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2 **Establishment of memory CD8⁺ T cells with live attenuated influenza**
3 **virus (LAIV) across different vaccination doses**
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31 **Keywords** CD8⁺ T cells, influenza infection, live attenuated, Vaccine

32 **Abbreviations** LAIV: live attenuated influenza virus; BAL: bronchoalveolar lavage;
33 hemagglutinin (HA) and the neuraminidase (NA); cold-adapted (ca); attenuated (att);
34 temperature sensitive (ts); neutralizing (NT); hemagglutinin inhibition (HI).

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39 **ABSTRACT**

40 FluMist has been used in children and adults for more than ten years. As pre-existing
41 CD8⁺ T-cell memory pools can provide heterologous immunity against distinct influenza
42 viruses, it is important to understand influenza-specific CD8⁺ T-cell responses elicited by
43 different LAIV regimens. In this study, we immunized mice intranasally (i.n.) with two
44 different doses of live-attenuated PR8 virus (PR8 ts, H1N1), low and high, and then
45 assessed protective efficacy by challenging animals with heterosubtypic X31-H3N2 virus
46 at 6 weeks post-vaccination. Different LAIV doses elicited influenza-specific CD8⁺ T-cell
47 responses in lungs and spleen, but unexpectedly not in bronchoalveolar lavage (BAL).
48 Interestingly, the immunodominance hierarchy at the acute phase after immunization
49 varied depending on the LAIV dose, however these differences disappeared at 6 weeks
50 post-vaccination, resulting in generation of comparable CD8⁺ T-cell memory pools. After
51 vaccination with either dose, sufficient numbers of specific CD8⁺ T-cells were generated
52 for recall and protection of mice against heterosubtypic H1N1->H3N2 challenge. As a
53 result, immunized mice displayed reduced weight loss, diminished inflammatory
54 responses and lower viral titres in lungs, when compared to unvaccinated animals.
55 Interestingly, the higher dose led to enhanced viral clearance on day 5 post-challenge,
56 though this was not associated with increased CD8⁺ T-cell responses, but with higher
57 levels of non-neutralizing antibodies against the priming virus. Our study suggests that
58 while different LAIV doses result in distinct immune profiles, even a low dose produces
59 sufficient protective CD8⁺ T-cell memory against challenge infection, though the high
60 dose results in more rapid viral clearance and reduced inflammation.

61

62 **INTRODUCTION**

63 Flumist, a live attenuated influenza virus (LAIV) vaccine, is used in healthy children and
64 adults (2 to 49 years of age) (Fiore *et al.*, 2009). It induces neutralizing serum antibodies
65 against the homosubtypic strain and also can stimulate strong heterosubtypic immunity via
66 T cell responses targeted to the shared regions of internal proteins (Powell *et al.*, 2007).
67 The six segments encoding internal proteins (PB2, PB1, PA, NP, M, and NS) of currently
68 used LAIV vaccines are derived from the donor strains (A/Ann Arbor/ 6/60 [AA60] H2N2
69 or B/Ann Arbor/1/66, AAca), and are reassorted with the hemagglutinin (HA) and the
70 neuraminidase (NA) from WHO recommended circulating strains by reverse genetics
71 (Chen *et al.*, 2006; Jin *et al.*, 2003). Four major loci (PB1 (1195) (K391E), PB1(1766)
72 (E581G), PB2(821) (N265S), and NP(146) (D34G)) are responsible for the characteristic
73 cold-adapted (ca), attenuated (att) and temperature sensitive (ts) phenotype (Jin *et al.*,
74 2003). The AAca virus was restricted in replication in the respiratory tract of mice and
75 ferrets (Chen *et al.*, 2010). These mutations introduced into the genetic background of
76 PR8 can also confer the phenotype of ca, att and ts in both ferrets and mice (Huber *et al.*,
77 2009; Jin *et al.*, 2004).

78 Since neutralizing (NT) or hemagglutinin inhibition (HI) antibodies are absent in
79 heterosubtypic challenge, it is believed that CD8⁺ T cells play a major role in
80 heterosubtypic immunity (Powell *et al.*, 2007; Thomas *et al.*, 2006; Wang *et al.*, 2015b)
81 (Grant *et al.*, 2016). T cell depletion experiments in both X31-primed and in ca.A/Alaska-
82 primed mice indicated that CD8⁺ T cells were the major contributors to protection as
83 measured at day 8 post-challenge, although CD4⁺ T cells also contributed to this process
84 (Powell *et al.*, 2007). It is, however, unknown whether the immunization dose of LAIV
85 affects the magnitude and functional quality of influenza-specific CD8⁺ T cell responses
86 and establishment of the memory pools. It is possible that high doses of LAIV can induce

87 numerically more CD8⁺ T cells and thus subsequently provide superior heterosubtypic
88 protection, or alternatively a low dose LAIV could recruit fewer CD8⁺ T cells to combat
89 the heterosubtypic influenza infection. Furthermore, could the LAIV immunization dose
90 affect the number of long-lasting tissue resident memory T (T_{RM}) cells in the lungs? In this
91 study, we immunized mice intranasally (i.n.) with different doses (1x10² FFU/mice and
92 2x10⁵ FFU/mice) of live-attenuated PR8 virus (PR8 ts, H1N1), which allowed us to model
93 different antigen doses provided by LAIV, compare the CD8⁺ T cell responses and the
94 resultant protective efficacy by challenging at 6 weeks post-vaccination with
95 heterosubtypic X31 (H3N2) virus.

96

97 **RESULTS**

98 **Live attenuated influenza virus (LAIV) has an attenuated phenotype in mice.**

99 Temperature-sensitive PR8 virus was generated as described previously (Huber *et al.*,
100 2009) and viable viral particles were rescued from allantoic fluid of inoculated eggs. PR8
101 ts demonstrated increased temperature sensitivity when eggs were incubated at either
102 33°C or 37°C (Fig. 1a), with significantly lower titres obtained at higher temperature
103 following 48 hours (h) incubation (p<0.05). When compared to the wild-type virus (PR8-
104 wt), PR8 ts displayed much slower growth kinetics at MOI 0.01, when grown in MDCK
105 monolayers at 37°C over 48 h (Fig. 1b). A low dose of 10² FFU and a high dose of
106 2.5x10⁵ FFU were used for *in vivo* intranasal infection of C57B6 mice, which confirmed
107 the attenuated phenotype of PR8 ts. At both doses, mice showed only transient body
108 weight loss of ~5% with quick recovery, whereas mice infected with 10² FFU of PR8-wt
109 lost on average 20% body weight within 9 days without signs of recovery (Fig. 1c). Since
110 PR8 ts virus cannot replicate efficiently in upper respiratory tract and in lungs, we

111 investigated how the viral dose of PR8 ts affects generation of CD8⁺ T cell responses and
112 subsequent protection from virulent challenge.

113

114 **Comparable primary or memory CD8⁺ T cell responses, but not immunodominance**
115 **hierarchy, with different vaccination doses.**

116 We investigated primary CD8⁺ T cell responses on d10 post-vaccination, and memory
117 responses at 6 weeks post-vaccination in order to determine whether vaccination dose can
118 lead to differences in specific anti-viral responses. On d10 post-vaccination, we assessed
119 the magnitude (number of tetramer⁺ CD8⁺ T cells, Fig. 2a and Fig. 2b) and functional
120 quality (number of interferon γ (IFN γ)-producing CD8⁺ T cells, Fig. 2c; or double positive
121 IFN γ ⁺TNF⁺ CD8⁺ T cells, Fig. 2d and Fig. 2e) to two immunodominant epitopes D^bNP₃₆₆
122 and D^bPA₂₂₄ (Bird *et al.*, 2015; Kedzierska *et al.*, 2006b). Our data show that efficient
123 CD8⁺ T cell responses can be detected in both lungs (the usual site of influenza virus
124 infection) and spleen (the secondary lymphoid organ). Unexpectedly, PR8 ts vaccination
125 did not induce detectable levels of influenza-specific CD8⁺ T cells in the bronchoalveolar
126 lavage (BAL), possibly due to the non-replicative nature of the LAIV in the lower
127 respiratory tract. However, the immunodominance hierarchy of D^bNP₃₆₆- and D^bPA₂₂₄-
128 specific CD8⁺ T cell responses varied between spleen and lungs. The higher PR8 ts
129 vaccination dose induced higher D^bPA₂₂₄⁺CD8⁺ T cell numbers in the lungs when
130 compared to the lower vaccination dose. However, no significant differences ($p > 0.05$)
131 were found for D^bNP₃₆₆⁺CD8⁺ T cells in lungs between different immunization doses.
132 Conversely, the lower vaccination dose induced significantly higher CD8⁺ T cell responses
133 to D^bNP₃₆₆ epitope in the spleen, as compared to the lower vaccination dose. Despite, a
134 high number of virus-specific tetramer⁺CD8⁺ T cells in lungs, only a limited number of
135 these cells appeared to secrete IFN γ or TNF α , as assessed by intracellular cytokine

136 staining (Fig. 2c,2d). It is important to note that the numbers of naïve antigen-specific
137 CD8⁺ T cells are around 68 ± 18 for naive D^bPA₂₂₄⁺CD8⁺ and 36 ± 21 for D^bNP₃₆₆⁺CD8⁺
138 T cells, as shown by our previous studies(La Gruta *et al.*, 2010; Valkenburg *et al.*, 2010)

139 We then investigated memory responses at 6 weeks after priming with PR8 ts (Fig.
140 3). No differences to either D^bNP₃₆₆⁺CD8⁺ or D^bPA₂₂₄⁺CD8⁺ T cell responses were
141 observed in the lungs, indicating that any differences that we observed at d10 post-priming
142 equalize over time when the effector CD8⁺ T cell populations contracted into memory.
143 However, the lower vaccination dose resulted in generation of significantly higher
144 numbers of virus-specific memory CD8⁺ T cells in the spleen. We also observed
145 significantly higher numbers of D^bNP₃₆₆-specific CD8⁺ T cells, and a modest but
146 significant difference in the numbers of D^bPA₂₂₄-specific CD8⁺ T cells (Fig. 3a).

147 We also enumerated resident memory CD8⁺ T cells (T_{RM}, CD69⁺CD103⁺CD8⁺) in
148 the lungs of vaccinated mice, but found no differences in the numbers of either virus-
149 specific or total T_{RM} cells between the two vaccination groups (CD103⁺CD69⁺CD8⁺) (Fig.
150 3b). These findings support our previous studies on the the early establishment of
151 influenza-specific CD8⁺ T cell memory during influenza virus infection (Bird *et al.*, 2015;
152 Kedzierska *et al.*, 2007).

153 To determine whether the numerically higher CD8⁺ T cell response induced by the
154 low dose immunization was due to higher inflammatory milieu, we measured cytokine
155 levels induced after primary immunization with low/high dose at d3 and d10. Our data
156 (Fig. 3d) showed that the cytokine levels induced by a high dose (2.5×10^5 FFU) in lungs at
157 d3 and d10 after vaccination were higher than those induced with the lower dose
158 (1×10^2 FFU), and were not associated with different numbers of antigen-specific CD8⁺ T
159 cell responses elicited by those doses.

160

161 **Priming with different doses of PR8 ts leads to similar efficient recall response.**

162 As described above, vaccination with a lower dose of 10^2 FFU of PR8 ts generated a
163 significantly larger pool of virus-specific memory $CD8^+$ T cells in the spleen (Fig. 3a). To
164 investigate whether this could affect secondary recall $CD8^+$ T cell responses following
165 virulent challenge, C57B6 mice were vaccinated intranasally with different doses of PR8
166 ts, and PBS-primed mice were included as a control. Six weeks following immunization,
167 all experimental groups were challenged with 10^4 PFU of X31 influenza virus. BAL and
168 spleen were examined for virus-specific $CD8^+$ T cell responses by measuring the
169 magnitude of D^bNP_{366} and D^bPA_{224} $CD8^+$ T cell responses on d5 and d8 post-challenge
170 (Fig. 4). The results showed that vaccinated animals could mount much quicker, efficient
171 secondary $CD8^+$ T cell response when compared to the control group. We detected
172 significantly higher numbers of both D^bNP_{366} - and D^bPA_{224} -specific $CD8^+$ T cells on d5 in
173 BAL and spleens of immunized animals compared to PBS controls (Fig. 4a). Those $CD8^+$
174 T cells also produced much higher levels of $IFN\gamma$ early in infection (Fig. 4b). Later during
175 the course of infection (d8), there was no difference between immunized and control
176 groups, and in the case of immunized animals, the recall pool of $CD8^+$ T cell already
177 started diminishing, suggesting earlier viral clearance and removal of antigenic stimulation.
178 However, both immunized groups mounted equivalent $CD8^+$ T cell recall responses
179 indicating that immunization dose does not affect the expansion of virus-specific memory
180 cells at the site of infection.

181 We also investigated whether different LAIV doses could reduce influenza-virus
182 mediated inflammation. To do this, the cytokine milieu at the site of infection was
183 assessed in lung homogenates using Cytokine Bead Array (CBA). A panel of cytokines
184 and chemokines, including interleukin (IL)-6, IL-10, IL-12, $IFN\gamma$, TNF and monocyte
185 chemotactic protein-1 (MCP-1, CCL-2) was evaluated on d3 (Fig. 5a) and d6 (Fig. 5b)

186 post-challenge. Our data indicate that immunized animals can control the inflammation
187 much better than unvaccinated control animals, as demonstrated by reduced levels of the
188 majority of cytokines tested (IL-6, IFN γ , TNF α and MCP-1 at d6, $p < 0.05$, Fig 5b),
189 particularly IL-6, which is a well-known factor promoting pulmonary inflammation.
190 Interestingly, only MCP-1 was significantly reduced differentially at d3 after infection in
191 high dose LAIV recipients, compared with those that received low dose LAIV, which was
192 consistent with the number of macrophages recruited to the site of infection at d3 (Fig. 5c).
193

194 **LAIV vaccination dose affects the rate of viral clearance.**

195 Consistent with limited differences in recall responses between groups of animals
196 vaccinated with different doses of LAIV, we did not observe any differences in clinical
197 symptoms (weight loss) between these two groups following challenge. Approximately 5-
198 7% body weight loss was observed in immunized groups regardless of the vaccine dose,
199 while the naïve group (PBS-primed) displayed 10-20% body weight loss and delayed
200 recovery (Fig. 6a). Mice vaccinated with a lower dose of 10^2 FFU of PR8 ts displayed
201 delayed viral clearance compared with the 2.5×10^5 FFU PR8 ts group (Fig. 6b). Viral load
202 was examined on d3, d5, d6 and d8 post-challenge in lung homogenates. At d5, the viral
203 titres in the lungs of vaccinated groups were significantly lower than those in control mice
204 ($P < 0.0001$), while on d6, the virus was undetectable in the lungs of immunized animals,
205 but still detectable in the control group. On d8 post-challenge, the majority of mice had
206 lung viral titres below the level of detection. On d5 post-challenge, mice vaccinated with
207 10^2 FFU LAIV had significantly increased levels of virus, compared with mice that
208 received 2.5×10^5 FFU ($P < 0.001$). Delayed viral clearance during antiviral response can
209 usually be attributed to delayed recruitment of influenza-specific CD8⁺ T cells to the site
210 of infection (Marois *et al.*, 2015), which was not observed in this case (Fig. 4). As the

211 magnitude of CD8⁺ T cell responses did not correlate with viral clearance across different
212 immunization doses, we investigated antibody levels triggered by different doses of PR8
213 ts vaccine. Our ELISA results show that antibody titres against homologous PR8 HA
214 protein were dose-dependent, with antibody titres induced by the higher 2.5x10⁵ FFU dose
215 detected at nearly 160-fold greater level than those induced by 10² FFU (Fig. 6c). We then
216 tested antibody titres against heterologous X31 HA protein, and found no difference
217 between doses of LAIV (Fig. 6d). It has been reported that antibodies raised against the
218 X31 virus do not significantly cross-react with the PR8 virus, nonetheless X31-specific
219 antibodies provide some protection against PR8 infection (Rangel-Moreno *et al.*, 2008).
220 Our data suggest that the same holds true when antibodies are raised against PR8.

221

222 **DISCUSSION**

223 In this study, we observed that different LAIV doses elicit distinct influenza-
224 specific CD8⁺ T cell responses in lungs and spleens, but unexpectedly not in
225 brochoalveolar lavage (BAL). Virus-specific CD8⁺ T cell responses typically fall into
226 reproducible hierarchies, with particular epitopes eliciting either immunodominant
227 (numerically high) or subdominant (numerically low) responses after the viral challenge.
228 In the B6 model of influenza virus infection, CD8⁺ T cell responses specific for epitopes
229 derived from the nucleoprotein (NP₃₆₆₋₃₇₄) and acid polymerase (PA₂₂₄₋₂₃₃) proteins
230 dominate during the primary acute immune response, with D^bNP₃₆₆⁺CD8⁺ T cells being
231 numerically larger on d10 after infection (Cukalac *et al.*, 2014; Kedzierska *et al.*, 2006a)
232 Interestingly, the immunodominance hierarchy at the acute time after immunization was
233 affected by the LAIV dose, however these differences disappeared at 6 weeks post-
234 vaccination. We found that sufficient numbers of specific CD8⁺ T cells were generated for
235 recall and protection of mice against heterosubtypic PR8 (H1N1)-> X31 (H3N2)

236 challenge (X31 virus shared the same six internal genes as PR8 virus). Thus, our study
237 indicates that immunization with even a low dose of LAIV can establish a protective
238 influenza-specific CD8⁺ T cell memory pool in lungs.

239 Currently, intranasal immunization with cold-adapted live attenuated influenza vaccine is
240 a very efficient way to induce cellular CD8⁺ T cell immunity against the virus (Carter &
241 Curran, 2011). Therefore, LAIV has been postulated to induce cross-protective immunity
242 against heterologous strains of influenza. Studies in mice demonstrated that priming with
243 LAIV induces heterosubtypic immunity, and while it does not lead to sterile immunity, it
244 results in faster viral clearance and protects against weight loss associated with infection
245 (Lanthier *et al.*, 2011; Powell *et al.*, 2007). CD8⁺ T cells are thought to be responsible for
246 the observed protection, with some involvement from CD4⁺ T cells, while B cells were
247 shown to be non-essential (Powell *et al.*, 2007). Studies in humans vaccinated with LAIV
248 indicated that the vaccine leads to development of Type 1 memory responses leading to T
249 cell recruitment via production of IFN γ and expression of chemokines (Lanthier *et al.*,
250 2011). More recently, Harty's group (Slutter *et al.*, 2013) showed that LAIV vaccination
251 leads to generation of cross-protective CD8⁺ T cells, but their numbers are inadequate to
252 control infection with heterosubtypic viral strain. This, however, can be rectified by
253 additional boosting that leads to increased numbers of virus-specific T cells and results in
254 memory pools sufficient to protect against heterologous challenge.

255 In our study, we have investigated whether different doses of LAIV can lead to
256 different numbers of virus-specific CD8⁺ T cells, and whether those T cells can effectively
257 protect against heterosubtypic challenge. A previous study showed that increasing a dose
258 of PR8 wt by an intraperitoneal injection route could not only expand the CD8⁺ T cell
259 response, but also change the immunodominance hierarchy (Luciani *et al.*, 2013). Two

260 different doses of LAIV used in this study led to generation of comparable numbers of
261 tetramer-positive CD8⁺ T cells in both lungs and spleens of vaccinated animals. The
262 notable difference was a slight change in immunodominance hierarchy 10 days following
263 priming, where upon immunization with a lower dose, increased numbers of D^bNP₃₆₆-
264 specific CD8⁺ T cells were induced in the spleen, while a higher dose led to increased
265 numbers of D^bPA₂₂₄-specific CD8⁺ T cells in the lungs of immunized animals. To some
266 degree, this is in agreement with the above mentioned study (Luciani *et al.*, 2013), where
267 increasing the viral dose via i.p. delivery led to a switch in immunodominance from NP₃₆₆
268 to PA₂₂₄ in the spleen. Interestingly, these differences were alleviated in the lungs at a later
269 time point (6 weeks post priming), while spleens of mice immunized with a lower dose of
270 LAIV showed higher numbers of NP₃₆₆-specific memory CD8⁺ T cells.

271 Despite the differences observed in the spleen, there was no difference in the
272 immunodominance hierarchy or numbers of virus-specific CD8⁺ T cells in the lungs 6
273 weeks post-immunization. Since the lung is the actual site of infection, we examined the
274 formation of resident memory CD8⁺ T cells. Lung T_{RM} cells provide the first line of
275 defence against respiratory viruses and are able to efficiently restrict early viral replication
276 by providing a rapid source of cytokines, particularly IFN γ (McMaster *et al.*, 2015).
277 Different doses of LAIV vaccine were able to induce formation of T_{RM} cells, but we did
278 not observe any differences between low and high immunization dose, either in numbers
279 of virus specific T_{RM} or overall T_{RM} numbers. At 6 weeks post-priming, the numbers of
280 virus-specific T_{RM} cells were relatively low, which is consistent with their non-
281 proliferative nature.

282 A recent report by Slutter *et al* (Slutter *et al.*, 2013) indicated that single
283 immunization of Balb/c mice with Flumist, 40 days prior to heterosubtypic challenge did
284 not induce sufficient numbers of cross-protective CD8⁺ T cells to control infection. Our

285 data indicate, that a single immunization with LAIV, either low or high dose, was able to
286 protect mice against non-homologous challenge (6 weeks post-immunization). This has
287 been demonstrated by a smaller and transient weight loss and accelerated viral clearance
288 from the lungs of immunized animals. A possible explanation for the observed difference
289 could be the vaccination-challenge strategies that differed between the two studies. While
290 Slutter *et al* primed with 2010-2011 Flumist vaccine containing H1N1, H3N2 and
291 influenza B strains and challenged with PR8, we used PR8 (H1N1)-based LAIV for
292 priming and challenged with X31 (H3N2) virus, which shared the same six internal genes
293 as PR8. Despite this difference, it was clear that sufficient numbers of virus-specific
294 heterosubtypic protective CD8⁺ T cells were induced in our study and protection was
295 achieved without a need for boosting. Interestingly, the higher immunization dose led to
296 more rapid virus clearance from lungs, which manifested itself on d5 post-challenge. This
297 phenomenon could not be linked to either increased numbers of virus-specific CD8⁺ T
298 cells or T_{RM} cells, increased levels of cytokines such as IFN γ , nor to higher levels of
299 heterosubtypic, neutralizing antibodies. None of these immunological parameters
300 significantly differed between the two immunization doses, though non-neutralizing
301 antibodies against the PR8 virus were found to be significantly higher in the high dose
302 immunized group. It seems that the higher LAIV vaccination dose also appears to induce
303 other factors, which contribute to further reduction in inflammation and viral load after the
304 secondary virus infection.

305 Powell *et al* reported that CD8⁺ T cells are thought to be mainly responsible for
306 heterosubtypic protection, with some involvement from CD4⁺ T cells, while B cells were
307 shown to be non-essential (Powell *et al.*, 2007). Conversely, others reported that the
308 antibodies against conserved regions of HA, NA, NP, M1 and M2 could also help cross-
309 reactive protection (Gerhard *et al.*, 1997; Khattak *et al.*, 1999; LaMere *et al.*, 2011;

310 Neiryneck *et al.*, 1999; Slepushkin *et al.*, 1995), which highlights the importance of B cells
311 and antibodies in the recovery of mice from influenza virus infection (Rangel-Moreno *et*
312 *al.*, 2008). Additionally, non-neutralizing antibodies could facilitate the expansion of
313 responding memory CD8⁺ T cells and promote recovery from lethal heterosubtypic
314 influenza challenge (Rangel-Moreno *et al.*, 2008). The intranasal immunization with
315 H5N1-att vaccine provided broad cross-protection against a range of H5N1 viruses in
316 mice and ferrets, mainly due to the protection specific to the H5 HA (Suguitan *et al.*,
317 2006), however, our data showed that the high dose and low dose induced similar cross-
318 reactive antibodies against X31 HA. Furthermore, there has been convincing evidence that
319 non-neutralizing antibodies are associated with the protection against heterosubtypic
320 influenza viruses (Carragher *et al.*, 2008; Rangel-Moreno *et al.*, 2008), potentially related
321 to the elimination of influenza virus via FcR-mediated phagocytosis (Huber *et al.*, 2001),
322 which might be a potential explanation for our observed differences in virus clearance and
323 inflammation reduction caused by different dose LAIV vaccination.

324

325 **METHODS**

326 **Infection of mice and characterization of PR ts in MDCK cells.** C57B6 mice were bred
327 and housed at Department of Microbiology and Immunology, University of Melbourne.
328 Experiments were approved and conducted under guidelines set by the University of
329 Melbourne Animal Ethics Committee. Mice were anaesthetised by inhalation of
330 methoxyflurane and intranasally infected with 1.0×10^2 and 2.5×10^5 plaque forming units
331 (FFU) of LAIV (PR8 ts; H1N1) influenza virus in 30 μ l of PBS. We based our definition
332 of the high (2.5×10^5 FFU) and low dose (10^2 FFU) amounts in this study on the clinical
333 dose of flumist (10^7 PFU) used in an adult human with an average weight of 75kg and
334 extrapolating this to a mouse with an average weight of 20g (10^3 PFU).

335

336 Mice were challenged intranasally 6 weeks later with 1×10^4 PFU of X31 (H3N2) virus in
337 30 μ l PBS. Lungs were harvested for viral load in a Madin-Darby canine kidney (MDCK)

338 and viral load were determined by plaque assay (Matrosovich *et al.*, 2006). As it is
339 difficult to observe plaque formation using PR8 ts in a standard plaque assay, we used a
340 focus assay as a means of measuring viral titres. Therefore, for the viral stock (PR8 ts)
341 titration and replication kinetics comparison between PR8 and PR8 ts in MDCK cells,
342 viral levels were determined by focus assay (focus forming assay), as previously described
343 (Wang *et al.*, 2010).

344

345 **Influenza virus-specific CTL responses.** Cells were recovered from spleen, lung and
346 bronchoalveolar lavage (BAL). Lymphocytes were stimulated with peptides representing
347 the H2D^b-restricted NP₃₆₆₋₃₇₄ (ASNENMTEM) or PA₂₂₄₋₂₃₃ (SSLENFRAYV) epitopes in a
348 intracellular cytokine secretion (ICS) assay (Bird *et al.*, 2015). Cells were also incubated
349 with D^bNP₃₆₆₋₃₇₄ or D^bPA₂₂₄₋₂₃₃ tetramers conjugated to PE or allophycocyanin (Bird *et al.*,
350 2015). Results were analysed using FlowJo software (Treestar, USA). Lungs for T_{RM}
351 analysis were processed as previously described (Kedzierski *et al.*, 2015; Quiñones-Parra
352 *et al.*, 2016; Wakim *et al.*, 2015).

353

354 **Cytometric Bead Analysis.** The CBA flex set (BD Bioscience) was used as per
355 manufacturer's instructions to determine the cytokine concentrations of BAL supernatant.
356 Data were acquired using FACSCantoII and analysis was by FCAP Array software (Soft
357 Flow Inc., Pecs, Hungary).

358

359 **Influenza-specific antibody.** Sera were prepared from blood taken 4 weeks after
360 immunisation. ELISA were performed in 96-well plates as previously described (Wang *et*
361 *al.*, 2015a).

362

363 **Statistical Analyses.** Analysis of variance and *p* values in this study were obtained using
364 one-way ANOVA non-parametric analyses and Tukey's post-hoc range tests or students t-
365 test (GraphPad Software, La Jolla, California USA).

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367

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372

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511
512
513 **FIGURE LEGENDS**

514
515 **Figure 1. Attenuated phenotype of PR8ts *in vitro* and *in vivo*.** (a) Significantly lower
516 ($P < 0.001$) viral titres were obtained when virus was grown in embryonated eggs at 37°C
517 when compared to 33°C. (b) Attenuated growth kinetics of PR8 ts compared to PR8-wt
518 over 48 h incubation period in MDCK monolayers, $n = 6$ per time point for PR8 wt. (c)
519 Comparison of body weight loss in C57B6 mice ($n = 5$ per group) infected intranasally with
520 either 10^2 FFU PR8 wt, 10^2 FFU PR8 ts or 2.5×10^5 FFU PR8 ts. Mean values were plotted,
521 error bars represent S.E.M, stastics between groups were performed using Mann-Whitney
522 test.

523
524 **Figure 2. Primary CD8⁺ T cell responses elicited by LAIV vaccination.** (a) Magnitude
525 of CD8⁺ T cell response in lungs, spleen and BAL on d10 post-vaccination. Virus-specific
526 CD8⁺ T cells were enumerated using MHC class I tetramers against immunodominant
527 D^bNP₃₆₆ and D^bPA₂₂₄ epitopes. (b) FACS analysis of tetramer positive CD8⁺ T cells in
528 lungs and spleen. Dot plots from individual mice were combined into a cumulative display
529 with mean frequencies included on the graph. Cytokine production by CD8⁺ T cell
530 stimulated with either NP₃₆₆ or PA₂₂₄ peptides. Absolute numbers of IFN γ ⁺ (c) and
531 polyfunctional IFN γ ⁺TNF⁺ (d) cells was assessed in lungs, spleen and BAL by FACS
532 following *ex vivo* ICS. Data from a representative experiment (of 2) were plotted, ($n = 5$ per
533 group) error bars represent S.E.M. (e) FACS analysis of IFN γ ⁺TNF⁺ CD8⁺ T cell
534 populations in the lungs and spleen. Dot plots from individual mice were combined into a
535 cumulative display with mean frequencies included on the graph. Stastics between groups
536 were performed using Mann-Whitney test.

537
538 **Figure 3. Memory formation following LAIV vaccination.** (a) Numbers of virus-
539 specific memory CD8⁺ T cells were assessed in spleen and lungs 6 weeks after initial PR8
540 ts administration (b) Numbers of tissue resident memory cells (CD103⁺CD69⁺) in the
541 lungs 6 weeks after PR8 ts vaccination. Mean values were plotted, error bars represent
542 S.E.M. Mice $n = 5$ for each group within one experiments (c) FACS analysis of memory
543 CD8⁺ T cell populations in the spleen and lungs. Dot plots from individual mice were
544 combined into a cumulative display. (d) Cytokines level were measured by CBA at d3 and
545 d10 after primary infection with low dose 10^2 FFU PR8 ts and high dose 2.5×10^5 FFU
546 PR8 ts ($n = 5$ per group within an experiment). Stastics between groups were performed
547 using Mann-Whitney test.

548
549 **Figure 4. Recall responses to heterosubtypic challenge with X31 (H3N2) influenza**
550 **virus.** (a) Total numbers of virus-specific memory CD8⁺ T cells were assessed in spleen
551 and BAL on day 5 and 8 following secondary X31 influenza challenge. (b) Capacity of
552 D^bNP₃₆₆- and D^bPA₂₂₄-specific CD8⁺ T cells to secrete IFN γ following challenge with
553 X31 influenza virus. Data from a representative experiment (of 2) were plotted, error bars
554 represent S.E.M, $n = 5$ for each group for each experiments.

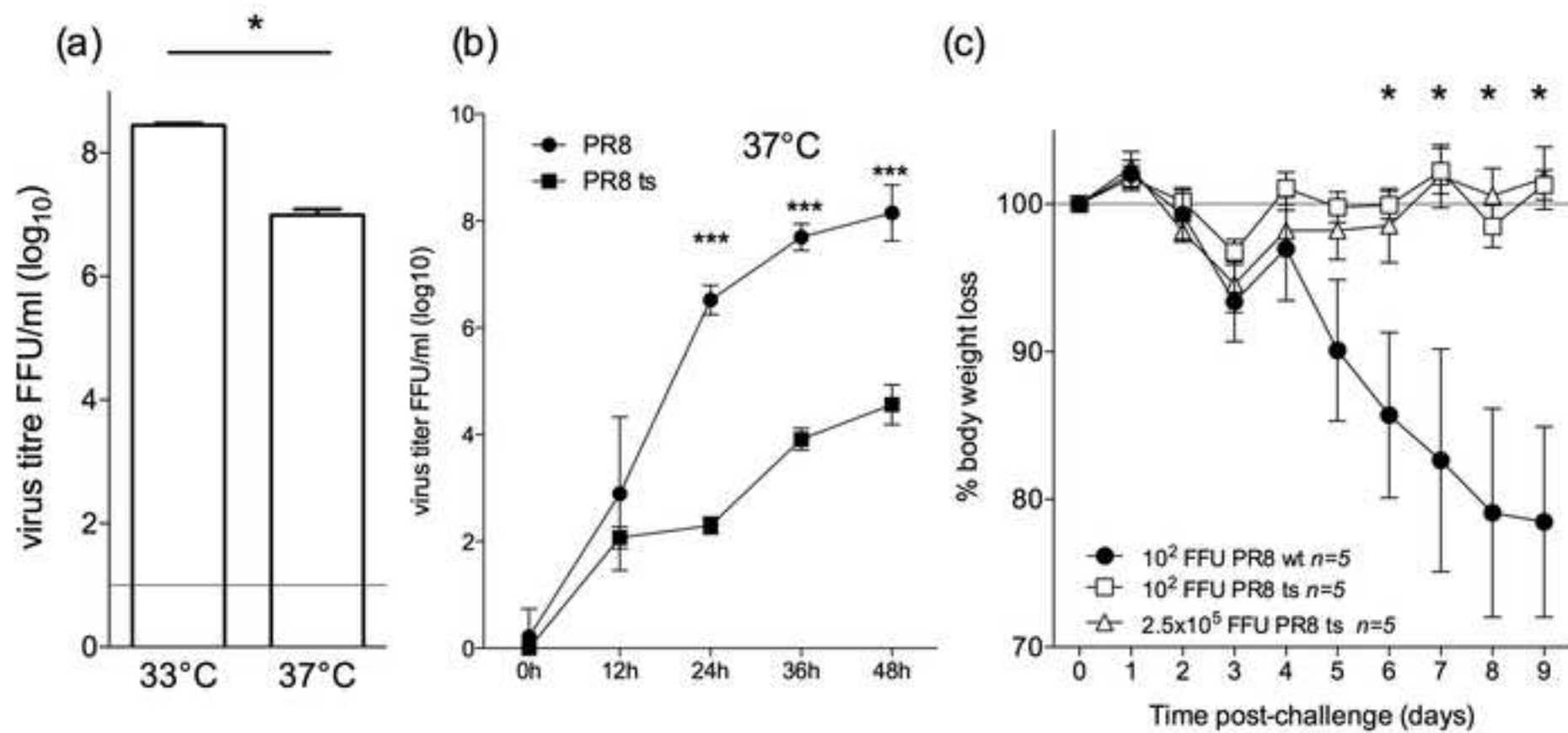
555
556 **Figure 5. Cytokine milieu and influx immune cells in the lungs of LAIV vaccinated**
557 **mice.** Cytokine levels (a,b) and influx cells (c) were analysed by CBA in lung
558 homogenates on d3 (a) and d6 (b) post-challenge with X31 virus. Mean values \pm S.E.M. are

559 shown for n=5 biological replicates within one experiments, stastics between groups were
560 performed using Mann-Whitney test.

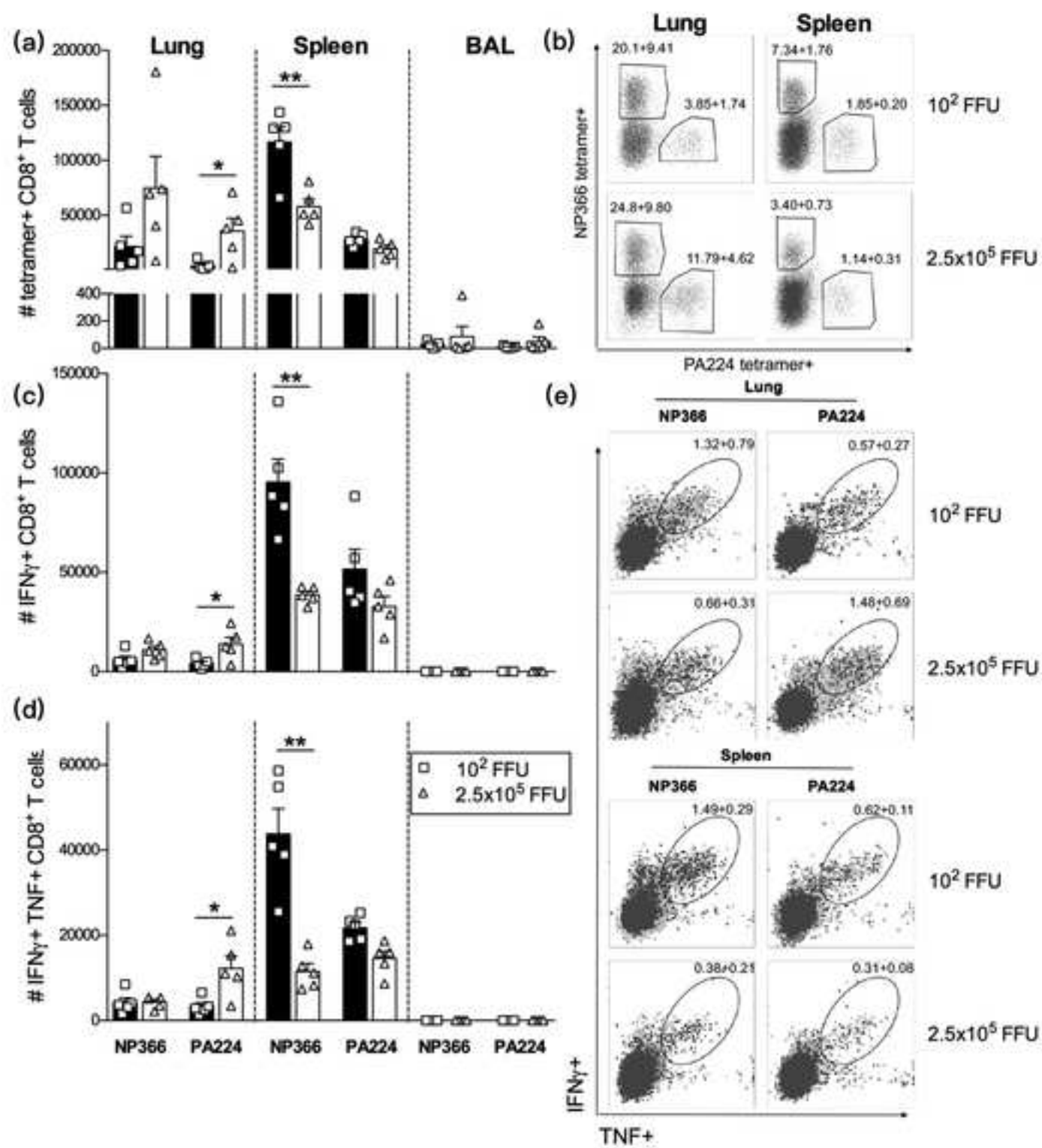
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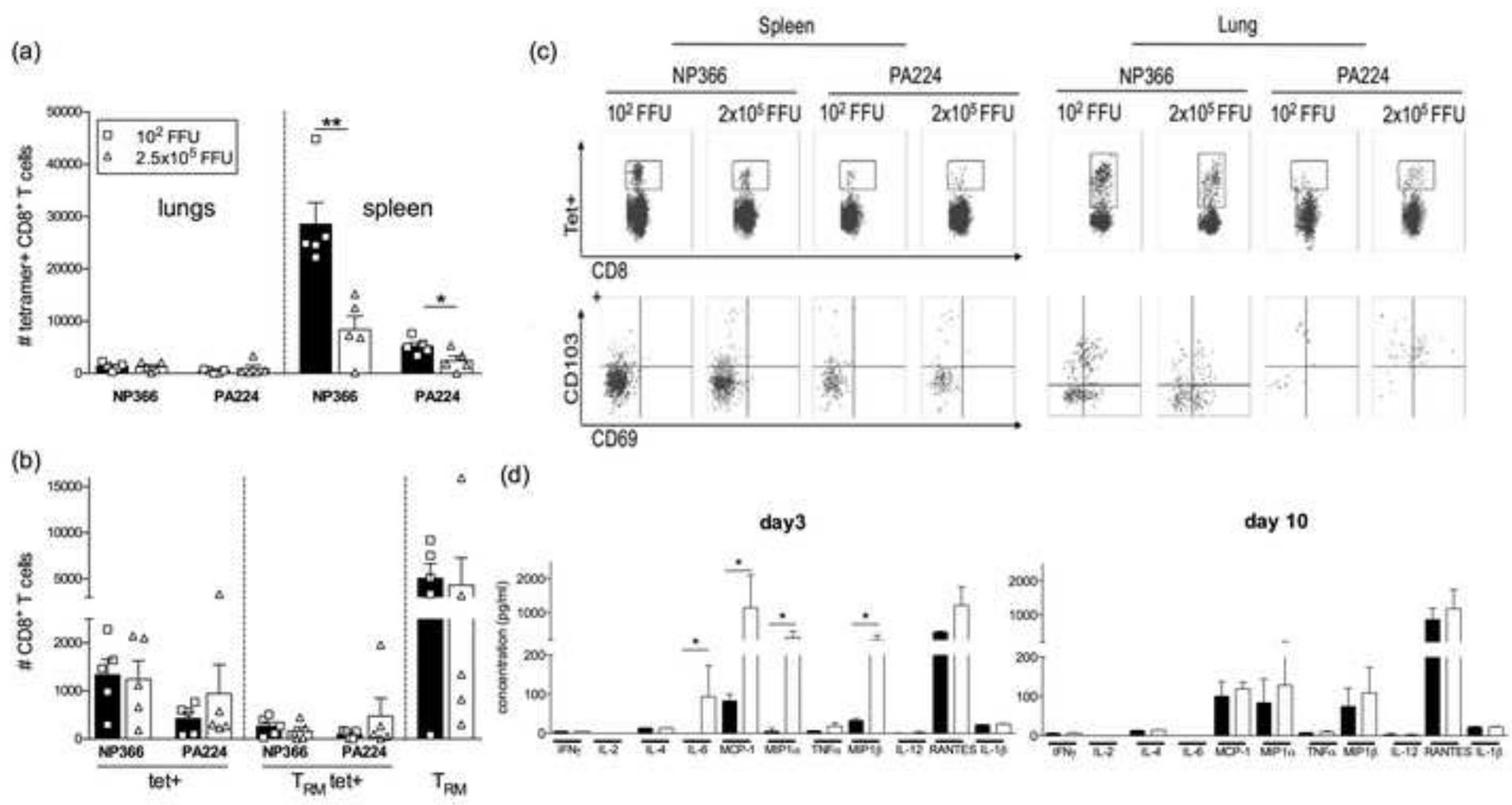
562 **Figure 6. LAIV vaccination protects mice from heterosubtypic challenge.** (a) Mice
563 were primed i.n. with either 10^2 FFU or 2.5×10^5 FFU PR8 ts or PBS (control group) and
564 challenged 6 weeks later with 10^4 PFU of X31 virus, weight loss was monitored for 8
565 days. (b) Comparison of lung viral titres between 10^2 FFU PR8 ts, 2.5×10^5 FFU PR8 ts or
566 control mice on d5, d6 and d8 post-challenge infection. Mean values \pm S.E.M. are shown.
567 Antibody titres following LAIV vaccination to either homologous PR8 HA (c) or
568 heterologous X31 HA (d). Mean values were plotted, error bars represent S.E.M., n=5
569 biological replicates within one experiments, stastics between groups were performed
570 using Mann-Whitney test.

571

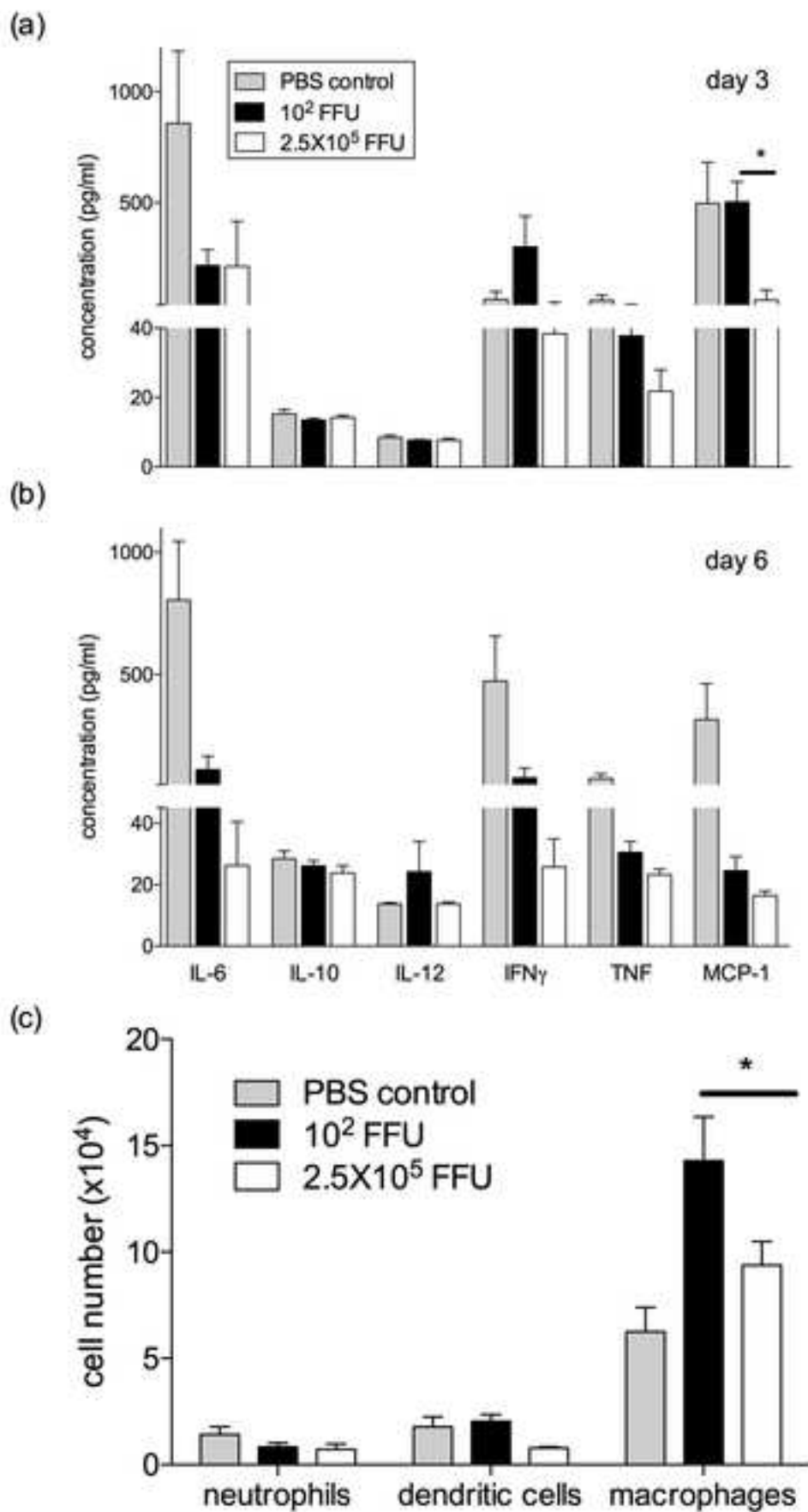


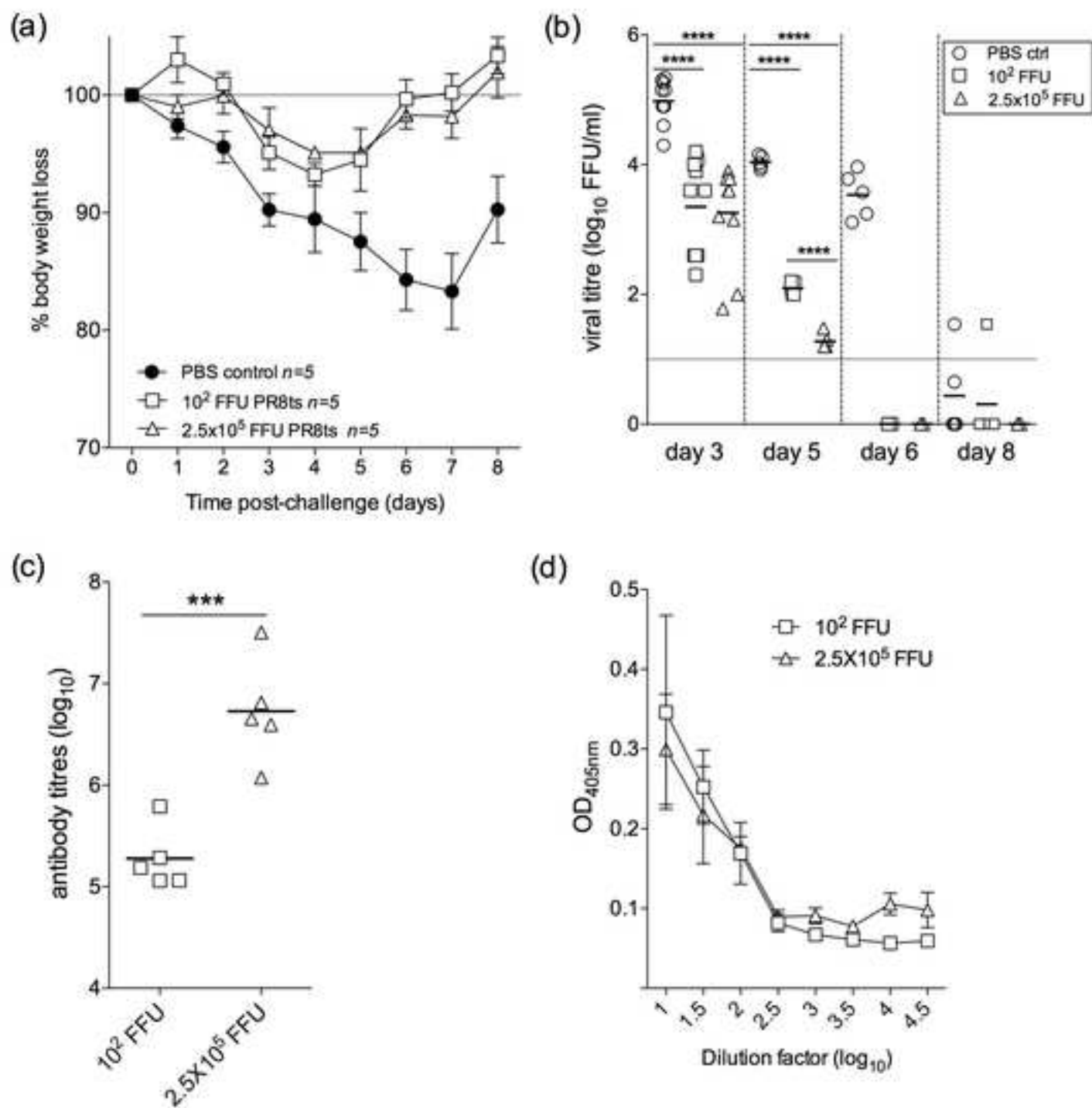
Wang et al. Fig. 1





Wang et al. Fig. 3





Wang et al. Fig. 6