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Establishment of memory CD8$^+$ T cells with live attenuated influenza virus (LAIV) across different vaccination doses

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

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ABSTRACT

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

INTRODUCTION

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

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

numerically more $CD8^+$ T cells and thus subsequently provide superior heterosubtypic protection, or alternatively a low dose LAIV could recruit fewer $CD8^+$ T cells to combat the heterosubtypic influenza infection. Furthermore, could the LAIV immunization dose affect the number of long-lasting tissue resident memory T (T_{RM}) cells in the lungs? In this study, we immunized mice intranasally (i.n.) with different doses ($1x10^2$ FFU/mice and $2x10^5$ FFU/mice) of live-attenuated PR8 virus (PR8 ts, H1N1), which allowed us to model different antigen doses provided by LAIV, compare the $CD8^+$ T cell responses and the resultant protective efficacy by challenging at 6 weeks post-vaccination with heterosubtypic X31 (H3N2) virus.

RESULTS

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

Temperature-sensitive PR8 virus was generated as described previously (Huber *et al.*, 2009) and viable viral particles were rescued from allantoic fluid of inoculated eggs. PR8 ts demonstrated increased temperature sensitivity when eggs were incubated at either 33°C or 37°C (Fig. 1a), with significantly lower titres obtained at higher temperature following 48 hours (h) incubation ($p<0.05$). When compared to the wild-type virus (PR8-wt), PR8 ts displayed much slower growth kinetics at MOI 0.01, when grown in MDCK monolayers at 37°C over 48 h (Fig. 1b). A low dose of 10^2 FFU and a high dose of $2.5x10^5$ FFU were used for *in vivo* intranasal infection of C57B6 mice, which confirmed the attenuated phenotype of PR8 ts. At both doses, mice showed only transient body weight loss of ~5% with quick recovery, whereas mice infected with 10^2 FFU of PR8-wt lost on average 20% body weight within 9 days without signs of recovery (Fig. 1c). Since 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 (IFNγ)-producing $CD8^+$ T cells, Fig. 2c; or double positive
121 $IFN\gamma^+TNF^+$ $CD8^+$ T cells, Fig. 2d and Fig. 2e) to two immunodominant epitopes D^bNP_{366}
122 and D^bPA_{224} (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^bNP_{366}- and D^bPA_{224}-
128 specific $CD8^+$ T cell responses varied between spleen and lungs. The higher PR8 ts
129 vaccination dose induced higher $D^bPA_{224}{}^+CD8^+$ 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 $D^bNP_{366}{}^+CD8^+$ T cells in lungs between different immunization doses.
132 Conversely, the lower vaccination dose induced significantly higher $CD8^+$ T cell responses
133 to D^bNP_{366} 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 IFNγ or TNFα, as assessed by intracellular cytokine

staining (Fig. 2c,2d). It is important to note that the numbers of naïve antigen-specific $CD8^+$ T cells are around 68 ± 18 for naive $D^bPA_{224}{}^+CD8^+$ and 36 ± 21 for $D^bNP_{366}{}^+CD8^+$ T cells, as shown by our previous studies(La Gruta *et al.*, 2010; Valkenburg *et al.*, 2010)

We then investigated memory responses at 6 weeks after priming with PR8 ts (Fig. 3). No differences to either $D^bNP_{366}{}^+CD8^+$ or $D^bPA_{224}{}^+CD8^+$ T cell responses were observed in the lungs, indicating that any differences that we observed at d10 post-priming equalize over time when the effector $CD8^+$ T cell populations contracted into memory. However, the lower vaccination dose resulted in generation of significantly higher numbers of virus-specific memory $CD8^+$ T cells in the spleen. We also observed significantly higher numbers of D^bNP_{366}-specific $CD8^+$ T cells, and a modest but significant difference in the numbers of D^bPA_{224}-specific $CD8^+$ T cells (Fig. 3a).

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

To determine whether the numerically higher $CD8^+$ T cell response induced by the low dose immunization was due to higher inflammatory millieu, we measured cytokine levels induced after primary immunization with low/high dose at d3 and d10. Our data (Fig. 3d) showed that the cytokine levels induced by a high dose ($2.5X10^5$FFU) in lungs at d3 and d10 after vaccination were higher than those induced with the lower dose ($1X10^2$FFU), and were not associated with different numbers of antigen-specific $CD8^+$ T cell responses elicited by those doses.

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

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

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

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

LAIV vaccination dose affects the rate of viral clearance.

Consistent with limited differences in recall responses between groups of animals vaccinated with different doses of LAIV, we did not observe any differences in clinical symptoms (weight loss) between these two groups following challenge. Approximately 5-7% body weight loss was observed in immunized groups regardless of the vaccine dose, while the naïve group (PBS-primed) displayed 10-20% body weight loss and delayed recovery (Fig. 6a). Mice vaccinated with a lower dose of 10^2 FFU of PR8 ts displayed delayed viral clearance compared with the $2.5x10^5$ FFU PR8 ts group (Fig. 6b). Viral load was examined on d3, d5, d6 and d8 post-challenge in lung homogenates. At d5, the viral titres in the lungs of vaccinated groups were significantly lower than those in control mice ($P<0.0001$), while on d6, the virus was undetectable in the lungs of immunized animals, but still detectable in the control group. On d8 post-challenge, the majority of mice had lung viral tires below the level of detection. On d5 post-challenge, mice vaccinated with 10^2 FFU LAIV had singinficantly increased levels of virus, compared with mice that received $2.5x10^5$ FFU ($P<0.001$). Delayed viral clearance during antiviral response can usually be attributed to delayed recruitment of influenza-specific $CD8^+$ T cells to the site of infection (Marois *et al.*, 2015), which was not observed in this case (Fig. 4). As the

magnitude of $CD8^+$ T cell responses did not correlate with viral clearance across different immunization doeses, we investigated antibody levels triggered by different doses of PR8 ts vaccine. Our ELISA results show that antibody titres against homologous PR8 HA protein were dose-dependent, with antibody titres induced by the higher $2.5x10^5$ FFU dose detected at nearly 160-fold greater level than those induced by 10^2 FFU (Fig. 6c). We then tested antibody titres against heterologous X31 HA protein, and found no difference between doses of LAIV (Fig. 6d). It has been reported that antibodies raised against the X31 virus do not significantly cross-react with the PR8 virus, nonetheless X31-specific antibodies provide some protection against PR8 infection (Rangel-Moreno *et al.*, 2008). Our data suggest that the same holds true when antibodies are raised against PR8.

DISCUSSION

In this study, we observed that different LAIV doses elicit distinct influenza-specific $CD8^+$ T cell responses in lungs and spleens, but unexpectedly not in brochoalveolar lavage (BAL). Virus-specific $CD8^+$ T cell responses typically fall into reproducible hierarchies, with particular epitopes eliciting either immunodominant (numerically high) or subdominant (numerically low) responses after the viral challenge. In the B6 model of influenza virus infection, $CD8^+$ T cell responses specific for epitopes derived from the nucleoprotein ($NP_{366–374}$) and acid polymerase ($PA_{224–233}$) proteins dominate during the primary acute immune response, with $D^bNP_{366}{}^+CD8^+$ T cells being numerically larger on d10 after infection (Cukalac *et al.*, 2014; Kedzierska *et al.*, 2006a) Interestingly, the immunodominance hierarchy at the acute time after immunization was affected by the LAIV dose, however these differences disappeared at 6 weeks post-vaccination. We found that sufficient numbers of specific $CD8^+$ T cells were generated for recall and protection of mice against heterosubtypic PR8 (H1N1)-> X31 (H3N2)

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

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

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

different doses of LAIV used in this study led to generation of comparable numbers of tetramer-positive $CD8^+$ T cells in both lungs and spleens of vaccinated animals. The notable difference was a slight change in immunodominance hierarchy 10 days following priming, where upon immunization with a lower dose, increased numbers of D^bNP_{366}-specific $CD8^+$ T cells were induced in the spleen, while a higher dose led to increased numbers of D^bPA_{224}-specific $CD8^+$ T cells in the lungs of immunized animals. To some degree, this is in agreement with the above mentioned study (Luciani *et al.*, 2013), where increasing the viral dose via i.p. delivery led to a switch in immunodominance from NP_{366} to PA_{224} in the spleen. Interestingly, these differences were alleviated in the lungs at a later time point (6 weeks post priming), while spleens of mice immunized with a lower dose of LAIV showed higher numbers of NP_{366}-specific memory $CD8^+$ T cells.

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

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

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

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

310 Neirynck *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 $1.0x10^2$ and $2.5x10^5$ plaque forming units
331 (FFU) of LAIV (PR8 ts; H1N1) influenza virus in 30µl of PBS. We based our definition
332 of the high ($2.5x10^5$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 30µl PBS. Lungs were harvested for viral load in a Madin-Darby canine kidney (MDCK)

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

Influenza virus-specific CTL responses. Cells were recovered from spleen, lung and bronchoalveolar lavage (BAL). Lymphocytes were stimulated with peptides representing the H2D^{b}-restricted NP$_{366-374}$ (ASNENMTEM) or PA$_{224-233}$ (SSLENFRAYV) epitopes in a intracellular cytokine secretion (ICS) assay (Bird *et al.*, 2015). Cells were also incubated with D^{b}NP$_{366-374}$ or D^{b}PA$_{224-233}$ tetramers conjugated to PE or allophycocyanin (Bird *et al.*, 2015). Results were analysed using FlowJo software (Treestar, USA). Lungs for T_{RM} analysis were processed as previously described (Kedzierski *et al.*, 2015; Quiñones-Parra *et al.*, 2016; Wakim *et al.*, 2015).

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

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

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

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FIGURE LEGENDS

Figure 1. Attenuated phenotype of PR8ts *in vitro* and *in vivo*. (a) Significantly lower ($P<0.001$) viral titres were obtained when virus was grown in embryonated eggs at 37°C when compared to 33°C. (b) Attenuated growth kinetics of PR8 ts compared to PR8-wt over 48 h incubation period in MDCK monolayers, n=6 per time point for PR8 wt. (c) Comparison of body weight loss in C57B6 mice (n=5 per group) infected intranasally with either 10^2 FFU PR8 wt, 10^2 FFU PR8 ts or $2.5x10^5$ FFU PR8 ts. Mean values were plotted, error bars represent S.E.M, stastics between groups were performed using Mann-Whitney test.

Figure 2. Primary $CD8^+$ T cell responses elicited by LAIV vaccination. (a) Magnitude of $CD8^+$ T cell response in lungs, spleen and BAL on d10 post-vaccination. Virus-specific $CD8^+$ T cells were enumerated using MHC class I tetramers against immunodominant D^bNP_{366} and D^bPA_{224} epitopes. (b) FACS analysis of tetramer positive $CD8^+$ T cells in lungs and spleen. Dot plots from individual mice were combined into a cumulative display with mean frequencies included on the graph. Cytokine production by $CD8^+$ T cell stimulated with either NP_{366} or PA_{224} peptides. Absolute numbers of $IFN\gamma^+$ (c) and polyfunctional $IFN\gamma^+TNF^+$ (d) cells was assessed in lungs, spleen and BAL by FACS following *ex vivo* ICS. Data from a representative experiment (of 2) were plotted, (n=5 per group) error bars represent S.E.M. (e) FACS analysis of $IFN\gamma^+TNF^+$ $CD8^+$ T cell populations in the lungs and spleen. Dot plots from individual mice were combined into a cumulative display with mean frequencies included on the graph. Stastics between groups were performed using Mann-Whitney test.

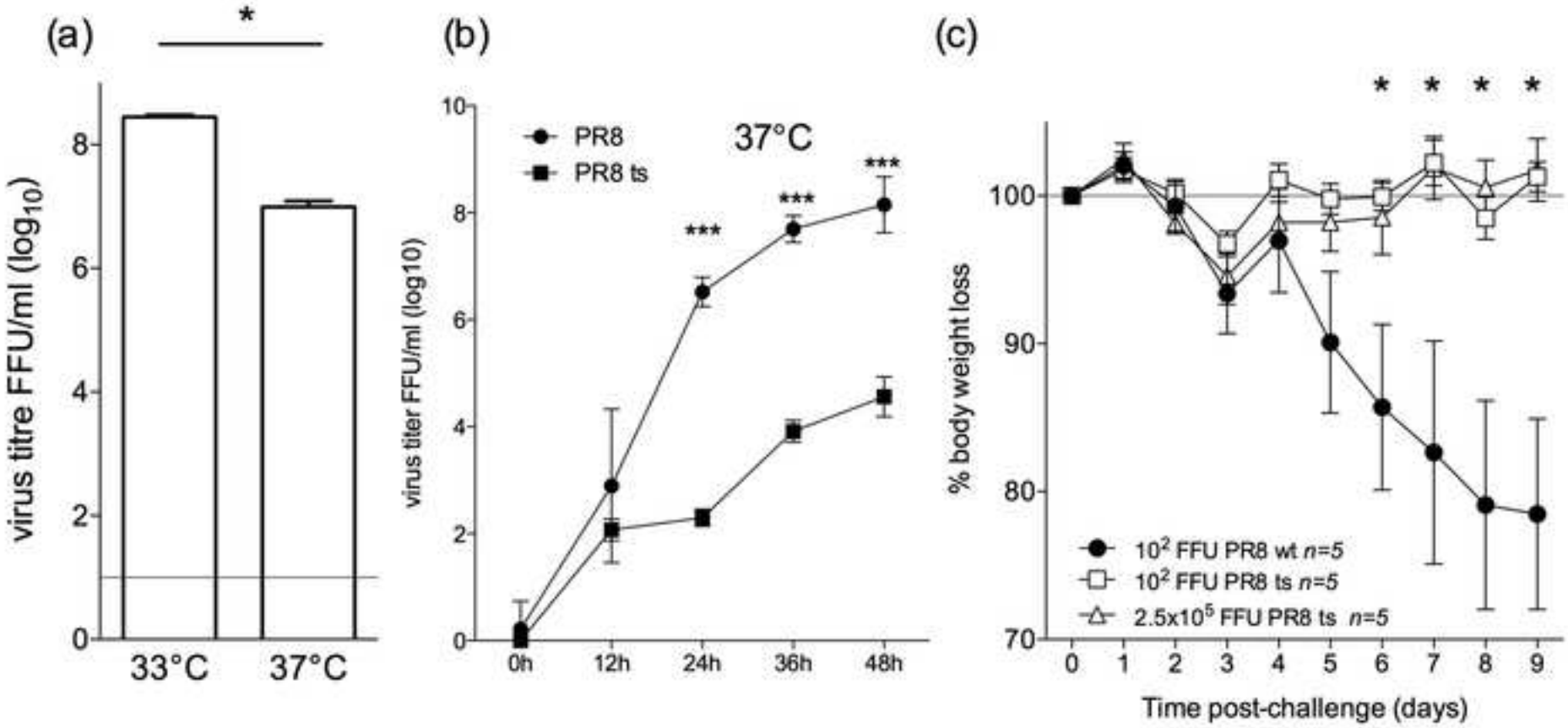
Figure 3. Memory formation following LAIV vaccination. (a) Numbers of virus-specific memory $CD8^+$ T cells were assessed in spleen and lungs 6 weeks after initial PR8 ts administration (b) Numbers of tissue resident memory cells ($CD103^+CD69^+$) in the lungs 6 weeks after PR8 ts vaccination. Mean values were plotted, error bars represent S.E.M. Mice n=5 for each group within one experiments (c) FACS analysis of memory $CD8^+$ T cell populations in the spleen and lungs. Dot plots from individual mice were combined into a cumulative display. (d) Cytokines level were measured by CBA at d3 and d10 after primary infection with low dose 10^2 FFU PR8 ts and high dose $2.5x10^5$ FFU PR8 ts (n=5 per group within an experiment). Stastics between groups were performed using Mann-Whitney test.

Figure 4. Recall responses to heterosubtypic challenge with X31 (H3N2) influenza virus. (a) Total numbers of virus-specific memory $CD8^+$ T cells were assessed in spleen and BAL on day 5 and 8 following secondary X31 influenza challenge. (b) Capacity of D^bNP_{366}- and D^bPA_{224}-specific $CD8^+$ T cells to secrete $IFN\gamma$ following challenge with X31 influenza virus. Data from a representative experiment (of 2) were plotted, error bars represent S.E.M, n=5 for each group for each experiments.

Figure 5. Cytokine milieu and influx immune cells in the lungs of LAIV vaccinated mice. Cytokine levels (a,b)and influx cells (c) were analysed by CBA in lung homogenates on d3 (a) and d6 (b) post-challenge with X31 virus. Meanvalues±S.E.M. are

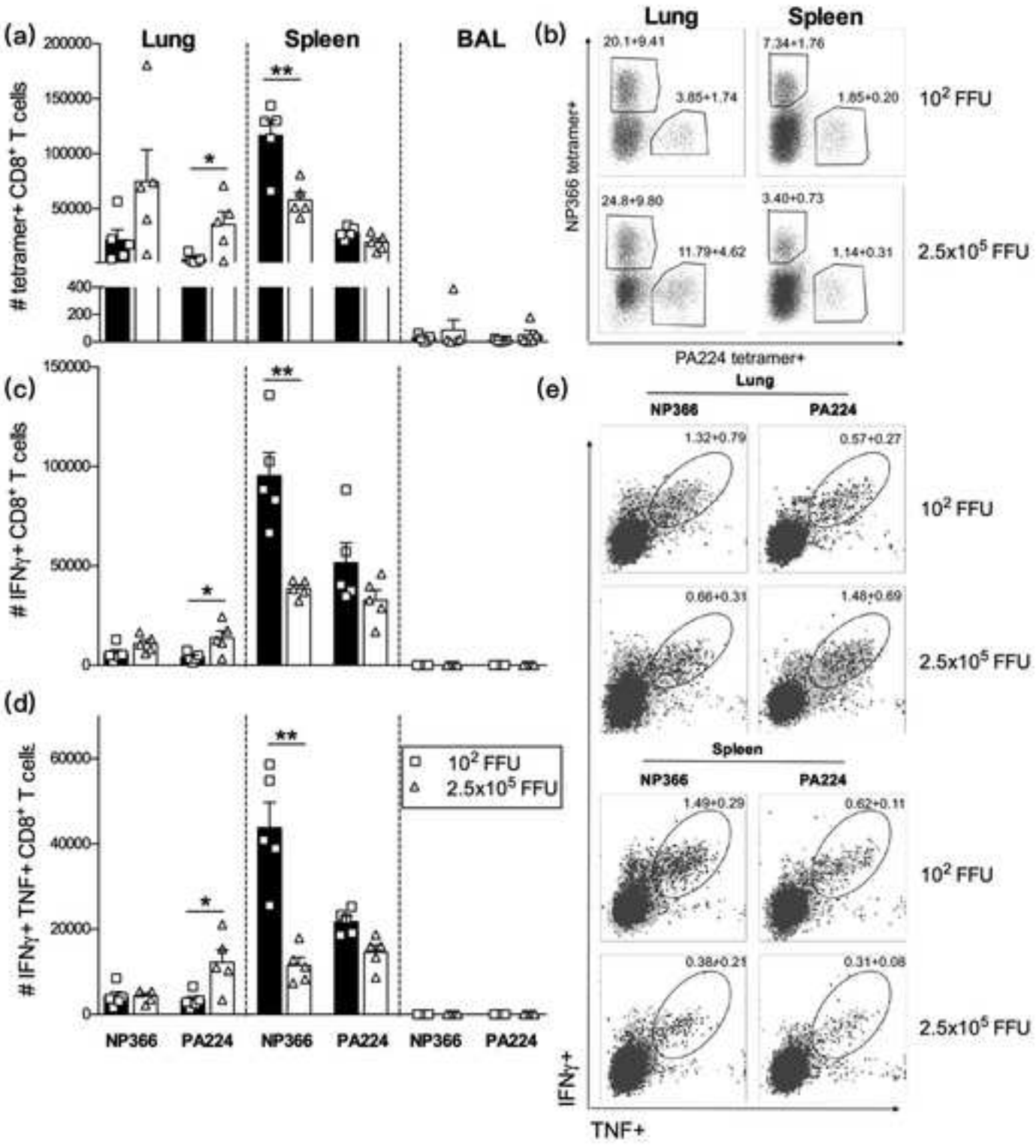
559 shown for n=5 biological replicates within one experiments, stastics between groups were
560 performed using Mann-Whitney test.
561
562 **Figure 6. LAIV vaccination protects mice from heterosubtypic challenge.** (a) Mice
563 were primed i.n. with either 10^2 FFU or $2.5x10^5$ FFU PR8 ts or PBS (control 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, $2.5x10^5$ FFU PR8 ts or
566 control mice on d5, d6 and d8 post-challenge infection. Mean values±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

Figure 1



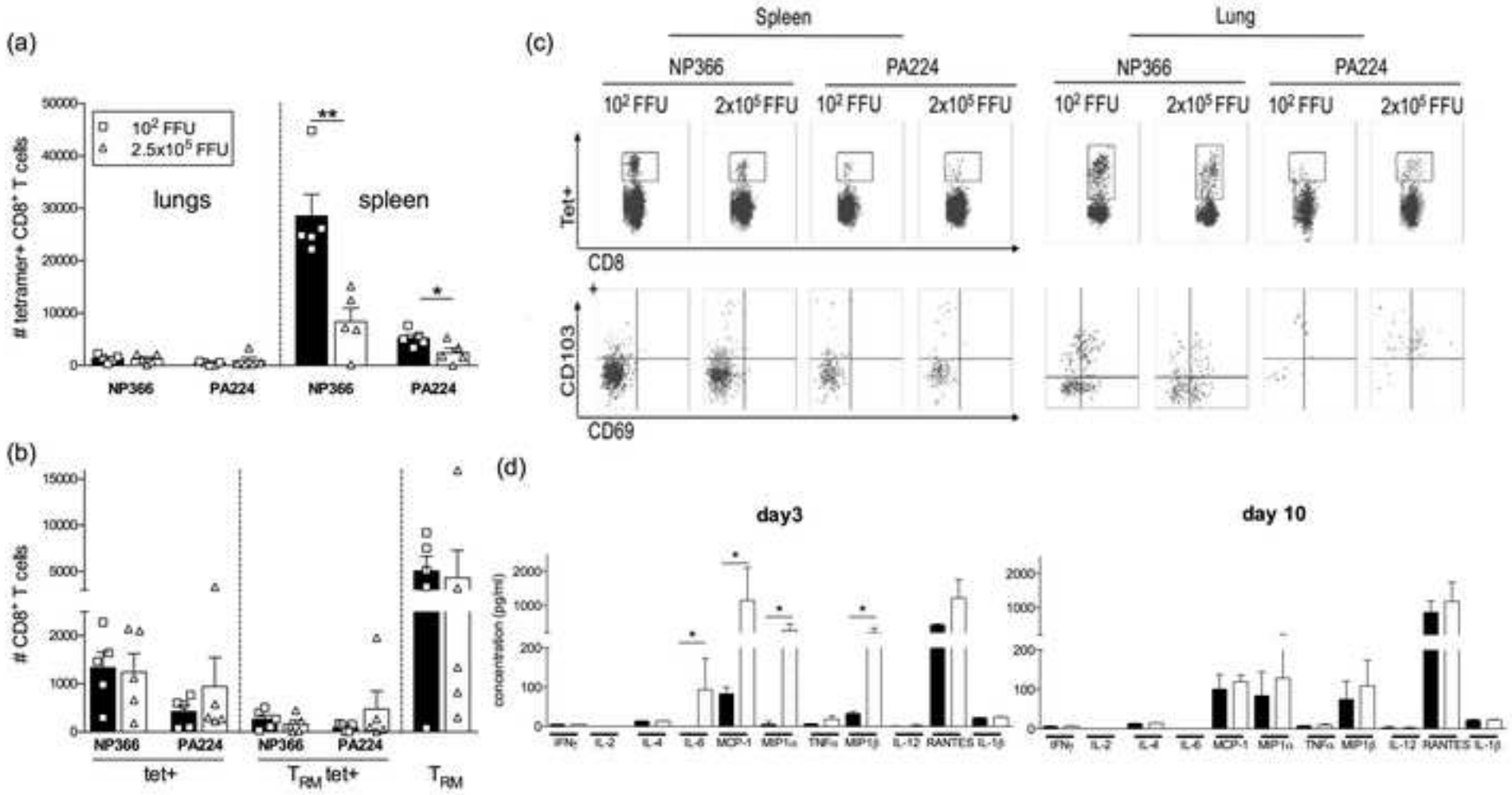
Wang et al. Fig. 1

Figure 2



Wang et al. Fig. 2

Figure 3



Wang et al. Fig. 3

Figure 4

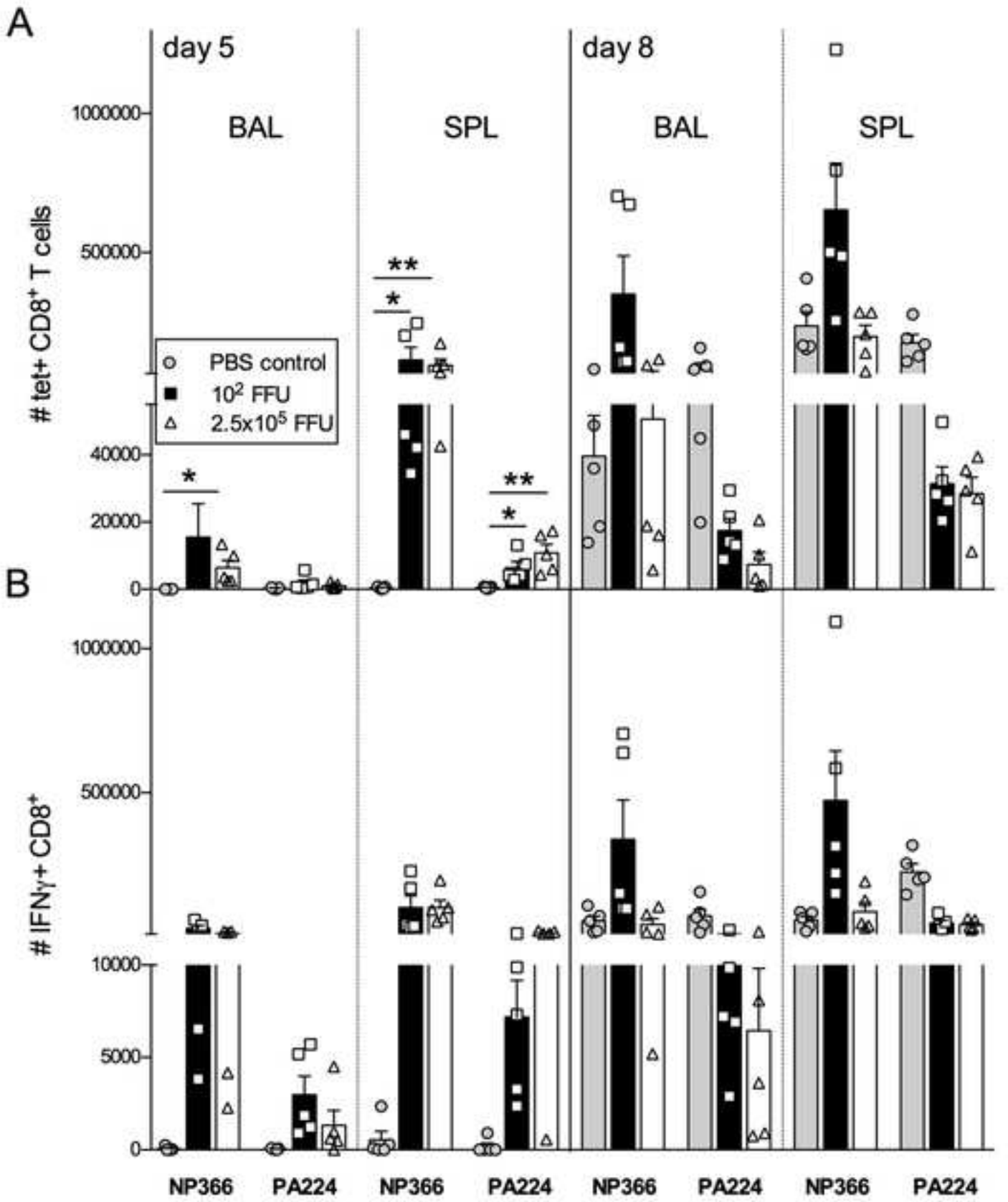

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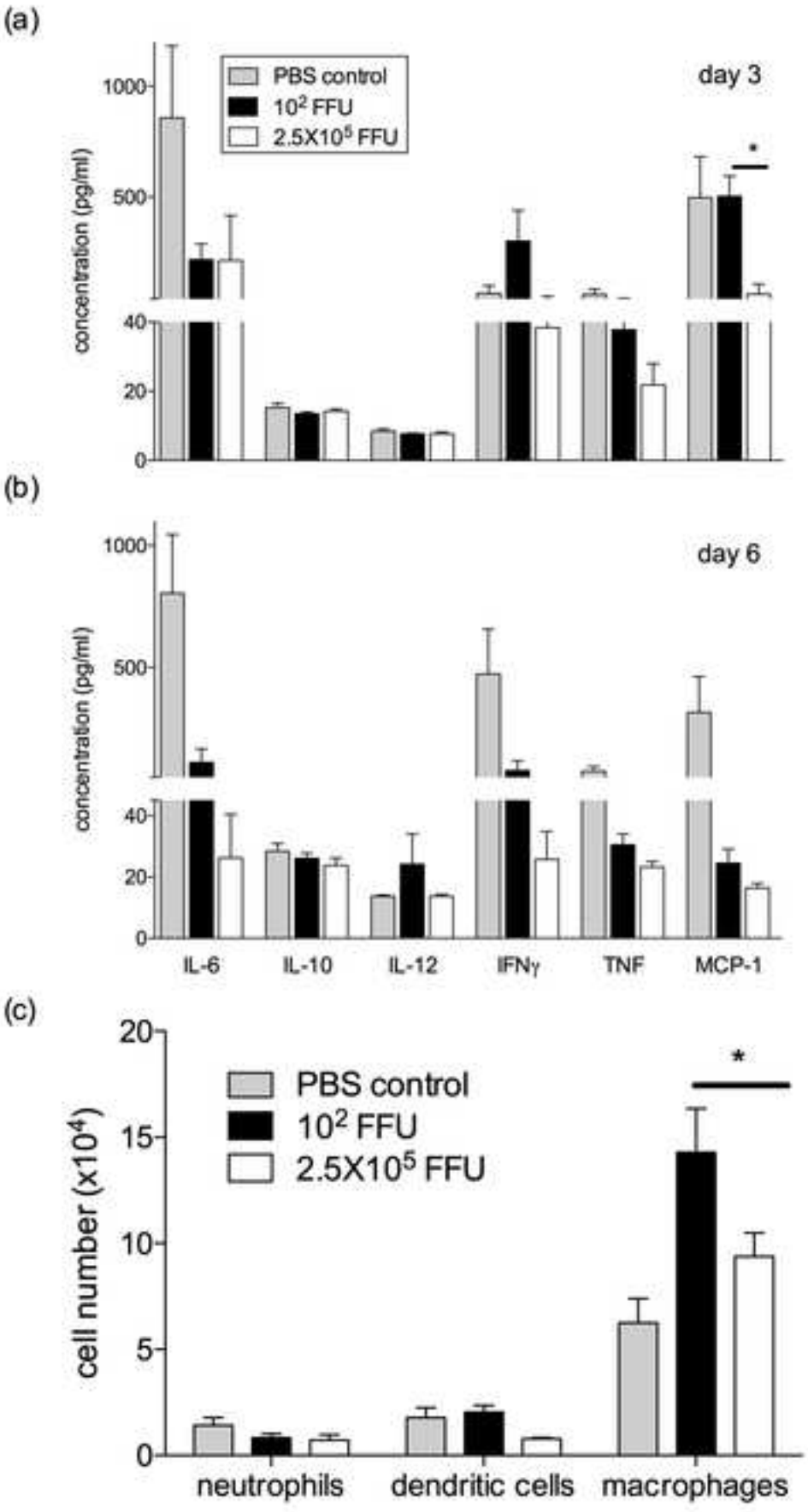
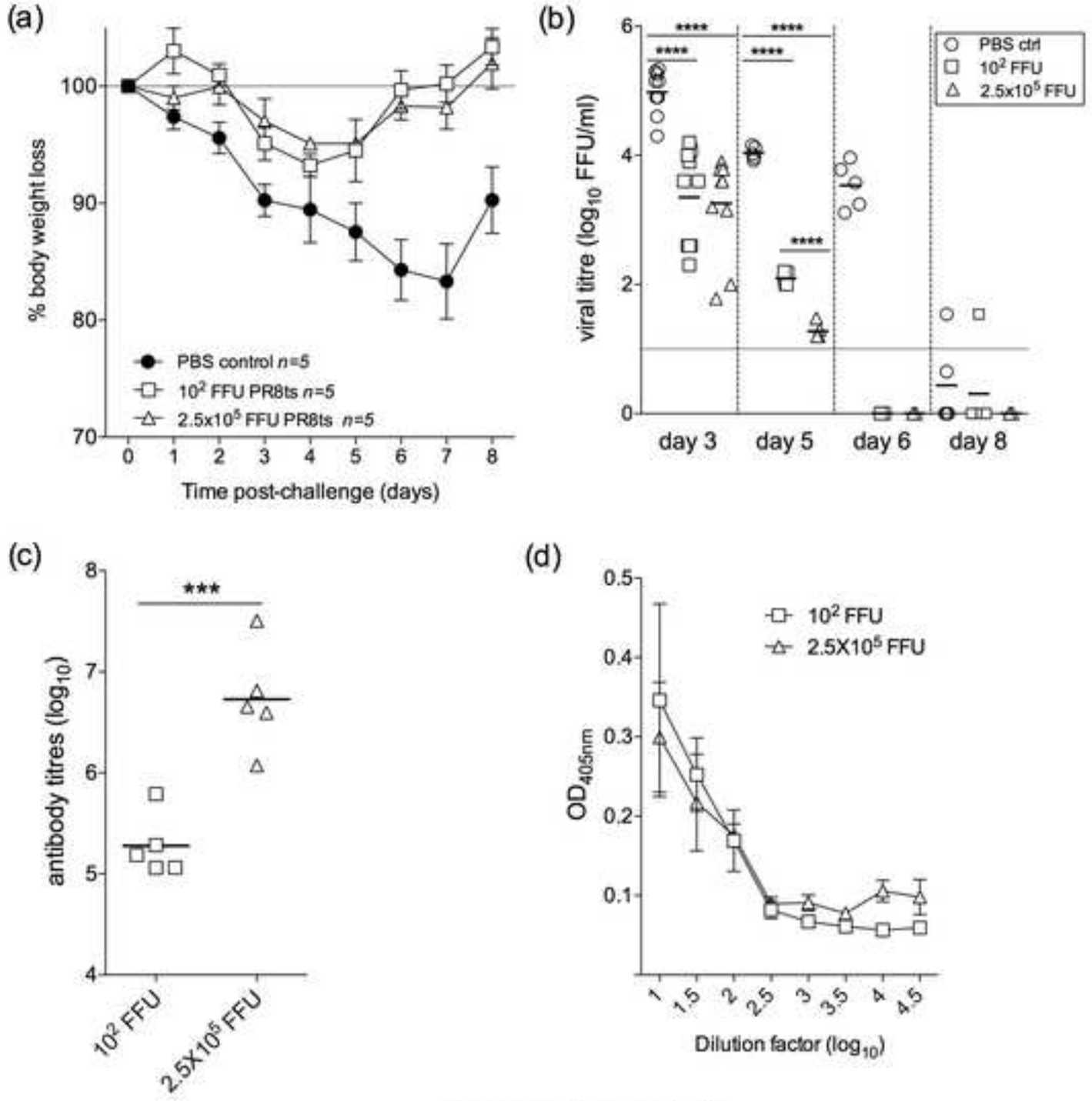
Figure 5



Wang et al. Fig. 5

Figure 6



Wang et al. Fig. 6