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Feline immunodeficiency virus (FIV) vaccine efficacy and FIV neutralizing antibodies:

Efficacy and potential prophylactic mechanism(s) of prototype and commercial FIV vaccines

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Abstract

A HIV-1 tier system has been developed to categorize the various subtype viruses based on their sensitivity to vaccine-induced neutralizing antibodies (NAbs): tier 1 with greatest sensitivity, tier 2 being moderately sensitive, and tier 3 being the least sensitive to NAbs (Mascola et al., *J Virol* 2005; 79:10103-7). Here, we define an FIV tier system using two related FIV dual-subtype (A+D) vaccines: the commercially available inactivated infected-cell vaccine (Fel-O-Vax[®] FIV) and its prototype vaccine solely composed of inactivated whole viruses. Both vaccines afforded combined protection rates of 100% against subtype-A tier-1 FIV_{Pet}, 89% against subtype-B tier-3 FIV_{FC1}, 61% against recombinant subtype-A/B tier-2 FIV_{Bang}, 62% against recombinant subtype-F'/C tier-3 FIV_{NZ1}, and 40% against subtype-A tier-2 FIV_{UK8} in short-duration (37–41 weeks) studies. In long-duration (76–80 weeks) studies, the commercial vaccine afforded a combined protection rate of at least 46% against the tier-2 and tier-3 viruses. Notably, protection rates observed here are far better than recently reported HIV-1 vaccine trials (Sanou et al., *The Open AIDS* 2012; 6:246-60). Prototype vaccine protection against two tier-3 and one tier-2 viruses was more effective than commercial vaccine. Such protection did not correlate with the presence of vaccine-induced NAbs to challenge viruses. This is the first large-scale (228 laboratory cats) study characterizing short- and long-duration efficacies of dual-subtype FIV vaccines against heterologous subtype and recombinant viruses, as well as FIV tiers based on *in vitro* NAb analysis and *in vivo* passive-transfer studies. These studies demonstrate that not all vaccine protection is mediated by vaccine-induced NAbs.

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Keywords

FIV; HIV-1; vaccine; neutralizing antibody tiers; efficacy; passive transfer

1. Introduction

The mechanism(s) of vaccine protection against AIDS lentiviruses such as human (HIV) and feline (FIV) immunodeficiency viruses are still unclear even after completion of four major Phase IIb-III trials on HIV-1 vaccines and after 10 years of commercial FIV vaccine release (Fel-O-Vax[®] FIV, Fort Dodge Animal Health (FDAH), Fort Dodge, IA) in the U.S. [1–6]. The failed Phase-III AIDSVAX vaccine trials tested the HIV-1 recombinant envelope (Env) protein vaccine for generating predominantly anti-HIV-1 antibody-mediated immunity [3], while the failed Phase-IIb STEP trial tested an adenovirus-vectored HIV-1 *gag/pol/nef* for inducing anti-HIV cell-mediated immunity (CMI) [2]. A more recent phase-III RV144 trial, consisting of canarypox virus-vectored HIV-1 *gag/pr/env* priming and AIDSVAX vaccine boosts, induced both CMI and humoral immunity and showed a modest overall vaccine efficacy of 31.2% [4]. However, these human trials did not use inactivated whole virus (IWV) approach due to safety concerns raised over potential incomplete inactivation [1,6]. The IWV approach is currently being used for commercial veterinary vaccines against retroviruses such as, feline leukemia virus, equine infectious anemia virus, and FIV [7–11]. No cases of breakthrough infections caused by incomplete inactivation of the FIV vaccine viruses have been reported for the Fel-O-Vax[®] FIV [11].

FIV causes a fatal acquired immunodeficiency syndrome (AIDS) in domestic cats and is an animal model for human AIDS [5,9]. Like HIV-1 with at least seven subtypes and numerous intersubtype recombinants [12], FIV has at least five subtypes (A–E, Fig. 1) with subtypes A and B being most prevalent globally followed by subtype C [9,13]. Thus, an effective FIV vaccine needs to confer protection against the predominant circulating FIV subtypes (A–C), as well as, the circulating recombinant forms (CRF) of FIV CRF-A/B, CRF-A/C, and CRF-B/C [13–15].

The prototype (dual-subtype IWVs) and Fel-O-Vax[®] FIV (dual-subtype IWVs plus infected cells) vaccines conferred protection against non-vaccine subtype-B viruses [16–18].

However, little is known about the duration, magnitude, and mechanism(s) of the vaccine protection against other subtype and recombinant viruses as well as the virus tiers based on virus neutralizing antibodies (NAbs) as described for HIV-1 [19]. Thus, the current studies assessed the efficacy of these vaccines and their vaccine-induced NAbs against virus strains from subtypes A, B, A/B, and F'/C.

2. Materials and methods

2.1. Animals

Specific pathogen free (SPF) cats were purchased from Liberty Research, Inc. (Waverly, NY), Harlan Sprague Dawley, Inc. (Madison, WI), Cedar River Laboratories (Mason City, IO), or were bred in the Laboratory of Comparative Retrovirology & Immunology at the University of Florida. Based on the Institutional Animal Care and Use Committee (IACUC)

policy to minimize the animal use, Study 8 used the vaccinated/protected cats from Study 3 (Group 3B). Cats challenged with FIV_{FC1} (subtype-B) were 8 weeks of age, while all other cats were 12–16 weeks old. All cats were maintained and utilized according to the policy and protocols approved by IACUC.

2.2. Immunization

The Fel-O-Vax[®] FIV vaccine is composed of 1.5×10^7 – 2.5×10^7 inactivated infected Fet-J cells plus 50 µg of inactivated whole viruses (IWVs), while the prototype FIV vaccine consisted of 500 µg IWVs (FIV_{Pet} plus FIV_{Shi}) at a 50/50 ratio of each strain, supplemented with cytokine(s) [5]. Both vaccines were formulated in the FDAH's (FD-1) adjuvant [5,20]. All prototype IWV vaccines were supplemented with one of the following cytokines or cytokine combinations at 5 µg/dose (Tables 1 and 2): human interleukin-12 (HuIL-12; Genetics Institute, Cambridge, MA), feline IL-12 (IL-12; R&D Systems, Minneapolis, MN), and feline IL-15 (IL-15) produced by our laboratory [21]. Some studies supplemented Fel-O-Vax[®] FIV with IL-12 and IL-15. The SPF cats received subcutaneous (SC) immunizations alone, or in combination with intradermal (ID) immunizations (SC/ID), in 3-week intervals.

2.3. FIV inoculum and challenge

Challenge viruses were classified into tiers of 1, 2, or 3 based on a previously defined HIV-1 tiering system [19]. Moreover, tier-1 FIVs are homologous vaccine strain(s) or those from the same subtypes as vaccine strain(s) that are readily neutralized by vaccine-induced NABs. Tier-2 FIVs are those from subtypes same as vaccine strain(s) but more resistant to vaccine-induced NABs, while tier-3 FIVs are those from subtypes different from vaccine strain(s) and are highly resistant to vaccine-induced NABs. Hence, FIVs from tiers 2 and 3 are the most difficult to protect.

All *in vivo*-derived inoculums were serially transferred from one cat to another, in a total of one-three cats, before final blood samples were collected from cats at peak viremia [17,18]. The *in vivo*-derived FIV_{Bang} (recombinant subtype-A/B) and FIV_{UK8} (subtype-A) consisted of cryopreserved pooled plasma from infected cats, while the *in vivo*-derived FIV_{FC1} (subtype-B) and FIV_{NZ1} (recombinant subtype-F/C) were cryopreserved pooled PBMC from infected cats. The *in vitro*-derived FIV_{Pet} consisted of culture fluid (<16 culture-passages) from the PBMC of infected cats as described [17]. The vaccinated and control cats were challenged intravenously (IV) 3–4 weeks following the final immunization [17,18].

2.4. Monitoring for FIV infection, T-cell status, and NABs

Challenged cats were considered positive for FIV infection when testing positive by immunoblot analysis, virus isolation, proviral PCR, and by a decrease in CD4⁺ T-cell counts and/or CD4⁺/CD8⁺ T-cell ratios [17,18]. Proviral PCR utilized primers targeting conserved FIV *env* sequences under previously described conditions [22]. The sequences were 5'-GAAATGTATAATATTGCTGG as forward primer and 5'-TTACATCCTAATTCTTGCATAG as reverse primer. The approximate amount of proviral DNA per cell was determined via semi-quantitative PCR using varying dilutions from a known number of cells as described [23]. These parameters were performed on PBMC at 3–

4, 6, 9, 12, 16, and 20–24 weeks post-challenge (wpc), or monthly thereafter until 52 wpc. Virus isolation was also performed on tissues at 20–52 wpc. Cats were deemed protected if testing negative for all the above parameters following vaccination and challenge. Since vaccine-induced FIV antibodies were already present, cats were considered antibody positive for infection when the antibodies to the FIV p24 and Env gp95/100 were enhanced, or remained elevated after challenge [17,18]. NAb analysis was performed as described for HIV-1 [24] with modifications. Briefly, heat-inactivated (56°C, 45 min) cat sera using the *in vitro*-derived FIV inoculums (<16 passages) were obtained as previously described [17,18,25] and varying serum dilutions were reacted with appropriate challenge virus for 1 hour at 37° C. These mixtures were then added *in vitro* to naïve feline PBMC taken from SPF cats, and culture supernatant was assessed for virus every 3 days over a 15 day period.

2.5. Passive-transfer studies

One passive-transfer study (Study PT1) was performed with heat-inactivated, pooled unpurified sera from Fel-O-Vax FIV-vaccinated SPF cats or from non-vaccinated/age-matched cats [25]. Each cat received cross-matched compatible serum equivalent to 30% of the recipient's total blood volume. Due to the large volume, cats received a 20% volume in the first IV transfer on day -1, with FIV challenge on day 0, followed by a 10% volume in the second transfer and monitored for infection for 24 weeks. Another two sets of SPF cats (Studies PT2 and PT3) similarly passively transferred with 6.5 mg immunoglobulin per kg body weight of purified pooled antibodies from either vaccinated or PBS-immunized SPF cats in the first transfer and 3.5 mg/kg in the second transfer. Antibodies purified by using caprylic acid purification followed by ammonium sulfate precipitation [26] had an IgG purity of >95% based on gel analysis (data not shown).

2.6. Statistics

Statistically significant differences were determined by the pair-wise Mann-Whitney Rank Sum test (SigmaPlot version 11.0, San Jose, CA), and Bonferroni's correction was also performed in Figure 2. All analyses were considered statistically significant when $p < 0.05$.

3. Results

3.1. Vaccine protection against heterologous-subtype viruses

In the short-duration Studies 1 and 2 against the recombinant subtype A/B FIV_{Bang} isolate (Table 1), statistically significant protection was observed with the prototype dual-subtype IWV (Groups 1A+2A vs. Groups 1B+2B, $p=0.001$) but not with the dual-subtype Fel-O-Vax[®] FIV. Normal CD4⁺ T-cell counts also confirmed this protection in the IWV-vaccinated/protected cats which were statistically different from the infected control cats (Fig. 2A, $p < 0.001$).

In another set of short-duration studies (Table 1, Studies 3 and 4), significant protection was observed with both Fel-O-Vax[®] FIV (Study 3, $p < 0.001$) and prototype IWV ($p=0.037$) against the heterologous (non-vaccine) subtype-B FIV_{FC1}. FIV_{FC1} belongs to heterologous-subtype B, unlike the recombinant FIV_{Bang} which is more closely related to the homologous (vaccine) subtype-A viruses except for the subtype-Benv_{V3-V9} [10]. Hence, the FIV_{FC1}

challenge is considered a more rigorous challenge in comparison to the subtype-A/B FIV_{Bang}. The combined result of Fel-O-Vax[®] FIV and IWV demonstrated high protection against FIV_{FC1} (Groups 3A+3B+4A+4B vs. Groups 3C+4C, $p<0.001$). This result was also supported by normal CD4⁺ T-cell counts and CD4⁺/CD8⁺ T-cell ratios (Fig. 2C and 2D) which were statistically different from the values of the infected control group (CD4⁺ T-cell counts, $p=0.015$; CD4⁺/CD8⁺ ratios, $p=0.001$).

In the study using a recombinant-subtype virus from New Zealand (FIV_{NZ1}) (Table 1, Study 5), both the IWV (6 of 8) and the Fel-O-Vax[®] FIV (2 of 5) conferred some protection, but the protection rate of only IWV group was statistically different from the infected control group ($p=0.008$). FIV_{NZ1} belongs to subtype-F'/C and has no subtype homology to either vaccine viruses (Fig. 1). The CD4⁺ T-cell counts and CD4⁺/CD8⁺ ratios of the vaccinated/protected groups were similar to those of the unchallenged control group but significantly higher than those of the infected control group (Fig. 2E and 2F, $p<0.001$).

3.2. Vaccine efficacy against homologous subtype-A strains

The next study determined whether the variation of vaccine efficacy observed against heterologous-subtype viruses also existed amongst the challenge strains from the same (homologous) subtype as the vaccine strain (FIV_{UK8}; Table 1, Study 6). The IWV afforded a significant combined protection rate of 40% (8 of 20) against challenge with the subtype-A FIV_{UK8}. Increasing the frequencies of vaccination from 3X (6 of 15) to 6X (2 of 5) did not affect the 40% protection rate. The vaccinated/protected cats had normal CD4⁺ T-cell counts and CD4⁺/CD8⁺ ratios which were significantly higher than those of the infected controls (Fig. 2G and 2H, both $p<0.001$). In contrast to the FIV_{UK8} study, the IWV vaccine conferred 100% protection against the *in vitro*-derived homologous-strain FIV_{Pet} (Table 1, Study 7) which showed an efficacy identical to those against *in vivo*-derived FIV_{Pet} [17].

3.3. Long-duration efficacy against heterologous-subtype viruses

A long-duration efficacy study with the Fel-O-Vax[®] FIV was performed with a slightly higher FIV_{FC1} challenge dose than Study 3 to ensure complete infection in all (>1 year old) cats (Table 2). The eight protected cats from Group 3B of Study 3 (after one-year confirmation of FIV-negative status) were given a one-year boost and subsequently challenged with FIV_{FC1} (Table 2, Groups 8A). An additional five cats (3X vaccinated one-year earlier without challenge, Group 8B) received a one-year boost, and were challenged 3 weeks later.

Six of 8 vaccinated/previously-protected cats in Group 8A were protected again at a slightly-higher FIV_{FC1} challenge dose, while all five previously-vaccinated/challenge-naïve Group 8B cats and all five PBS-immunized cats became infected. Four of 6 protected cats belonged to Group 8A1, which were boosted with the Fel-O-Vax[®] FIV/IL-12+IL-15, while the remaining two protected cats from Group 8A2 were boosted with Fel-O-Vax[®] FIV alone. This suggests that the cytokines in the one-year boost enhanced vaccine protection (Group 8A1 vs. Group 8A2+8B, $p=0.046$).

In another long-duration study (Table 2, Study 9), 2 of 4 cats vaccinated 3X with the Fel-O-Vax FIV[®] in the first year (without challenge), followed by a one-year boost, were protected against the *in-vivo*-derived FIV_{Bang} at the same challenge dose and timing as Study 1.

3.4. Vaccine-induced NABs as the mechanism of vaccine protection

The importance of vaccine-induced NABs in the prophylactic protection observed above were next evaluated. Cats vaccinated 3X with IWV or Fel-O-Vax[®] FIV (before challenge) developed strong NAB titers to FIV_{Pet} and FIV_{Shi}, while substantially less to FIV_{Bang} and minimal-to-no titers to FIV_{UK8}, FIV_{FC1}, and FIV_{NZ1} (Table 3). After three vaccinations the Fel-O-Vax[®] FIV (without cytokines) induced NABs in more cats than the IWV, except for the NABs to FIV_{Pet} and FIV_{Shi}.

The vaccine protection against the homologous FIV_{Pet} (Table 1, Study 7) correlated with vaccine-induced NAB titers to FIV_{Pet} (Table 3), while no such correlation was observed against the subtype-A FIV_{UK8} (Tables 1 and 3). Although both vaccines induced minimum-to-no NAB titers to heterologous subtype-F/C FIV_{NZ1} (Table 3), 8 of 13 (62%, combined result) vaccinated cats were protected against FIV_{NZ1} (Table 1, Study 5). Furthermore, both vaccines conferred 75–100% protection against a heterologous-subtype FIV_{FC1}, even though these vaccines induced minimal-to-no anti-FIV_{FC1} NAB titers (Tables 1 and 3). In contrast, varying anti-FIV_{Bang} NAB titers (<10–1000) were induced by vaccination (Table 3, only average titers), although these titers did not correlate with the protection observed against the subtype-A/B FIV_{Bang} (individual data not shown).

The addition of IL-12 and IL-15 to the Fel-O-Vax[®] FIV did not significantly enhance the magnitude or frequency of the NAB titers to heterologous-subtype strains following the initial vaccinations or the annual boost (Table 3). However in long-duration Study 8 (Table 2), the vaccine/cytokines group had a significantly higher protection rate compared to the non-cytokine group, suggesting that these cytokines may enhance vaccine protection memory without enhancing the NAB titer.

3.5. Vaccine-induced NABs to define FIV tiers

Based on the definition of virus tiers modified from those described for HIV-1 [19], both vaccines induced moderate-to-high NAB titers to tier-1 viruses (e.g., vaccine strains and heterologous NAB-sensitive strains) but minimal NAB titers to tier-3 viruses that are most resistant to NABs (e.g., NAB-resistant strains generally different from vaccine subtypes) (Table 3). FIV_{Bang} is placed under tier 2 because the majority of its expressed Env regions belong to subtype B and not to the vaccine subtypes A and D [18], and due to the low anti-FIV_{Bang} NAB titers induced by Fel-O-Vax[®] FIV. Thus, our definition of tier-2 strains are heterologous strains from the same vaccine subtype or heterologous-subtype Env sequence, which are more resistant to Fel-O-Vax[®] FIV than to prototype IWV.

3.6. Passive-transfer studies to determine the mechanism of prophylaxis and the FIV tiers

The role of vaccine-induced antibodies in the protection observed were further evaluated via passive-transfer studies using the antibodies generated from the Fel-O-Vax[®] FIV-vaccinated cats before FIV_{Pet} or FIV_{FC1} challenge (Table 4, Studies PT1, PT2, and PT3). In Study PT1,

the pooled vaccine serum with anti-FIV_{Pet} NAb titer of 2000/mL conferred protection against FIV_{Pet} in all four recipients but not in controls immunized with saline or non-vaccine serum. All recipients of the pooled vaccine serum had NAb titers to FIV_{Pet} ranging from 50–1000 at 1 wpc (Study PT1, only average titers). All recipients of the vaccine serum, except for one, completely lost the anti-FIV_{Pet} NABs by 24 wpc, while two control cats tested (other two untested) developed infection-induced anti-FIV_{Pet} NAb titers.

In Studies PT2 and PT3 (Table 4), passive transfer with purified vaccine-induced antibodies protected 7 of 8 recipients against FIV_{Pet} and 0 of 5 against FIV_{FC1}, while all 12 control cats became infected. In Study PT2, all recipients of vaccine antibodies had anti-FIV_{Pet} NAb titers ranging from 10–1000 at 1 wpc, but only one cat had an anti-FIV_{FC1} NAb titer (50 NAb) at 1 wpc. This study supports the notion that the vaccine induced antibodies, but not other serum factors, were involved in the passive protection against the homologous tier-1 FIV_{Pet}. In Study PT3, the five recipients of the purified vaccine-induced antibodies had anti-FIV_{Pet} NAb titers ranging from 50–1000 at 1 wpc, while only two cats had anti-FIV_{FC1} NAb titers of 10–50 at 1 wpc (Table 4, only average titers). Except for one, all unprotected cats as well as infected control cats had undetectable anti-FIV_{FC1} NAb titers at 14 and 24 wpc. Thus, these results suggest that the homologous FIV_{Pet} is a tier-1 virus, while heterologous-subtype FIV_{FC1} is a tier-3 virus.

4. Discussion

The dual-subtype FIV vaccines conferred a statistically significant cross-subtype protection rate of 89% against a subtype-B virus, which was better than those against recombinant subtype-A/B and -F'/C viruses. Other FIV researchers have also described the efficacy of the Fel-O-Vax[®] FIV vaccine, which ranged from high efficacies against the subtype-B Japanese FIV_{AO2} [16], subtype-A FIV_{FD/US} [27], and subtype-A FIV_{FD/DutchA} [28] viruses, to no sterilizing efficacy against the subtype-A FIV_{UK8} virus [29,30]. Based also on the work of Dunham et al. [29,30], the prototype IWV with the IL-12 supplement was generally more effective against heterologous isolates than the Fel-O-Vax[®] FIV. Collectively, these studies suggest the prototype IWV is better than Fel-O-Vax[®] FIV. This may be due to the fact that the prototype IWV consists of only inactivated whole virus, while Fel-O-Vax[®] FIV consists of inactivated infected cells [5].

The addition of IL-12 and IL-15 to the vaccines was meant to enhance CMI such as T-helper 1 (TH1) activities with IL-12 [31–34] and T-cell memory with IL-15 [35,36]. The addition of cytokines to the Fel-O-Vax[®] FIV had no effect on short-duration vaccine efficacy since both groups conferred 100% protection (Table 1, Group 3B vs. Group 4B). More importantly, the addition of FeIL-12+FeIL-15 to the Fel-O-Vax[®] FIV retained the long-duration protective efficacy more than those boosted with the vaccine alone ($p=0.046$, Table 2). The addition of such cytokines to enhance vaccine immunity has been reported in rodent models with HIV vaccines [37,38], in macaque models with SIV vaccines [39,40], and in cat model with HIV-1 p24 vaccine for cross-protection [21].

A major objective of our studies was to determine whether the vaccine-induced NABs alone mediate the observed vaccine protection. As expected, the vaccine-induced NABs had the

highest neutralizing activities against the vaccine viruses, considerably less against the recombinant tier-2 FIV_{Bang}, and minimal-to-none against tier-2 FIV_{UK8} and tier-3 strains (FIV_{FC1} and FIV_{NZ1}). This lack of a clear correlation between vaccine protection and vaccine-induced NAb against tier-2 and -3 viruses suggests that CMI may be essential for vaccine-induced immunity. In support of this, the prototype IWV has been reported to also induce strong anti-FIV CMI [17,20].

Our results demonstrate that the Fel-O-Vax[®] FIV induces high frequencies of NAb responders against a broad spectrum of FIV isolates (Table 3). A recent study showed that immunization with FIV_{Pet} Env-expressing autologous T cells conferred protection against a homologous challenge and correlated with immunization-induced NAb [41]. In line with this, both active and passive immunizations in our studies conferred protection against homologous tier-1 FIV_{Pet}. Our results also suggest that the Fel-O-Vax[®] FIV can induce sufficient levels of FIV-specific CMI, based on the lack of correlation between high vaccine protection rate (Table 1) and vaccine-induced NAb to tier-3 FIV_{FC1} (Table 3), and the lack of passive protection with vaccine antibodies against FIV_{FC1} (Table 4). A similar resistance to vaccine-induced NAb was also observed with the tier-2 FIV_{UK8} and the tier-3 FIV_{NZ1} (Table 3). Hence these results suggest that the vaccine-induced CMI is critical towards the protection against the heterologous-subtype tier-3 viruses, as well as, to the homologous subtype-A tier-2 FIV_{UK8}.

In the current studies, the dual-subtype vaccine approach had a broad efficacy when tested against global isolates from the U.S. (California, Massachusetts, and Florida), the United Kingdom, and New Zealand using challenge doses substantially higher than those of natural transmission [42]. These studies also used *in vivo*-derived challenge viruses from tiers 2 and 3, which are considered to be more difficult to protect by vaccination due to their resistance to NAb [19], and due to the high virus quasi-speciation of such inoculums [43–45]. As circulating FIV and HIV-1 subtype recombinants are becoming increasingly globally prevalent [13–15,46–48], our findings provide insights into the nature of the challenge inocula needed for second-generation FIV and first-generation HIV-1 vaccines, the levels of prophylactic efficacy needed against global isolates, and the immune mechanisms of protection.

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Highlights

- Prototype (IWV) & commercial (Fel-O-Vax[®] FIV) FIV vaccines against high-dose FIVs.
- IWV and Fel-O-Vax[®] FIV conferred 40–100% protection rates against all FIV tiers.
- These protection rates were much better than reported Phase IIb-III HIV-1 trials.
- Protection against NAb-based tiers -2 and -3 viruses require cellular immunity.
- IWV vaccine was more effective than Fel-O-Vax[®] FIV against tier-2 & -3 viruses.

Figure 1. FIV *gag* phylogenetic distribution of the vaccine and challenge viruses

The subtype designations of the inoculum and vaccine viruses (subtype-A FIV_{Pet} and subtype-D FIV_{Shi}) were previously determined by proviral sequence and phylogenetic comparisons [49,50] of the FIV *env* [10], *gag* (Fig. 1), and *pol* (data not shown). As shown for the first time, FIV_{NZ1} is a recombinant that belongs to a new subtype F' at Gag (Gag-p24 shown; GenBank accession: GQ406242) and Pol (data not shown; GenBank accession: GQ996603), while its envelope (GenBank accession: GQ406243) has previously been described to belong to subtype C [10,15]. The full sequence analysis demonstrates FIV_{Bang} to belong to subtype A (Gag-p24 shown for the first time) except for the envelope V4-V9 which is subtype B [18]. FIV Gag-p24 phylogeny is based on 58 sequences derived from GenBank FIV strains with accession number: Petaluma (M25381), Bangston (AY684181), TM2 (E03581), FC1 (DQ365596), UK8 (GU055218), BM3070 (AF474246), Shizuoka

(AY679785), NZ1 (GQ406242), *Yokohama* (D37819), *Aomori1* (D37823), *Fukuoka* (D37822), MD (AF361320), C36 (AY600517), PPR (M36968), *Sendai1* (D37820), *Sendai2* (D37821), SwissZ1 (X57002), Wo (L06311), *ATESb20* (AF531049), *ATESd03* (AF531050), *ATNOc07* (AY196330), *ATSTb30* (AF531054), *ATSTc01* (AF531058), *ATVIa85* (AF531061), *ATVIa90* (AF531059), *ATVib31* (AF531063), *ATVib97* (AF531055), *ATVId02* (AF531075), *CHSHa10* (AF31069), *DEBAb91* (AF531069), *DEBA d58* (AF531070), *DEBE d72* (AF531051), *DEBWa06* (AF531048), *DEF Rd68* (AF531071), *DEW d60* (AF531072), Dutch113 (X68019), USIL24897B (U11820), *ITRO d76* (AF531052), *ITRO d78* (AF531053), *M2* (Y13867), *M3* (Y13866), NCSU1 (I64733), TN1 (DQ365589), TN2 (DQ365590), TN3 (DQ365591), TN4 (DQ365592), TN6 (GQ422126), TN7 (GQ422127), TN8 (DQ365595), *TX77* (AY139106), *TX78* (AY139107), *TX84* (AY139108), *TX120* (AY139105), *TX132* (AY139112), *TX200* (AY139110), *LP3* (AB027302), *LP20* (AB027303), *LP24* (AB027304), *Aomori2* (D37824)]. Twenty-three sequences are full length (224 aa), and the remaining 35 sequences in italics are partial sequences ranging from 190–221 aa, with exception of the three subtype-C viruses (LP3, LP20, LP24), which have only 113 aa.

Figure 2. The effect of vaccination on the CD4⁺ T-cell counts and the CD4⁺/CD8⁺ T-cell ratios after homologous and heterologous challenges

Results from Study 1 and Studies 3–6 cats evaluated for CD4⁺ T-cell counts and CD4⁺/CD8⁺ T-cell ratios are shown under the corresponding challenge FIV strain: subtype-A/B FIV_{Bang} (A, B), subtype-B FIV_{FC1} (C, D), subtype-A FIV_{NZ1} (E, F), and subtype-F'/C FIV_{UK8} (G, H). CD4⁺ T-cell counts at 1000/μL are the y-axes for leftmost subfigures A, C, E, and G, and CD4⁺/CD8⁺ T-cell ratios are the y-axes for rightmost subfigures B, D, F, and H. The designated symbols represent the cats immunized with Fel-O-Vax[®] FIV (●), Fel-O-Vax[®] FIV plus prototype IWV (□), prototype IWV (3X or 6X) (▲), FeT-J (◆), adjuvant (FD-1) control (◇), PBS control (×), and unchallenged/non-vaccinated age-matched control cats (⋈). The y-axis for FIV_{FC1} challenge (C, D) is set higher than the others due to the higher CD4⁺ T-cell counts caused by the use of younger cats. Note that some of the sera were pre-absorbed with uninfected FeT-J (vaccine cells) before NAb testing, and were tested side-by-side with unabsorbed sera due to the potential augmentation of NAb titers with anti-cellular antibodies [51]. No difference in NAb titers were observed between the FeT-J absorbed and unabsorbed sera.

Table 1

Short-duration efficacies against homologous and heterologous subtype challenges.

Study-Group	Vaccine Immunogens ^a	Cytokine Supplement ^a	Imm Freq ^b	Challenge Strain ^c (Subtype, Tier)	# Protected ^d # Total (%)	p value ^e
HETEROLOGOUS CHALLENGE STUDIES						
1A	IWV ^f	HuIL-12	3X	Bang (A/B, 2) [25]	10/14 (71%)	0.004
1B	Adjuvant (n=8) ^f & PBS (n=2)	HuIL-12	3X	Bang (A/B, 2) [25]	0/10 (0%)	
2A	Fel-O-Vax	None	3X	Bang (A/B, 2) [25]	1/4 (25%)	--
2B	Adjuvant (n=2) ^f & PBS (n=2)	None	3X	Bang (A/B, 2) [25]	0/4 (0%)	
^e Combined result of Vaccinated Groups 1A + 2A:						
					11/18 (61%)	0.001
^c Combine result of Control Groups 1B + 2B:						
					0/14 (0%)	
3A	IWV ^f	HuIL-12	3X	FC1 (B, 3) [15]	3/4 (75%)	0.037
3B	Fel-O-Vax	None	3X	FC1 (B, 3) [15]	8/8 (100%)	<0.001
3C	FeT-J (n=2) ^{fg} & PBS (n=8)	None	3X	FC1 (B, 3) [15]	0/10 (0%)	
4A	Fel-O-Vax + IWV ^f	IL-12+IL-15	3X	FC1 (B, 3) [15]	3/4 (75%)	--
4B	Fel-O-Vax	IL-12+IL-15	3X	FC1 (B, 3) [15]	3/3 (100%)	--
4C	Adjuvant (n=2) ^f & PBS (n=2)	IL-12+IL-15 ^h & None	3X	FC1 (B, 3) [15]	0/4 (0%)	
^e Combined result of Vaccinated Groups 3A + 3B + 4A + 4B:						
					17/19 (89%)	<0.001
^e Combined result of only Fel-O-Vax-Vaccinated Groups 3B + 4B:						
					11/11 (100%)	<0.001
^c Combine result of Control Groups 3C + 4C:						
					0/14 (0%)	
5A	Fel-O-Vax	None	3X	NZ1 (F'/C, 3) [50]	2/5 (40%)	--
5B	IWV ^f	IL-12	3X	NZ1 (F'/C, 3) [50]	6/8 (75%)	0.008
5C	PBS	None	3X	NZ1 (F'/C, 3) [50]	0/10 (0%)	
^e Combined result of Vaccinated Groups 5A + 5B:						
					8/13 (62%)	0.014
^c Combine result of Control Group 5C:						
					0/10 (0%)	
HOMOLOGOUS SUBTYPE-A CHALLENGE STUDIES						
6A	IWV ^f	HuIL-12	3X	UK8 (A, 2) [10]	6/15 (40%)	0.046
	IWV ^f	HuIL-12	6X	UK8 (A, 2) [10]	2/5 (40%)	--
6B	FeT-J ^{fg}	None	3X-6X	UK8 (A, 2) [10]	0/7 (0%)	

Study-Group	Vaccine	Immunogens ^a	Cytokine Supplement ^a	Imm Freq ^b	Challenge Strain ^c (Subtype, Tier)	# Protected ^d		<i>p</i> value ^e
						# Total (%)	# Total (%)	
	PBS	HuIL-12	None	3X-6X	UK8 (A, 2) [10]	0/3 (0%)		
	PBS			3X-6X	UK8 (A, 2) [10]	0/10 (0%)		
7A	IWV ^f	HuIL-12	None	<i>e</i> Combined result of Vaccinated Group 6A:		8/20 (40%)		0.030
				Combine result of Control Group 6B:		0/20 (0%)		
7B	PBS	None	None	3X	Pet (A, 1) [25]	6/6 (100%)		0.004
				3X	Pet (A, 1) [25]	0/6 (0%)		
				<i>e</i> Combined result of only IWV-Vaccinated Groups from Studies 1-7:		31/47 (66%)		<0.001
				Combine result of Control Groups from Studies 1-7:		0/51 (0%)		
				<i>e</i> Combined result of only Fel-O-Vax-Vaccinated Groups from Studies 1-7:		14/20 (70%)		<0.001
				Combine result of Control Groups from Studies 1-7:		0/28 (0%)		

^a Some of the inactivated dual-subtype whole-FIV viruses (IWV) or the commercial Fel-O-Vax[®] FIV vaccine (Fel-O-Vax) were supplemented with cytokine(s): human IL-12 (HuIL-12), feline IL-12 (IL-12), or feline IL-12 plus feline IL-15 (IL-12+IL-15).

^b Immunization frequencies (Imm Freq).

^c Subtype-A/B FIVBang, subtype-B FIVFC1, subtype-F/C FIVNZ1, subtype-A FIVUK8, or subtype-A FIVPet were used as challenge virus at the designated mean cat infectious dose [CID50].

^d Protected cats were negative for FIV infection based on the criteria set in Methods 2.4.

^e Statistically significant difference between individually vaccinated group and its control group (last group in each study) and between the combined results of the vaccinated groups and that of the control groups is also shown with *p*-value in italics when *p*<0.05 and with (–) when not significant.

^f The adjuvant used was FD-1 adjuvant, which is the adjuvant for Fel-O-Vax[®] FIV.

^g Uninfected FeT-J cells (FeT-J) (the cells used to produce the Fel-O-Vax[®] FIV and prototype IWV vaccines) were immunized at a total cell number (2×10⁷ cells) similar to the cell number found in the Fel-O-Vax[®] FIV.

^h Only adjuvant, but not PBS, was supplemented these cytokines.

Table 2

Long-duration efficacies against heterologous-subtype challenges.

Study-Group	Vaccine	Immunogens ^a	Cytokine Supplement ^a	Imm Freq ^b	Challenge Strain ^c (Subtype, Tier)	# Protected # Total (%) ^d	p value ^e
8A1	Fel-O-Vax ^f	IL-12+IL-15 ^f		3X+1X ^g	FC1 (B, 3) [25] ^h	4/4 (100%)	0.02
8A2	Fel-O-Vax ^f	None		3X+1X ^g	FC1 (B, 3) [25] ^h	2/4 (50%)	--
8B	Fel-O-Vax	None		3X+1X ^g	FC1 (B, 3) [25] ^h	0/5 ⁱ (0%)	--
8C	PBS	None		3X+1X ^g	FC1 (B, 3) [25] ^h	0/5 (0%)	--
^e Combined result of Vaccinated Groups 8A1 + 8A2 + 8B:							6/13 (46%)
^e Combined result of Vaccinated Groups 8A1 + 8A2:							6/8 (75%)
^e Combined result of Control Groups 8C:							0/5 (0%)
^j Vaccinated Group 8A1:							4/4 (100%)
^e Combined result of vaccinated Groups 8A2 + 8B:							2/9 (22%)
9A	Fel-O-Vax FIV	None		3X+1X ^g	Bang (A/B, 2) [25] ^h	2/4 (50%)	--
9B	PBS	None		3X+1X ^g	Bang (A/B, 2) [25] ^h	0/3 (0%)	--
^e Combined result of Vaccinated Groups 8A1 + 8A2 + 8B + 9A:							8/17 (47%)
^e Combined result of Control Groups 8C+ 9B:							0/8 (0%)

^{a-e} Same as corresponding footnotes a-e from Table 1 except that only Fel-O-Vax[®] FIV (Fel-O-Vax) was tested against FIV_{FC1} or FIV_{Bang}.

^f Eight vaccinated/protected cats from Group 3B of Study 3 were transferred to Study 8 as Group 8A1 and 8A2. They received a one-year boost, followed by a second FIV_{FC1} challenge 3 weeks later. Group 8A1 cats were boosted with the Fel-O-Vax[®] FIV supplemented with FeIL-12+FeIL-15 (IL-12+IL-15), while Group 8A2 cats were boosted with Fel-O-Vax[®] FIV without cytokine.

^g Three SC immunizations in year-1, followed by one SC boost in year-2 (3X+1X).

^h First (Groups 8B, 8C, 9A, and 9B) challenge at 3 or second (Groups 8A1 and 8A2) FIV_{FC1} week after the one-year boost with a vaccine or PBS.

ⁱ Two of 5 cats showed an 8-week delay in virus detection.

^j Comparison between the result from vaccinated Group 8A1 with one-year vaccine boost containing IL-12+IL-15 and the combined results from vaccinated Groups 8A2 and 8B with one-year boost without IL-12+IL-15.

Table 3

NAb titers of the IWV or Fel-O-Vax[®] FIV vaccination compared to the protection rates.

Vaccine Immunogens ^a	Cytokine Supplement ^a	Imm Freq ^b	# of Animals ^c	Pet/A	Average NAb titer (% Responder) ^d					
					Shi/D	UK8/A	FCI/B	Bang/A/B	NZ1/F/C	
IWV	HuIL-12	3X	112-23	118 (83)	96 (78)	10 (12)	10 (13)	73 (61)	0	
IWV	HuIL-12	6X	4	1000 (100)	78 (100)	10 (25)	10 (50)	400 (75)	ND	
Fel-O-Vax	none	3X	15-27	216 (63)	60 (74)	10 (30)	10 (30)	22 (70)	10 (20)	
Fel-O-Vax	IL-12+IL-15	3X	3	10 (67)	55 (67)	ND	10 (33)	40 (100)	ND	
Fel-O-Vax	none	3X+1X	9	25 (67)	370 (33)	ND	0	33 (100)	ND	
Fel-O-Vax	IL-12+IL-15	3X+1X	4	700 (75)	55 (50)	0	0	70 (75)	0	
NAb-based FIV Tier ^e :				1 ^f	1	2	3 ^f	2	3	
Protection Rate of IWV:				100%	ND	40%	75%	71%	75%	
Protection Rate of Fel-O-Vax:				ND	ND	ND	100%	25%	40%	
Combined Protection Rate:				100% ^g	ND	40% ^g	89%	61%	62%	

^a Same as footnote a from Table 1.

^b Pre-challenge sera from cats with immunization frequency (Imm Freq) of 3X, 6X, or 3X+1X (3X in year-1 with a boost in year-2).

^c Total number of cats analyzed for vaccine groups with IWV/HuIL-12 or Fel-O-Vax[®] FIV alone varies between 12-23 or 15-27 animals respectively, depending on which virus is used for the NAb assay. Cat sera from the current studies and previous studies [9,17] were analyzed. The nine cats immunized 3X with Fel-O-Vax[®] FIV in year-1 with a Fel-O-Vax[®] FIV boost in year-2 (3X+1X) were from Groups 8A2 and 8B of Study 8 before challenge. The four cats immunized 3X with Fel-O-Vax[®] FIV in year-1 with a Fel-O-Vax[®] FIV/IL-12+IL-15 boost in year-2 were from Group 8A1 of Study 8 before challenge.

^d The strain and subtype of the FIV used in the NAb assays are FIV_{Pet} (Pet/A), FIV_{Shi} (Shi/D), FIV_{UK8} (UK8/A), FIV_{FC1} (FC1/B), FIV_{Bang} (Bang/A/B), and FIV_{NZ1} (NZ1/F/C). Dilution titers of 10 are considered positive. Results are shown as the average NAb to the designated strain, and the percentage of the number of cats responding (% Responder). All FIV isolates designated as ND are not analyzed. The groups with no NAb responses are shown with a "0".

^e FIV tiers are based on vaccine-induced NAb titers from three bolded rows and in combination with the subtype origin of the Env sequence, similar to those described for Nab-based tiers against HIV-1 [19].

^f Virus tier for FIV_{Pet} and FIV_{FC1} also confirmed by passive-transfer studies with vaccine-induced antibodies in cats (Table 4).

^g Based only on IWV protection rate.

Table 4

Passive transfer (PT) studies against homologous subtype-A FIV_{Pet} or heterologous subtype-B FIV_{FC1}.

Study-Group	Vaccine and Antibody Sources <i>a</i>	Challenge Strain [CID50] <i>b</i>	Average Anti-Pet NAb (# of Responder) ^c				Average Anti-FC1 NAb (# of Responder) ^c				# Protected # Total (%) ^d	<i>p</i> -value ^e
			-3 wpc	1 wpc	14 wpc	24 wpc	-3 wpc	1 wpc	14 wpc	24 wpc		
PT1 A	Fel-O-Vax® FIV vaccinated cat serum	Pet [25]	0	513 (4)	ND	500 (1)	0	0	ND	0	4/4 (100%)	0.034
PT1 B	non-vaccinated cat serum, saline	Pet [25]	0	0	1000 (2) ^f	1000 (2) ^f	0	0	0	0	0/4 (0%)	
PT2 A	Fel-O-Vax® FIV vaccinated cat Ab	Pet [25]	0	639 (4)	0	1000 (1)	0	50 (1)	0	0	7/8 (87%)	0.004
PT2 B	Fet-J vaccinated Ab, non-vaccinated cat Ab, saline	Pet [25]	0	0	670 (6)	1000 (7)	0	0	0	0	0/7 (0%)	
<i>e</i> Combined result of groups PT1A+PT2A: 11/12 (92%)												
PT3 A	Fel-O-Vax® FIV vaccinated cat Ab	FC1 [25]	0	630 (5)	0	0	0	30 (2)	0	1000 (1)	0/11 (0%)	--
<i>e</i> Combined result of groups PT1B+PT2B: 0/5 (0%)												
PT3 B	Fet-J vaccinated cat Ab, non-vaccinated cat Ab, saline	FC1 [25]	0	0	0	0	0	0	0	0	0/5 (0%)	
<i>e</i> Combined result of groups PT1A+PT2A: 11/12 (92%)												
<i>e</i> Combined result of group PT3A: 0/5 (0%)												

^aThe cats from passive-transfer studies (Studies P-T1-P-T3) were IV transfused with saline, pooled serum (stock at 2000 NAb titers to FIV_{Pet} and 10 to FIV_{FC1}) or purified antibodies (Ab) (stock at 5000 NAb titer to FIV_{Pet} and 50 to FIV_{FC1}). The latter two were derived from either Fel-O-Vax-vaccinated cats, Fel-J-immunized cats, or non-vaccinated cats.

^bMean cat infectious dose [CID50].

^cbefore challenge (-3 weeks post Shows the average serum NAb titers to FIV_{Pet} and FIV_{FC1} challenge, wpc) and after challenge (1, 14, and 24 wpc) of each group and the number of the NAb responders (# of Responder). The average NAb titers of 30–639 at 1 wpc are due to the antibodies from passive transfer, while titers at 14 and 24 wpc are most likely due to infection except for one cat in PT1 group A. This cat had the highest NAb titer (>1000) from passive transfer at 1 wpc which was completely lost by 30 wpc. The groups with no NAb responses are shown with a “0”.

^dProtected cats were negative for FIV infection based on the criteria set in Methods 2.4.

^eStatistically significant differences between individual vaccine-antibody group and its control group are shown with *p*-values, while those without significance are shown as (--). Statistical comparison between the combined results is presented in italics.

^fOnly 2 of 4 control cats were tested for NAb to FIV_{Pet}.

^gAdditional comparison is made between vaccine-antibody group with FIV_{Pet} challenge and vaccine-antibody group with FIV_{FC1} challenge.