Recombinant influenza virus expressing HIV-1 p24 capsid protein induces mucosal HIV-specific CD8 T-cell responses

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1. Introduction

HIV infection in women is primarily acquired across the cervico-vaginal mucosa. Establishing strong CD8 T-cell immunity in mucosal tissue may form an effective barrier against infection [1]. Numerous human and non-human primate models have illustrated the importance of CD8 T-cell responses in controlling HIV infections. CD8 T-cell depletion of SIV-infected macaques resulted in elevated levels of viremia [2]. The appearance of viral escape mutants illustrate the selective pressures that are exerted by CD8 T-cell responses mounted in infected individuals [3,4]. CD8 T-cells targeting HIV epitopes appear prior to peak viremia and wane as viral loads decrease to set point [5–7]. The bridging of this temporal gap between early HIV replication and cell-mediated host immunity is a fundamental aim for CD8 T-cell-based vaccines [8].

Influenza virus is a strong stimulator of CD8 T-cell responses. Macaque studies previously employing a heterosubtypic prime-boost regimen with influenza viruses encoding short (9–10 amino acids) SIV epitopes demonstrated induction of CD8 T-cell responses, but the elicitation of narrow responses resulted in the evolution of escape SIV variants [9]. Whole heterologous protein expression will enable a broader coverage of epitopes and impede viral escape [10]. The influenza A virus genome consists of eight negative-sense, single-stranded RNA segments that encode more than 10 viral proteins. Here, we adopt a previously described model that incorporated a heterologous gene in the NS-segment [11]. We generated recombinant influenza viruses that expressed the HIV-1 p24 capsid protein as a model for a live mucosal HIV vaccine.
We demonstrated that recombinant influenza viruses expressing whole HIV-1 p24 capsid stimulated HIV-specific CD8 T-cell responses in the lungs and vaginal mucosa of mice immunized with a prime-boost regimen.

2. Materials and methods

2.1. Construction of recombinant influenza NS-segment

Influenza sequences were derived from influenza A/Puerto Rico/8/34 (PR8) Mount Sinai (GenBank AF389122.1) and the HIV-1 p24 sequence from HIV-1 vector pNL4-3 (GenBank AF324493.2). Porcine teschovirus-1 (PTV-1) 2A, Thosae asigna virus (TAV) 2A, NS-segment splice mutations and linker sequences were adapted from previous studies [12,13]. The recombinant NS1-p24-NEP DNA sequence was constructed synthetically (Life Technologies) and cloned into the phW2000 plasmid (St. Jude Children’s Hospital) [14]. A GFP-plasmid variant was constructed with sequences from *Pontellina plumata* (GenBank AY268072.1). Plasmids were verified by Sanger sequencing.

2.2. Rescue and propagation of viruses

Recombinant influenza viruses were reverse engineered using the eight-plasmid system described previously [14,15]. PR8 viruses (H1N1) were created using recombinant NS-segments on a PR8 plasmid background. For X31 (H3N2) viruses, the HA and NA plasmids were substituted with A/Hong Kong/68 genes. Viruses isolated were amplified and quantitated as described previously [15,16].

2.3. Stability of heterologous DNA inserts in passaged viruses

Stability of the heterologous DNA inserts was determined by serially passaging each recombinant virus four times in embryonated eggs. Total RNA was isolated from virus-infected allantoic fluid using the QIAamp Viral RNA Mini Kit (Qiagen). Heterologous DNA inserts were amplified using a one-step RT-PCR kit (Invitrogen) with primers corresponding to the p24 sequence.

2.4. In vitro p24 expression assays

A549 cells were propagated to >90% confluency in TC75 flasks with RF10 media (RPMI 1640, 10% FCS, 1× penicillin-streptomycin-glutamine; Life Technologies). Cells were washed twice with PBS and infected with recombinant influenza virus at an MOI = 10 for 1 h at 37 °C/5% CO2. Cells were washed thrice with RF10 and incubated in RF10 for an additional 12 h at 37 °C/5% CO2. Adherent cells were trypsinized and resuspended in RF10 prior to transfer of 10^6 cells into 5 mL polystyrene FACs tubes. Cells were fixed with 1% formaldehyde (Polysciences), permeabilized (BD 340973) and stained with influenza anti-NP (431; Abcam) and HIV-1 anti-p24 (KC57; Beckman Coulter) antibodies for 1 h at RT. Cells were fixed again in 1% formaldehyde.

2.5. Mice immunizations

All animal procedures were approved by the University of Melbourne Animal Ethics Committee. Groups of 4–5 BALB/c female mice at 8 weeks of age were anaesthetized by isoflurane inhalation and intranasally immunized with influenza virus doses ranging from 50 to 10^3 p.f.u. in 50 μL PBS. Five days before intravaginal immunization, mice were injected subcutaneously with 2 mg of medroxyprogesterone acetate (Pharmacia and Upjohn) in 100 μL PBS. Mice were immunized intravaginally by atraumatic instillation of 3 × 10^5 p.f.u. virus in 10 μL PBS.

2.6. Lymphocyte isolation

Isolated cells were processed in RF7 media (RPMI1640, 7% FCS, 10 mM HEPES, 1 mM sodium pyruvate, 1× penicillin-streptomycin-glutamine; Life Technologies). Whole blood was pooled by tail vein bleeding (100 μL/mouse). Bronchoalveolar lavages (BAL) were pooled from each treatment group. Three lavages of 1 mL each were performed with PBS on individual mice. Adherent cells were removed by incubating BAL on petri dishes at 37 °C for 1 h. Single cell suspensions of spleens were prepared by mechanical homogenization through a 70 μm cell strainer. Red blood cell lysis was performed with Pharm Lyse™ (BD) for 5 min at RT under dark conditions. Vaginal tissues were pooled and digested with 0.4 mL/tissue of RF7 media containing 2 mg/mL collagenase (Sigma C2139) and 1.25 mg/mL DNase I (Sigma). Tissues were digested for 1 h at 37 °C on a 200 r.p.m. orbital shaker. Undigested tissue debris was removed by a 70 μm cell strainer and 40%/70% Percoll gradient purification (Sigma) was performed for lymphocyte isolation.

2.7. Tetramer staining

Cells were stained with a viability dye for 30 min (Biolegend), washed once and incubated for 1 h at RT with PE-conjugated MHC-I H2-K^b^ A9 tetramer (University of Melbourne). Post-tetramer staining, surface staining of cells was performed for 30 min at RT using the following surface antibodies: CD3 (17A2; Biolegend), CD4 (GK1.5; BD), CD8 (53–6.7; BD), CD44 (IM7; BD) and CD45 (30–F11; BD). Cells were washed twice with PBS and fixed with Stabilizing Fixative solution (BD).

2.8. Intracellular cytokine staining

10^6 cells were aliquoted into round bottom 96-well plates. GolgiPlug (BD), GolgiStop (BD), CD107a antibody (1D4B; BD) and 0.5 mg/mL DNase I (Worthington) were added to the cell mix. Cells were activated with 1 μg/mL of A9 peptide or 1/20 diluted DMSO solution, and incubated at 37 °C/5% CO2 for 6 h. As above, cells were stained with viability dye and surface antibodies. Cells were fixed in 1% formaldehyde (Polysciences) for 10 min at RT. Cells were permeabilized (BD 340973) and cytokine staining was performed for 30 min at RT using antibodies for TNF (MP6-XT22; BD), IL-2 (JES6-5H4; BD) and IFNγ (XMG1.2; BD). Cells were washed once and fixed with Stabilizing Fixative solution (BD).

2.9. Data analysis

Flow cytometry data were acquired with a BD LSRFortessa and analyzed with FlowJo 9.8.3, Pesteel 1.7 and SPICE 5.35. Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc.). Data in Fig. 4 were analyzed by a Kruskal–Wallis test, followed by Mann–Whitney tests. A Bonferroni adjustment was used to set a new p-value level of 0.0166 for these analyses; p-values <0.0166 were considered significant.

3. Results

3.1. Generation of recombinant influenza viruses

In an effort to induce mucosal immunity, we engineered recombinant influenza viruses encoding whole heterologous proteins in the NS-segment. The wild type NS-segment encodes NS1 and NEP, with NEP expressed from spliced mRNA (Fig. 1A). The insertion of GFP into the NS-segment has been described previously by use of a multi-cistronic expression system based on the PTV 2A ribosomal skip sequence [11]. Our attempts to clone the HIV-1 p24 capsid gene into the same vector system, however, did not yield virus. The
NS-segment was redesigned to include a second PTV 2A sequence between the NS1 and HIV-1 p24 capsid gene junction, but this resulted in an unstable recombinant virus displaying deletion of the p24 gene (Fig. 1B and C). Replacing the PTV 2A sequence between the NS1 and HIV-1 p24 capsid gene junction with an alternative TAV 2A sequence yielded recombinant influenza viruses encoding HIV-1 p24 protein (Flu-p24) that grew to high titers in embryonated eggs (Fig. 1D). Average titers obtained from three plaque assays for Flu(PR8)-p24 and Flu(X31)-p24 strains were 6.5 × 10^8 and 3.3 × 10^8 p.f.u./mL, respectively. For controls in subsequent immunogenicity studies, we rescued GFP-encoding recombinant influenza viruses (Flu-GFP).

The stability of the heterologous genes was assessed by four serial passages of the viruses in embryonated eggs (Fig. 1C). RT-PCR showed that the p24 gene insert was stable in Flu(X31)-p24. Minor deletion variants (<0.5 kb) were observed for Flu(PR8)-p24 in the 3rd and 4th passages. Viruses used for immunization studies were derived from the 1st passage in eggs.

### 3.2. In vitro expression of p24 from recombinant influenza viruses

The ability of these viruses to express the heterologous protein was investigated by infection of the human alveolar basal epithelial A549 cell line (Fig. 2). Cells were co-stained for intracellular expression of the influenza nucleoprotein (NP) and HIV-1 p24 capsid. Both Flu(PR8)-p24 and Flu(X31)-p24 viruses induced intracellular p24 expression. Influenza infection was confirmed in most cells by expression of NP, with the p24+ population essentially confined to the NP+ population.

### 3.3. In vivo attenuation of recombinant influenza viruses

The level of attenuation and safety of the viruses were investigated in BALB/c mice in a dose escalation study. Groups of five mice were immunized intranasally with 10^3, 10^4 and 10^5 p.f.u. of the Flu(PR8)-p24 virus (Fig. 3A). These groups displayed no significant weight loss or any clinical signs of disease. In contrast, 50 p.f.u. of wild type PR8 administered via intranasal route induced significant weight loss. Additional groups of five mice were immunized intranasally with 10^3, 10^6 and 10^7 p.f.u. of the Flu(X31)-p24 virus (Fig. 3B). Doses of 10^5 p.f.u. and 10^6 p.f.u. did not induce significant weight loss. The dose of 10^7 p.f.u. induced significant weight loss, but not more than 20% of initial body weight. The data suggested that dose ranges of 10^3–10^5 and 10^5–10^6 for the Flu(PR8)-p24 and Flu(X31)-p24 viruses, respectively, were attenuated and safe in mice. We repeated immunizations a further three times for Flu(PR8)-p24 and Flu(X31)-p24 at 10^7 and 10^5 p.f.u., respectively, in groups of 5 mice and no weight loss over the 10 days post-infection.
immunized group (7.5% and 16.6% from repeat experiments) and i.n./i.vag Flu-p24 virus immunized group (4.2% and 4.3% from repeat experiments). In mice primed intranasally by recombinant influenza vectors (Flu-p24 and Flu-GFP), boosting by the i.n. route increased BAL CD8 T-cells, which is indicative of lung inflammation (Fig. 4C). Substantial lung-infiltration was not observed in i.vag. boosted mice, despite recall of HIV-specific CD8 T-cell responses.

Spleens from individual mice were analyzed for systemic HIV-specific CD8 T-cell responses (Fig. 4D). A significantly elevated response was observed for the Flu-p24 i.n./i.n. immunized group. Although some mice in the Flu-p24 i.n./i.vag. group displayed low HIV-specific CD8 T-cell responses, the group response was not significantly increased compared to the mock PBS group.

Vaginal tissues were pooled 13 days post-boost (Fig. 4E). HIV-specific CD8 T-cell responses in the vaginal mucosa were elevated for the i.n./i.vag immunized group (2.7% and 6.0% from repeat experiments). I.n./i.n. immunization with PBS, Flu-GFP or Flu-p24, did not induce an HIV-specific CD8 T-cell response in the vaginal mucosa.

3.5. Polyfunctionality of vaccine-induced HIV p24-specific CD8 T-cells

CD8 T-cells expressing multiple cytokines are associated with effective immunity against HIV-1 [18]. We studied an additional group of five mice i.n./i.n. immunized with 10^6 p.f.u. of Flu(PR8)-p24 followed by 10^5 p.f.u. of Flu(X31)-p24. The surrogate degradation marker, CD107a, and cytokines IL2, TNF and IFNγ were measured in A9 peptide-stimulated CD8 T-cells. Flu-p24 immunization produced CD8 T-cells with multiple cytokine responses in BAL and spleen (Fig. 5A and B). Polyfunctionality was assessed in BAL samples (Fig. 5C). The most common functional profile of A9-specific CD8 T-cells expressed either TNFα/IFNγ/CD107a (26.1% of total A9-specific CD8 T-cells) or TNFα/IFNγ (22.5%). We confirmed this data in pooled BAL samples from three additional sets of 5 mice each using Flu(PR8)-p24/Flu(X31)-p24 doses of 10^5/10^6, 10^6/10^7, 10^7/10^8 p.f.u. and found that in each case the TNFα/IFNγ/CD107a* (26.9–38.2%) and TNFα/IFNγ* (25.3–38.5%) expression populations were the two most common populations. In spleen, ~50% of A9-specific CD8 T-cells expressed 2–4 cytokine and degradation markers (Fig. 5D).

4. Discussion

Heterologous protein expression in influenza has been achieved by incorporation of coding sequences into the NA- [9] and HA-segments [19,20]. In these studies, however, expression was confined to short coding sequences for epitopes of 7–12 amino acids. Insertion of larger genes have been achieved using truncated forms of the NS1 gene in the NS-segment, but these recombinant viruses were too severely attenuated to grow in vivo [17,21]. Other segments of the genome, including the PB2- and NA-segments, have been modified for the incorporation of a sizeable sequence encoding the 185 amino acid Gaussia luciferase protein [22,23]. These studies illustrate the versatility of the influenza genome, warranting the investigation of incorporating a whole HIV-1 protein. From the perspective of a vaccine development pipeline, recombinant influenza virus-based vaccines could readily leverage on established manufacturing capabilities utilized for the production of live attenuated influenza virus vaccines [24,25].

The recombinant influenza viruses here were conceptually derived from a construct published previously [11]. The 2A ‘cleavage’ sequences result in the ribosomal skipping between the C-terminus of the 2A peptide and the downstream protein, effectively facilitating multi-cistronic translation [26]. Using this was observed. Subsequent immunogenicity studies in mice were performed within these margins of safety.

3.4. Immunogenicity

To induce mucosal immunity, serial intranasal prime-boost immunizations (i.n./i.n.) or an intranasal prime followed by an intravaginal boost immunization (i.n./i.vag.) were investigated (Fig. 4A). Immunizations were performed with a PR8 (H1N1) prime followed by a heterosubtypic X31 (H3N2) boost, as it was previously shown that the opposite regimen (i.e. H3N2-H1N1) was less effective at inducing secondary CD8 T-cell responses [17]. CD8 T-cell responses to HIV-1 p24 were measured with an A9 tetramer.

Whole blood from groups of immunized mice were pooled 10 days post-boost (Fig. 4B, upper row). Control groups immunized via i.n./i.n. instillation with PBS or with recombinant influenza expressing an irrelevant protein, Flu-GFP, lacked HIV-specific CD8 T-cells as expected. The i.n./i.vag. Flu-p24 regimen induced a moderate HIV-specific CD8 T-cells response (0.4% and 0.2% from repeat experiments). The i.n./i.n. Flu-p24 immunized group displayed the highest frequency of circulating HIV-specific CD8 T-cells (1.6% and 2.5% from repeat experiments). influenza-specific CD8 T-cell responses were measured simultaneously by use of NP147 tetramer staining. The i.n./i.n. Flu-p24 immunization induced 4.0% NP147 Tet+ CD8 T-cells in duplicate experiments, approximately 2.5-fold higher than the HIV-specific CD8 T-cells.

BAL samples were pooled from each group 13 days post-boost (Fig. 4B, bottom row). The HIV-specific CD8 T-cell responses in BAL were substantially elevated in the i.n./i.n. Flu-p24 virus

**Fig. 3.** Weight loss in mice infected with recombinant influenza viruses. (A) Mice infected with 10^4, 10^5, 10^6 p.f.u. of Flu(PR8)-p24 or 50 p.f.u. of wild type PR8. (B) Mice infected with 10^6, 10^7 or 10^8 p.f.u. of Flu(X31)-p24. Groups of five BALB/c mice were administrated viruses by intranasal inoculation. Body weight is displayed as a percentage of day 0 weight. Error bars indicate mean ± SEM. *Culling of mice with body weight of <80%.
Fig. 4. In vivo immunogenicity of recombinant influenza viruses. (A) Immunization regimen of BALB/c mice receiving an intranasal PR8 prime followed by an intranasal or intravaginal X31 boost. (B) HIV-specific (AI9) CD8 T-cell frequencies in blood (day 31) and BAL (day 34) samples pooled from each treatment group; plots shown are from one of two independent experiments. (C) CD8 T-cell counts of BAL samples; data points represent a pooled BAL response from groups of 5 mice. (D) Frequency of HIV-specific (AI9) CD8 T-cells in spleen of individual mice. (E) Frequency of HIV-specific (AI9) CD8 T-cells in vaginal mucosa pooled from each treatment group; plots shown are from one of two independent experiments. HIV-specific (AI9) CD8 T-cells were gated from live singlet cells expressing CD45 and CD3.
In a prototypical design, we did not rescue recombinant virus encoding HIV-1 p24 as a fusion to NS1. We rationalized that dissociation of the p24 protein from NS1 may be required for the rescue of viable recombinant viruses. To achieve this, the PTV 2A sequence was duplicated to allow for separate p24 expression from NS1 (Fig. 1B). We, however, observed deletion of the p24 gene in the NS-segment of the rescued recombinant virus (Fig. 1B and C). Sequencing of this deletion displayed a single PTV 2A sequence linking the NS1 and NEP genes, indicating likely deletion by homologous recombination of the two direct PTV 2A repeat sequences. To avoid deletion of the heterologous gene insert, we engineered a heterologous TAV 2A sequence in subsequent recombinant influenza constructs (Fig. 1D).

Although the 2A sequences promote expression of individual NS1, p24 and NEP proteins from the NS-segment, the fidelity of the 2A ‘cleavage’ mechanism may not be fully efficient. PTV and TAV 2A sequences are highly active amongst the repertoire of 2A sequences, albeit exhibiting relatively low levels of incomplete cleavage activity [26,27]. This could result in accumulation of fusion by-products (e.g. p24-NEP, NS-p24 or NS-p24-NEP), leading to potential loss of function of influenza NS1 or NEP and virus attenuation. Heterologous GFP fused onto NS1 has been shown to attenuate the influenza virus by 100-fold [11]. The attenuation we observed here might also be contributed by the insertion of the sizable HIV-1 p24 gene. Packaging of the NS-segment may be affected due to the increase in size of the NS-segment. In addition, the recombinant NS-segment was rearranged into a single ORF to accommodate the heterologous gene insert, in contrast to overlapping spliced ORFs for the WT NS-segment. NS1:NEP expression ratios are likely to differ between recombinant and WT influenza viruses resulting in differential levels of pathogenicity. Nevertheless, attenuation of viral vaccines is required for safety while maintaining strong immunogenic properties. Indeed, these vectors are immunogenic at 2–3 orders of magnitude below the dose that induces weight loss. The influenza vectors described here in a pre-clinical mouse model fulfill both criteria of safety and ability to stimulate CD8 T-cell responses.

In contrast to epitope-based vaccines, we developed these vectors to express the whole HIV-1 p24 capsid protein. The Gag protein is a suitable candidate for T-cell-based vaccines, as it displays high level sequence conservation amongst HIV-1 isolates, particularly for the p24 sequence [28]. Mutations derived from CD8 T-cell responses against Gag result in reduced viral fitness [29,30]. Furthermore, p24-specific CD8 T-cell responses in HIV-infected subjects correlate with decreased viral load and elevated

![Image](https://example.com/image.png)
CD4 T-cell count [31–33]. We previously assessed immunization of pigtail macaques (Macaca nemestrina) with recombinant influenza viruses encoding various SIV CD8 T-cell epitopes in the NA stalk protein [9]. Although substantial SIV-specific CD8 T-cell responses were induced, this did not reduce levels of viremia when challenged with SIVmac251 owing to the rapid emergence of escape mutants. This study, however, alluded to the potential for broader immunity in controlling escape mutants by simultaneously inducing SIV-specific CD8 T-cell responses against multiple epitopes. With our vectors now expressing whole heterologous HIV-1 protein, we are now poised to re-assess this in a non-human primate model using influenza vectors expressing multiple whole SIV proteins from a cocktail of 2 or more recombinant viruses. We have also expressed other proteins of HIV-1 (e.g. p17 matrix) from recombinant influenza viruses. We hypothesize that the expression of whole heterologous proteins will induce broader responses [10].

To induce high level HIV-specific immunity in the genital mucosa, we evaluated a series of prime-boost strategies via two mucosal routes (i.e. intranasal or intravaginal). Heterosubtypic influenza prime-boost immunization has been demonstrated to be effective in boosting CD8 T-cell responses by circumventing neutralizing antibodies against surface HA or NA epitopes generated after the primary immunization [17,34,35]. We similarly employed this strategy to boost HIV-specific CD8 T-cells, demonstrating increased in vivo immunogenicity. As expected with the use of similar vector backbones in the prime-boost regimen, immunized mice also displayed influenza-specific CD8 T-cells responses. Future experiments detailing both HIV- and influenza-specific responses at various time-points would be of importance in assessing the immuno-dominance of the two responses, which could impact the efficacy of the vaccine. Alternatively, heterologous prime-boost regimens employing dissimilar vectors alongside these recombinant influenza viruses may improve levels of HIV-specific immunogenicity while avoiding competing vector-specific responses.

Mucosal immunization has been shown to induce recirculating T-cell responses between the lung and vaginal mucosa draining lymph nodes [17,19,36–38]. CD8 T-cells induced by mucosal immunization can have increased avidity toward cognate epitopes in contrast to parenteral immunization [39]. We, however, did not detect HIV-specific CD8 T-cells in the distal iliac lymph node, which drains the female reproductive tract, following intranasal immunization (data not shown). In part, this may be reconciled by observations that prime-boost immunizations increase the $T_{EM}/T_{CM}$ ratio, as opposed to single immunization, and anamnestic responses skew the anatomical localization of CD8 T-cells to non-lymphoid tissues as opposed to lymph nodes [40]. We hypothesize that this skewing would be beneficial in inducing a protective HIV-specific mucosal CD8 T-cell response in non-lymphoid tissues such as the vaginal mucosa. We note, however, that an HIV-specific CD8 T-cell response was not detected in the vaginal mucosa of mice that were prime-boost immunized via strictly intranasal routes. In intravaginal HSV-2 infection, increase in IFN$\gamma$ subseqently upregulated VCAM-1 and ICAM-1 addresses in vaginal tissue [41]. VCAM-1 and ICAM-1 interactions with VLA-4 (α4β1) and LFA-1 (αLβ2) integrins, respectively, facilitate recruitment of T-cells into the vaginal mucosa [42]. Although these recruitment factors were not investigated in our studies, it would be conceivable that the intravaginal Flu-p24 booster increased recruitment of HIV-specific CD8 T-cells into the vaginal mucosa by induction of similar addressin-integrin interactions. Intranasal influenza infection increases expression of both VLA-4 and LFA-1 in activated CD8 T-cells [43], suggesting that intranasal Flu-p24 boosting would similarly induce CD8 T-cells capable of homing to the vaginal mucosa. However, the absence of vaginal inflammation and addressin upregulation in intranasally boosted mice would result in negligible vaginal mucosa T-cell recruitment despite the observed increase in systemic HIV-specific CD8 T-cells.

Intravaginal boosting with an influenza virus in mice previously primed by intranasal immunization can induce heightened recruitment of CD8 T-cells into the vaginal mucosa [34,35]. We recapitulated these findings here, demonstrating that HIV-specific CD8 T-cells can be induced to high frequencies in the vaginal mucosa. Intravaginal immunization, however, may not be a practical human vaccine regimen. Recent findings of ‘prime-pull’ regimens demonstrated the ability to establish long-lived tissue resident memory ($T_{RM}$) CD8 T-cells within the vaginal mucosa with mild inflammatory stimulus or chemokines locally administered to the vaginal mucosa post-immunization [44,45]. We are currently investigating a ‘pull’ approach combined with prime-boost intranasal immunization utilizing the Flu-p24 vectors described here. Infection of intraepithelial CD4 T-cells has been implicated as the initial point of entry for HIV-1 infection [46]. We speculate that an HIV-1 vaccine generating immunity by $T_{RM}$ CD8 T-cells can act as a rapid first line of defense against new infections within the vaginal mucosa.

In summary, influenza vectors encoding the HIV-1 p24 capsid are safe and immunogenic in mice. We demonstrate the potential of these recombinant influenza viruses as mucosal HIV-1 vaccines with the capacity to encode whole heterologous HIV-1 p24 capsid protein as opposed to the use of short epitopes.

**Conflicts of interest**

None.

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