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

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Running title: CD8+ T cells and LAIV vaccination

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

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

We investigated primary CD8+ T cell responses on d10 post-vaccination, and memory
responses at 6 weeks post-vaccination in order to determine whether vaccination dose can
lead to differences in specific anti-viral responses. On d10 post-vaccination, we assessed
the magnitude (number of tetramer+ CD8+ T cells, Fig. 2a and Fig. 2b) and functional
quality (number of interferon γ (IFNγ)-producing CD8+ T cells, Fig. 2c; or double positive
IFNγ+TNF+ CD8+ T cells, Fig. 2d and Fig. 2e) to two immunodominant epitopes DbNP366
and DbPA224 (Bird et al., 2015; Kedzierska et al., 2006b). Our data show that efficient
CD8+ T cell responses can be detected in both lungs (the usual site of influenza virus
infection) and spleen (the secondary lymphoid organ). Unexpectedly, PR8 ts vaccination
did not induce detectable levels of influenza-specific CD8+ T cells in the bronchoalveolar
lavage (BAL), possibly due to the non-replicative nature of the LAIV in the lower
respiratory tract. However, the immunodominance hierarchy of DbNP366- and DbPA224-
specific CD8+ T cell responses varied between spleen and lungs. The higher PR8 ts
vaccination dose induced higher DbPA224+CD8+ T cell numbers in the lungs when
compared to the lower vaccination dose. However, no significant differences (p>0.05)
were found for DbNP366+CD8+ T cells in lungs between different immunization doses.
Conversely, the lower vaccination dose induced significantly higher CD8+ T cell responses
to DbNP366 epitope in the spleen, as compared to the lower vaccination dose. Despite, a
high number of virus-specific tetramer+CD8+ T cells in lungs, only a limited number of
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 DβPA224<sup>+</sup>CD8+ and 36 ± 21 for DβNP<sub>366</sub>+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 DβNP<sub>366</sub>+CD8+ or DβPA224<sup>+</sup>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 DβNP<sub>366</sub>-specific CD8+ T cells, and a modest but significant difference in the numbers of DβPA224-specific CD8+ T cells (Fig. 3a).

We also enumerated resident memory CD8+ T cells (TRM, CD69<sup>+</sup>CD103<sup>+</sup>CD8+) in the lungs of vaccinated mice, but found no differences in the numbers of either virus-specific or total TRM cells between the two vaccination groups (CD103<sup>+</sup>CD69<sup>+</sup>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<sup>5</sup>FFU) in lungs at d3 and d10 after vaccination were higher than those induced with the lower dose (1X10<sup>5</sup>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$^b$NP$_{366}$ and D$^b$PA$_{224}$ 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$^b$NP$_{366}$- and D$^b$PA$_{224}$-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 IFN$\gamma$ 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, IFN$\gamma$, 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 titres below the level of detection. On d5 post-challenge, mice vaccinated with 10^2 FFU LAIV had significantly 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 doses, 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\textsuperscript{b}NP\textsubscript{366}-
specific CD8+ T cells were induced in the spleen, while a higher dose led to increased
numbers of D\textsuperscript{b}PA\textsubscript{224}-specific CD8+ T cells in the lungs of immunized animals. To some
degree, this is in agreement with the above mentioned study (Luciani \textit{et al.}, 2013), where
increasing the viral dose via i.p. delivery led to a switch in immunodominance from NP\textsubscript{366}
to PA\textsubscript{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\textsubscript{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\textsubscript{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 IFN\textsubscript{γ} (McMaster \textit{et al.}, 2015).
Different doses of LAIV vaccine were able to induce formation of T\textsubscript{RM} cells, but we did
not observe any differences between low and high immunization dose, either in numbers
of virus specific T\textsubscript{RM} or overall T\textsubscript{RM} numbers. At 6 weeks post-priming, the numbers of
virus-specific T\textsubscript{RM} cells were relatively low, which is consistent with their non-
proliferative nature.

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

METHODS

Infection of mice and characterization of PR ts in MDCK cells. C57B6 mice were bred
and housed at Department of Microbiology and Immunology, University of Melbourne.
Experiments were approved and conducted under guidelines set by the University of
Melbourne Animal Ethics Committee. Mice were anaesthetised by inhalation of
methoxyflurane and intranasally infected with 1.0x10^2 and 2.5x10^5 plaque forming units
(FFU) of LAIV (PR8 ts; H1N1) influenza virus in 30µl of PBS. We based our definition
of the high (2.5x10^5 FFU) and low dose (10^2 FFU) amounts in this study on the clinical
dose of flumist (10^7 PFU) used in an adult human with an average weight of 75kg and
extrapolating this to a mouse with an average weight of 20g (10^3 PFU).

Mice were challenged intranasally 6 weeks later with 1x10^4 PFU of X31 (H3N2) virus in
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 an 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 TRM 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 10² FFU PR8 wt, 10² FFU PR8 ts or 2.5x10³ 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 D³NP₃₆₆ and D³PA₂₂₄ 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 NP₃₆₆ or PA₂₂₄ peptides. Absolute numbers of IFNγ⁺ (c) and polyfunctional IFNγ⁺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 IFNγ⁺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 10² FFU PR8 ts and high dose 2.5x10⁵ 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 D³NP₃₆₆⁻ and D³PA₂₂₄-specific CD8⁺ T cells to secrete IFNγ 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
shown for n=5 biological replicates within one experiments, stastics between groups were performed using Mann-Whitney test.

**Figure 6. LAIV vaccination protects mice from heterosubtypic challenge.** (a) Mice were primed i.n. with either $10^2$ FFU or $2.5 \times 10^5$ FFU PR8 ts or PBS (control group) and challenged 6 weeks later with $10^4$ PFU of X31 virus, weight loss was monitored for 8 days. (b) Comparison of lung viral titres between $10^2$ FFU PR8 ts, $2.5 \times 10^5$ FFU PR8 ts or control mice on d5, d6 and d8 post-challenge infection. Mean values±S.E.M. are shown. Antibody titres following LAIV vaccination to either homologous PR8 HA (c) or heterologous X31 HA (d). Mean values were plotted, error bars represent S.E.M., n=5 biological replicates within one experiments, stastics between groups were performed using Mann-Whitney test.
Wang et al. Fig. 1
Wang et al. Fig. 2
Figure 3

(a) 

(b) 

(c)  

(d) 

Wang et al. Fig. 3
Figure 4

A

Day 5

BAL

SPL

Day 8

BAL

SPL

# tet+ CD8+ T cells

PBS control

10^2 FFU

2.5x10^5 FFU

# IFNγ+ CD8+

NP366

PA224

NP366

PA224

NP366

PA224

NP366

PA224
Figure 5

(a) Concentration (pg/ml) of various cytokines measured on day 3. 
(b) Concentration (pg/ml) of various cytokines measured on day 6. 
(c) Cell number (x10^4) of neutrophils, dendritic cells, and macrophages measured on day 6.

Wang et al. Fig. 5
Wang et al. Fig. 6