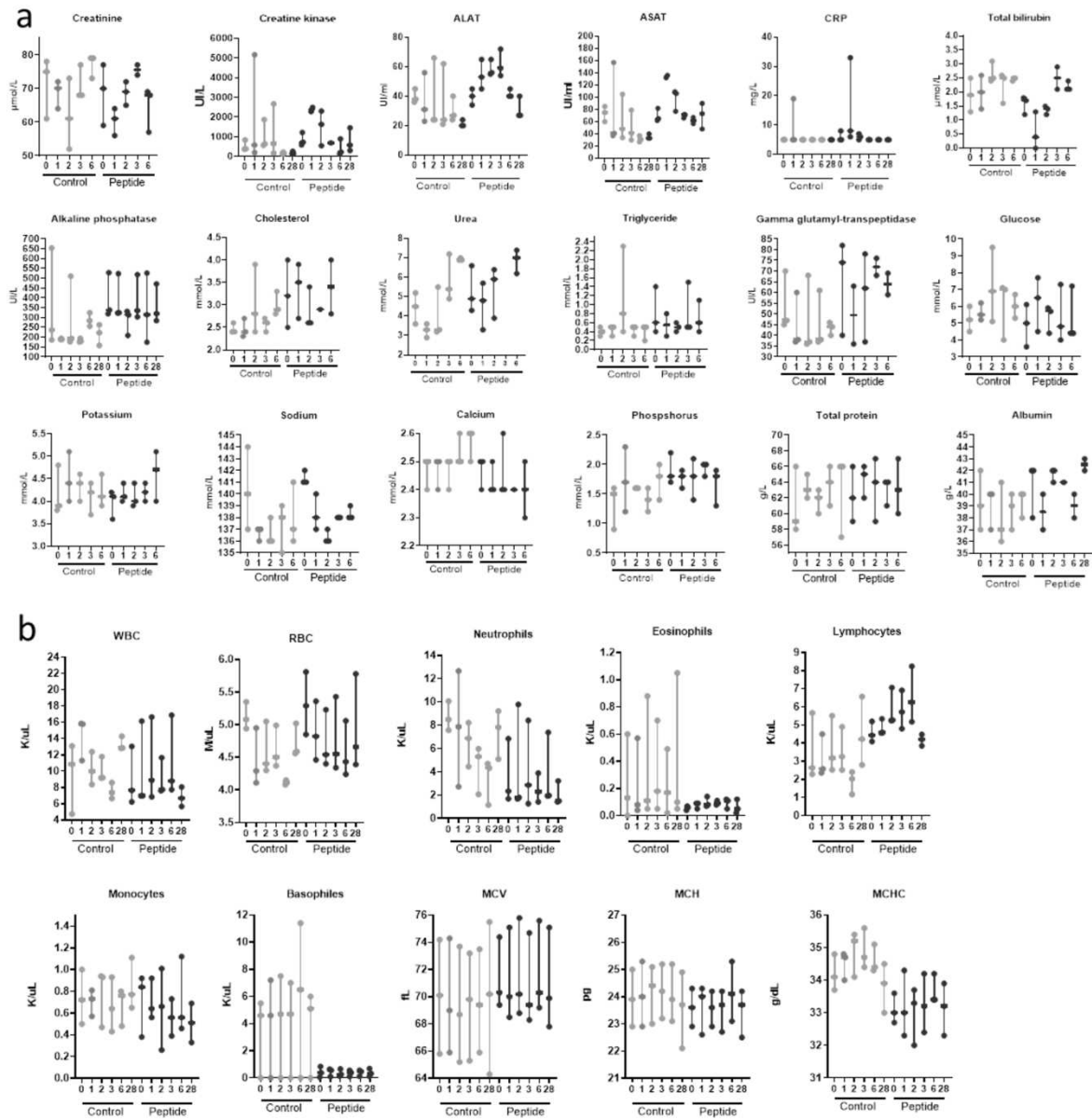
890 viability after 96 h by MTT assay.

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893 **Fig. 3. Delivery of the aerosol into macaques' lungs.** (a) Representative scintigraphy imaging
 894 of different regions of interest in a cynomolgus monkeys nebulized with ^{99m}TC -DTPA tracer (74
 895 Mbq) in 3 ml NaCl 0.9%, using a prototype mesh nebulizer and measured by E-cam gamma
 896 camera. Mean values \pm SD of the distribution of aerosol deposition were determined from the
 897 digitalized images obtained in four experiments. (b) Lung localization of the aerosolized HRC4
 898 peptide analyzed in cranial lobe lung sections from monkeys nebulized under mechanical
 899 ventilation for the indicated time: 15 min (n=1), 24 h (n=2) or 16 h (n=1), prior to euthanasia.
 900 Staining was performed with rabbit anti-HRC peptide and goat anti-rabbit Alexa 555 (orange
 901 staining) and DAPI was used to stain nuclei (blue staining) (Scale bar: 20 μm). (c) Quantification
 902 of HRC4 in the NHPs' serum after peptide nebulization, by ELISA. (d) Determination of the
 903 presence of anti-HRC4 antibodies (IgG, IgA and IgM) by ELISA in the serum of peptide-nebulized
 904 macaques, from either HRC4 biodistribution or MeV-infection study (values obtained at day 0 are
 905 subtracted from those at D28 for each individual animal). Negative control (C-) corresponds to
 906 serum of a naïve macaque and positive control (C+) to rabbit anti-HRC4 antiserum.



907

908 **Fig. 4. Evolution of biochemical (a) and hematological (b) parameters in blood of cynomolgus**
 909 **monkeys following nebulization of either saline (control) or HRC4 (peptide).** (a)

910 Concentration of indicated biochemical parameters in blood of monkeys, measured on days 0, 1,

911 2, 3, 6 and 28 after nebulization of either saline solution (0.9% NaCl, n=3) or HRC4 peptide (4

912 mg/kg, n=5); (b) Haematological parameters measured at days 0, 1, 2, 3, 6 and 28 after nebulisation

913 of either saline solution (0.9% NaCl, n=3) or HRC4 peptide (4 mg/kg, n=5). ALAT: Alanine

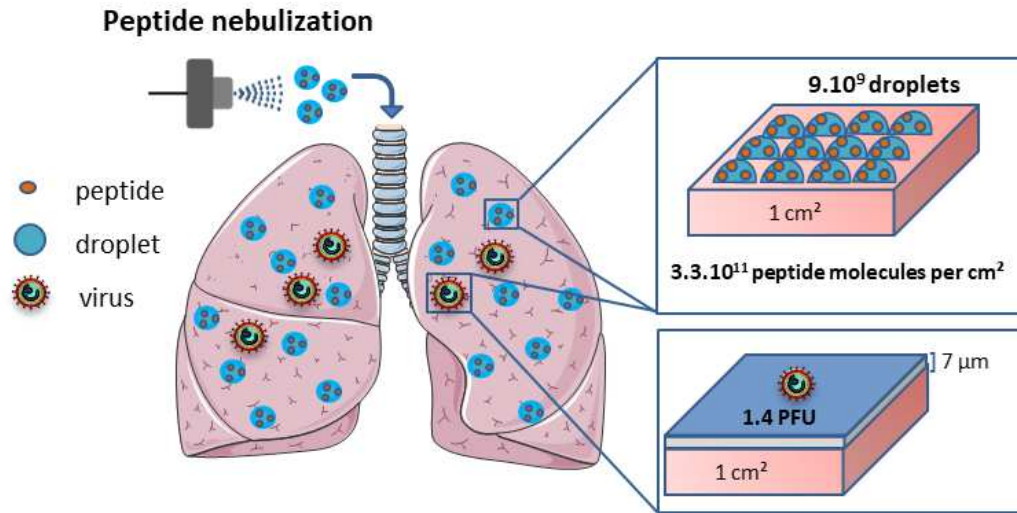
914 aminotransferase; ASAT: Aspartate aminotransferase; CRP: C-reactive protein; MCV: Mean

915 corpuscular volume; WBC: white blood cells; RBC: red blood cells; MCH: Mean corpuscular;

916 MCHC: Mean corpuscular haemoglobin concentration.

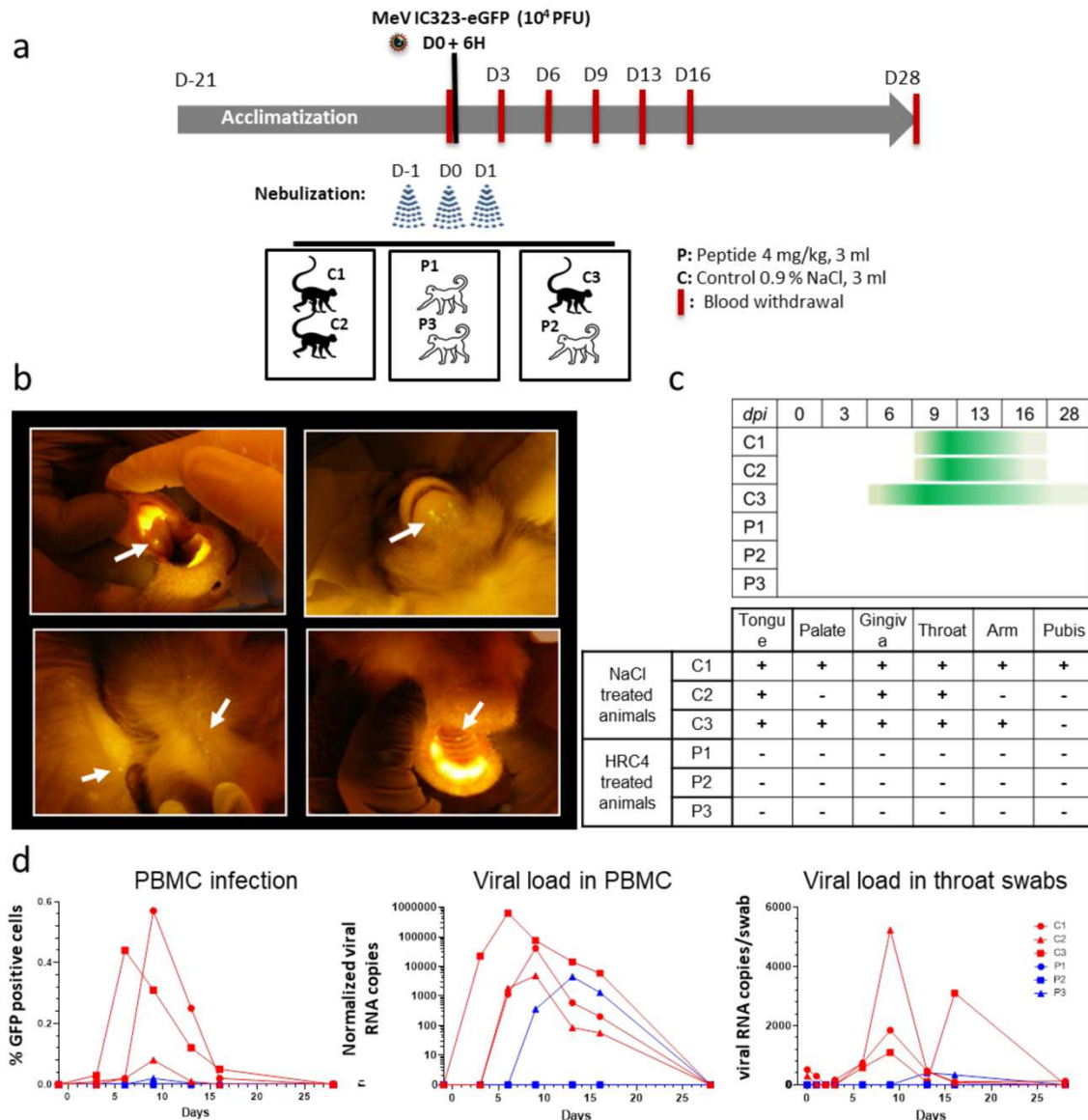
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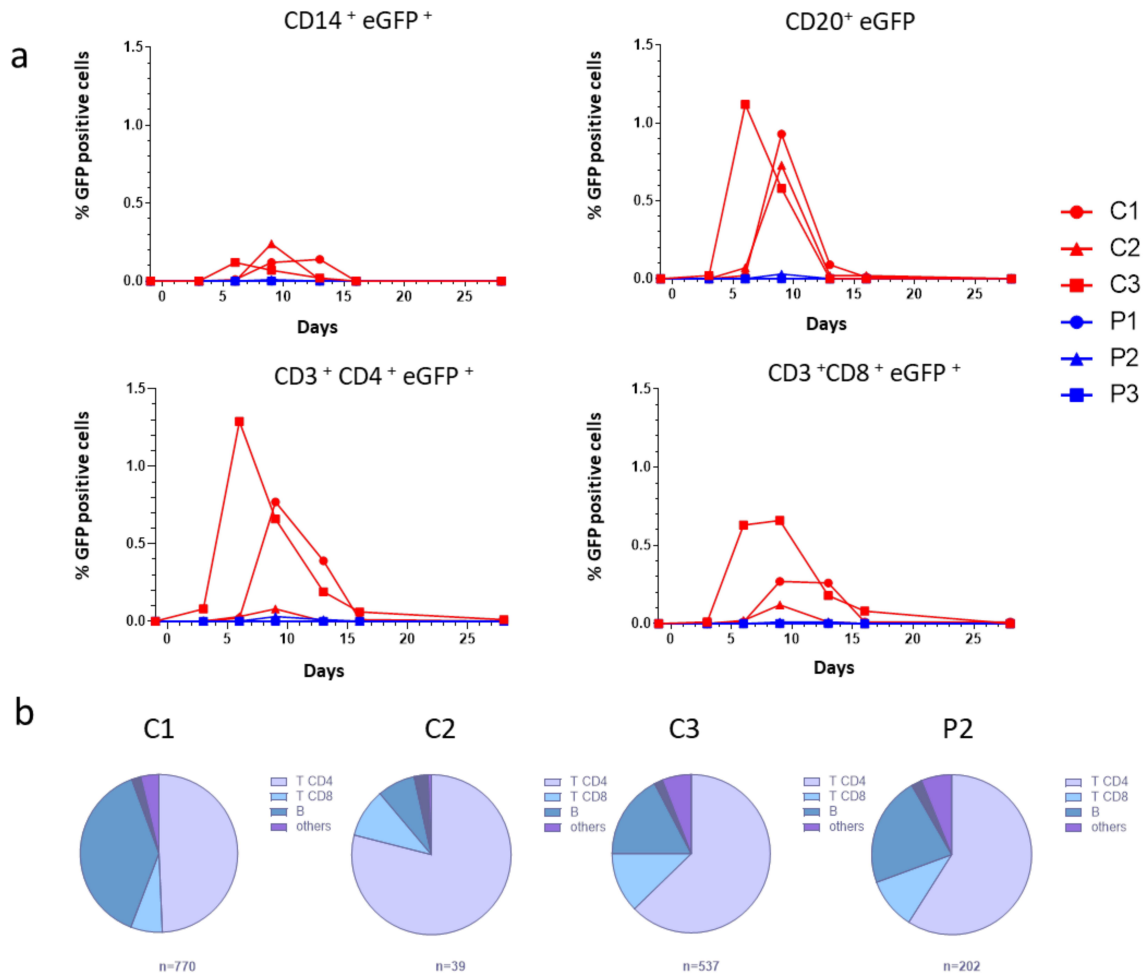
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Fig. 5. Schematic presentation of the peptide and virus deposition in the lungs. The calculation based on the estimation of the peptide dose per unit of lung internal surface, taking into consideration the number of droplets formed following the administration and the peptide molecules into the pulmonary area. Following the nebulization, 11% of the formed droplets reached the lungs (as shown in Fig 1A), representing a density of 9×10^9 droplets/cm² of lung internal surface and containing 3×10^{11} peptide molecules/cm². Instillation of the viral inoculum (10^4 Plaque Forming Units, PFU in 5 ml) via endotracheal tube leads to the virus dispersion in the lung conductive airways in the form of 7 µm thin liquid layer (presented in blue color, covering the maximum surface of 7140 cm²). This represents 3.5 % of the total lung surface area, giving the density of infectious particles of 1.4 PFU per cm² of airways and estimated ratio between the peptide and the virus is 2×10^{11} per cm² of the lung surface.



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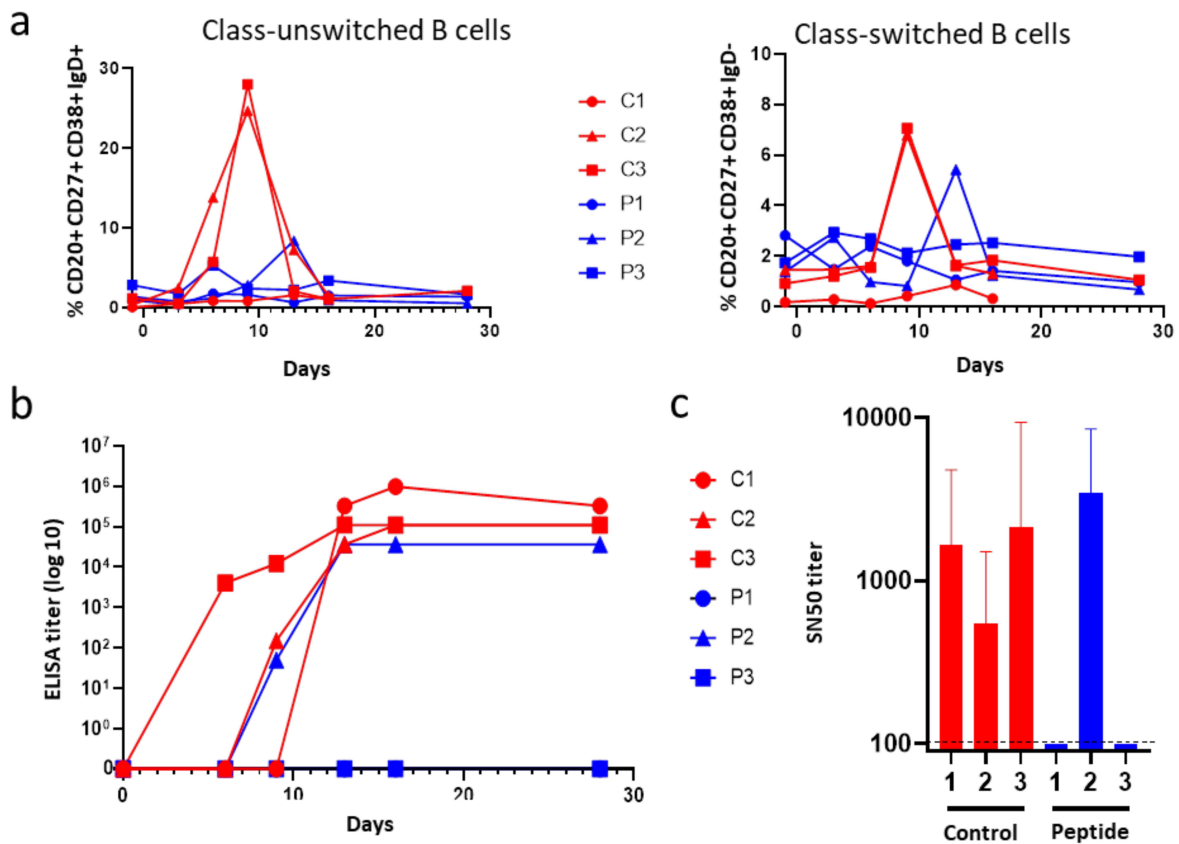
941 **Fig. 6. HRC4 nebulization protects monkeys from clinical manifestation of MeV**
 942 **infection.** (a) Experimental design: cynomolgus monkeys (3 animals/group) were either nebulized
 943 with 3 ml of NaCl 0.9% (control group, C) or with 3 ml HRC4 peptide 4 mg/ml (experimental
 944 group, P), 24 h and 6 h before and 24 h after intra-tracheal infection with 10^4 PFU MeV IC323
 945 eGFP. Blood samples were taken every 3 days for the first 16 days and fluorescence of the skin
 946 and mucosa tested at that time points. (b) Macroscopic manifestation of MeV infection, typical
 947 fluorescent rash observed on tongue, skin (back and chin) and palate (marked with white arrows),
 948 monitored under anesthesia using a blue light with orange filter. (c) Duration of the clinical signs
 949 in MeV-infected animals followed daily (dpi: days post infection). (d) Analysis of viremia by
 950 quantification of the percentage of GFP⁺ peripheral blood mononuclear cells (PBMC) in the blood
 951 by flow cytometry and MeV-specific RNA in PBMCs and in throat swabs by RT-qPCR, during
 952 the course of infection.



953

954

955 **Fig. 7. Nebulization of HRC4 peptide protects PBMCs from MeV infection.** (a) Quantification
 956 of MeV eGFP positive cells in indicated PBMC subpopulations by flow cytometry: CD14⁺
 957 monocytes, CD4⁺, CD8⁺ and CD20⁺ lymphocytes in MeV-infected cynomolgus monkeys by flow
 958 cytometry, following the nebulization of either 0.9% NaCl (C) or HRC4 peptide (P). CD4⁺ T
 959 lymphocytes were characterized as CD3⁺CD8⁻, and CD8⁺ T lymphocytes were characterized as
 960 CD3⁺/CD8⁺; B-lymphocytes were characterized as CD3⁻/CD20⁺ cells. (b) Analysis of the
 961 contribution of each lymphocyte subpopulation among infected PBMCs; results are presented as
 962 the percentage of each analyzed cell population among the infected cells on the day of peak of
 963 MeV infection (day 6 for C3 and day 9 for C1, C2 and P2). Numbers below the graphs correspond
 964 to the number of analyzed cells for each presented animal. Data were acquired on a MACSQuant®
 965 10 flow cytometer (Miltenyi).



966

967 **Fig. 8. Establishment of the humoral immune response in animals that develop MeV**

968 **infection. (a)** Analysis of the presence of class-unswitched IgM secreting B cells (CD20⁺ CD27⁺

969 CD38⁺ IgD⁺) and switched memory B cells secreting IgG, IgA or IgE (CD20⁺ CD27⁺ CD38⁺ IgD⁻

970) in the peripheral blood of NHPs, in following the nebulization of either 0,85% NaCl (C) or HRC4

971 peptide (P) and MeV infection; **(b)** Quantification of total MeV-specific immunoglobulin by

972 ELISA; plotted values present the reciprocal values of last serum dilution with detectable optical

973 density measure. **(c)** Sero-neutralization assay performed using plaque reduction test, following

974 the infection of Vero-hSLAM cells with MeV IC323-eGFP (50 PFU/well). SN₅₀ values were

975 calculated by regression using Prism software (Nonlinear fitting, variable slope, R²: 0.87-0.98);

976 dashed line represents detection limit and error bars represent confidence interval, variable slope,

977 R²: 0.87-0.98); dashed line presents detection limit.

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