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An initial examination of the potential role of T-cell immunity in protection against feline immunodeficiency virus (FIV) infection

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Abstract

The importance of vaccine-induced T-cell immunity in conferring protection with prototype and commercial FIV vaccines is still unclear. Current studies performed adoptive transfer of T cells from prototype FIV-vaccinated cats to partial-to-complete feline leukocyte antigen (FLA)-matched cats a day before either homologous FIV_{Pet} or heterologous-subtype pathogenic FIV_{FC1} challenge. Adoptive-transfer (A–T) conferred a protection rate of 87% (13 of 15, $p < 0.001$) against FIV_{Pet} using FLA-matched T cells, whereas all 12 control cats were unprotected. Furthermore, A–T conferred protection rate of 50% (6 of 12, $p < 0.023$) against FIV_{FC1} using FLA-matched T cells, whereas all 8 control cats were unprotected. Transfer of FLA-matched T and B cells demonstrated that T cells are needed to confer A–T protection. In addition, complete FLA-matching and addition of T-cell numbers $> 13 \times 10^6$ cells were required for A–T protection against FIV_{FC1} strain, reported to be a highly pathogenic virus resistant to vaccine-induced neutralizing-antibodies. The addition of FLA-matched B cells alone was not protective. The poor quality of the anti-FIV T-cell immunity induced by the vaccine likely contributed to the lack of protection in an FLA-matched recipient against FIV_{FC1}. The quality of the immune response was determined by the presence of high mRNA levels of cytolytic (perforin) and cytotoxic (granzymes A, B, and H) and T helper-1 cytokines (interferon- γ [IFN γ] and IL2). Increased cytokine, cytolytic and cytotoxic production was detected in the donors which conferred protection in A–T studies. In addition, the CD4⁺ and CD8⁺ T-cell proliferation and/or IFN γ responses to FIV p24 and reverse transcriptase increased with each year in cats receiving 1X–3X vaccine boosts over 4 years. These studies demonstrate that anti-FIV T-cell immunity induced by vaccination with a dual-subtype FIV vaccine is essential for prophylactic protection against AIDS lentiviruses such as FIV and potentially HIV-1.

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Keywords

Feline immunodeficiency virus; vaccine; T cell immunity; adoptive transfer

1. Introduction

The development of an effective HIV-1 vaccine for prophylaxis and immunotherapy is critically needed in light of the inability of the antiretroviral therapy alone to cure HIV-1 infection [1–6]. After the failure of the STEP trial [7] which was designed to target cell-mediated immunity, the major emphasis in phase-II to phase-III clinical HIV-1 vaccine trials focused on vaccines formulated to induce antibody immunity against HIV-1, and in particular HIV-1 neutralizing antibodies (NAbs) (IAVI Report, clinical trials database, <http://www.iavireport.org/Trials-Database/Pages/default.aspx>, accessed 05/13/2015). Even though the focus has moved away from the generation of T-cell immunity, many of the known HIV NAbs consists of the IgG isotype. Therefore, the importance of T-cell immunity cannot be ignored since the production of IgG antibodies generally requires CD4⁺ T-cell helper-2 (TH2) activity via production of the appropriate cytokines [8]. Only token efforts in developing a T-cell based HIV-1 vaccine have been made without major success (reviewed in [9]). However, numerous findings evaluating HIV-1 infected subjects indicated the importance of T-cell immunity, especially CD8⁺ T-cell immunity, in the control of HIV-1 infection [10–12]. Similar findings were also demonstrated in the simian immunodeficiency virus (SIV)/macaque model where CD8⁺ T cells were important in the control of SIV load in SIV-infected macaques [13–15]. More recently, a vaccine consisting of a SIV *gag/pol/env* construct in a CMV vector conferred 50% protection with homologous SIV challenge in rhesus macaques [16,17]. The mechanism of such protection was reported to be mediated by an unconventional major histocompatibility complex (MHC)-II restricted CD8⁺ T-cell immunity rather than anti-SIV NAb immunity [17]. Furthermore, the importance of antiviral T-cell immunity against feline immunodeficiency virus (FIV), the feline counterpart of HIV, has been demonstrated [18–20].

In the FIV/cat model of HIV/AIDS, the prototype (inactivated whole virus [IWV]) and the commercial (inactivated whole cell lysate) dual-subtype FIV vaccines, composed of subtypes A and D, conferred protection against the heterologous subtype-B FIV_{FC1} isolate [21]. Furthermore, this FIV isolate was resistant to vaccine-induced FIV NAbs based on *in vitro* testing and an *in vivo* passive-transfer study using vaccine-induced purified antibodies. Hence, the most likely mechanism of such protection was reported to be the vaccine-induced cellular immunity such as T-cell immunity [18–21]. This observation was also supported by an earlier study which determined high levels of T-cell immunity generated by cats vaccinated with the prototype FIV vaccine [19]. In addition, complete protection against FIV challenge was observed in 36% (4 of 11) of recipients of adoptive transfer with Ab-free peripheral blood mononuclear cells (PBMC) from vaccinated parental donors prior to homologous FIV challenge [18]. Since no vaccine antibodies were transferred, such protection was thought to be mediated by cellular immunity such as antiviral T-cell immunity [19].

Current studies have been undertaken to decisively determine if feline leukocyte antigen (FLA)-restricted T-cell immunity induced by the prototype FIV vaccine is indeed conferring protection against a challenge with vaccine-induced NAb-resistant, pathogenic FIV_{FC1}. The following studies utilized adoptive transfer of T-cell preparations from vaccinated cats to FLA-matched and unmatched naïve cats a day before challenge. The A-T approach is based on a well-established concept that T cells are presented with viral peptides by MHC-restricted antigen presenting cells and/or MHC-restricted virus-infected cells [22]. Thus, the protection conferred between vaccinated donors and MHC-matched A-T T-cell recipients further confirms that the vaccine immunity is mediated by anti-FIV T-cell immunity.

2. Materials and methods

2.1. MHC-matched animals and adoptive-transfer (A–T) studies

In order to develop MHC-matched laboratory cats, three lines of semi-inbred cats were developed over 15 years (described in [20,23]). Each donor-recipient pair in the A-T study was first matched by mixed leukocyte reaction (MLR) [18] from semi-inbred cats of the same colony. Donors were vaccinated subcutaneously (400 µg) and intradermally (100 µg) with the prototype dual-subtype FIV vaccine 4X in the first year and 1X-3X per year thereafter. For example, a 2-year vaccinated donor refers to any cat that received the prototype dual-subtype FIV vaccine 4X in the first year and 1X-3X in second year, placing the total number of vaccinations at 5X-7X for a 2-year vaccinated donor. The prototype FIV vaccine consists of 250 µg each of inactivated whole viruses (IWV) of subtype-A FIV_{Pet} and subtype-D FIV_{Shi} in FD-1 adjuvant (kindly provided by Fort Dodge Animal Health, Fort Dodge, IO) supplemented with 5 µg of recombinant feline IL2 (FD-1 adjuvant/FeIL12) (R&D Systems, Minneapolis, MN).

The control group in these studies was represented by any cat that did not receive T cells from vaccinated donors, and thus consisted of T-cell or PBMC transfer from non-vaccinated cats, B-cell transfer from vaccinated cats, and/or only PBS. Controls in previous studies directly immunized with uninfected vaccine cell line (e.g., FeT-J cell lysate as non-specific antigen) in adjuvant alone and adjuvant/HuIL12 afforded no protection [21]. Therefore, the addition of a control group consisting of recipients of A-T of T cells from donors vaccinated with non-specific antigen, such as uninfected vaccine cell antigen devoid of FIV antigen, was not included.

In Studies 3, 4 and 5, the protected recipient cats from previous A-T study, Study 2, were vaccinated and used as A-T donors. A-T studies were performed with small group sizes in order to perform the blood collection from the donors, analysis of the donor cells, T-cell purifications, and adoptive transfers in a single day. A total of five AT studies using T-cell preparations were performed. The recipients of A-T were challenged with homologous (i.e., vaccine strain) FIV_{Pet} in the first three studies and heterologous-subtype FIV_{FC1} in the last two studies. The T-cell preparation was administered intravenously (IV) 24 hours prior to IV challenge with 25× median 50% cat infectious doses (CID₅₀) of either *in vitro*-derived FIV_{Pet} or *in vivo*-derived pathogenic FIV_{FC1} as previously described [21]. As a final confirmation of FLA-matching, both the protected and unprotected recipients and their corresponding donors were further evaluated for FLA class-I and -II matching by an FLA-

specific PCR method. All procedures on cat studies including inbreeding and maintenance were completed in accordance with the University of Florida IACUC.

2.2. Purification of T-cell enriched population

The T-cell enriched population was produced by sorting PBMC using the DynaMag™-15 magnetic separation system (Life Technologies, Oslo, Norway). Briefly, ficoll-gradient purified PBMC from vaccinated A-T donors [18] were treated with rabbit polyclonal Abs to feline IgG and IgM (Bethyl Laboratories, Montgomery, TX), and subsequently mixed with magnetic beads conjugated with goat anti-rabbit IgG (Life Technologies) and then passed twice through a magnetic field to deplete B cells. The resulting flow through was used as the T-cell enriched population. The B-cell population for A-T Study 1 consisted of the positively selected B cells eluted from the magnetic beads. To obtain the CD8⁺ T-cell enriched population (CD8⁺ T-cell population) the T-cell enriched population was incubated with a combination of murine IgG MAbs to feline CD4 (hybridoma clone kindly provided by Dr. Nazareth Gengozian, described in [24]) and to canine CD21 for further B-cell depletion (AbD Serotec, Raleigh, NC; cross-reactivity to feline CD21 described in [25]). Cells were then washed 3X with PBS, and treated with magnetic beads conjugated anti-mouse IgG before exposure to the magnetic field. To obtain the CD4⁺ T-cell enriched population (CD4⁺ T-cell population), the murine IgG MAb to feline CD4 was replaced with murine IgG MAb to feline CD8 (SouthernBiotech, Birmingham, AL). The final T-cell preparations were washed 3X and resuspended in PBS at total concentrations of 5–10×10⁶ cells/mL. The phenotypes of these cell preparations were determined by fluorescence-activated cell sorting (FACS) as previously described [21].

2.3. PCR-based MHC matching

MHC-matching was determined by PCR using primer sets specific for FLA class-I and class-II alleles as previously described [26] (Table 1, Supplemental Table 1). All amplified products were sequenced by Eurofins MWG Operon LLC (Louisville, KY) and determined to be specific for the sequence of the corresponding FLA allele.

2.4. Monitoring for FIV infection

FIV infection of the challenged cats was determined by virus isolation and proviral PCR of PBMC at 3, 6, 9, 12, and 21–27 weeks post challenge (wpc) [21]. These analyses were also performed on the bone marrow, lymph node, and thymus at the termination of each study. The sera collected at corresponding timepoints were tested for FIV antibodies by immunoblot analysis as previously described [27]. As the focus of these studies were to determine the ability of T cells from vaccinated donors to confer complete protection, plasma and proviral virus loads were not measured.

2.5. Monitoring cellular immunity of the vaccinated donors

The donor cats were evaluated for vaccine-induced cellular immunity 6–8 weeks after the blood collection for the A-T procedure. This evaluation of anti-FIV cellular immunity was delayed to allow donor cats to recover from blood draws used entirely for the preparation of T-cell enriched populations for A-T to maximize T cells transferred. The feline IFN γ and

IL-2 ELISpot analyses of PBMC to overlapping peptide pools for FIV p24 and reverse transcriptase (RT) were performed as previously described [28]. CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cell proliferation was determined by FACS using the carboxyfluorescein diacetate succinimide ester (CFSE) method as previously described [29]. Cytokine and cytotoxin mRNA analysis was performed using PBMC and FACS-sorted CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells from donors as previously described [19].

2.6. Statistics

The various groups in the A-T studies were compared using pairwise Mann-Whitney Rank Sum test (SigmaPlot version 11.0, San Jose, CA). The recipients in the experimental group received T-cell enriched populations from cats vaccinated with prototype FIV vaccine. However, the recipients in the control group did not receive T-cell transfer from cats vaccinated with non-specific antigen in identical vaccine formulations devoid of FIV antigen, but rather consisted of T-cell or PBMC transfer from non-vaccinated cats, B-cell transfer from vaccinated cats, and/or only PBS. All statistical results are shown in the right most column on Tables 2 and 3.

3. Results

3.1. A-T studies against homologous FIV_{Pet} challenge

Adoptive transfer studies began with Study 1 (Table 2), which was a small pilot study to determine if A-T of the T-cell enriched population from vaccinated semi-inbred cats could confer protection in the semi-inbred siblings against homologous FIV_{Pet} challenge. These donor-recipient pairs were FLA matched based solely on MLR analysis. Although not statistically significant, A-T protection was observed in 75% (3 of 4) of T-cell recipients, whereas no (0 of 2) A-T protection was observed in B cell recipients.

In Study 2 (Table 2), T cells were separated into CD4⁺ and CD8⁺ T cells to determine which population confers A-T protection. Complete protection was observed in all T cells recipients from vaccinated/MHC-matched siblings. In comparison, only 2 of 3 (67%) recipients receiving either CD4⁺ T cells or CD8⁺ T cells were protected. Notably, the unprotected cats received the lowest number of CD4⁺ (24×10^6) or CD8⁺ (14×10^6) T-cells and were either partially matched (CD8⁺ T-cell recipient [BDK]) in both FLA class-I and -II or completely FLA matched (CD4⁺ T-cell recipient [BDJ]). Additionally, partially-to-completely FLA-matched recipients of CD8⁺ or CD4⁺ T cells in these groups were protected against the same challenge (Table 2, Study 2A, [BDH, BDD, QVF, and QVG]).

Studies 1 and 2 used 4X vaccinated donors in Year 1, whereas Study 3 (Table 2) used vaccinated donors which were immunized annually for 5 years (1X-3X per year). Six of 7 recipients of the T-cell enriched population from vaccinated donors were protected against homologous FIV_{Pet} whereas all control cats were infected. The one unprotected cat (DVA) had only 50% and 25% compatibility with donor at FLA class-I and class II, respectively. In contrast, the protected cat (VSF), which received more donor T cells than the unprotected cat (DVA), had 50% FLA class-I and 33% FLA class-II compatibility with the donor. Nevertheless, these three studies demonstrated that T cells are important in dual-subtype

vaccine protection against homologous FIV_{Pet}. Moreover, such anti-FIV T-cell immunity was conferred by donor cats receiving 1X-3X boosts per year for 5 years.

3.2. A-T studies against heterologous-subtype FIV_{FC1} challenge

In order to determine that T cells are essential in conferring protection against both homologous and heterologous subtype viruses, two A-T studies against vaccine-induced NAb-resistant, heterologous-subtype FIV challenge were performed using a highly-divergent heterologous subtype FIV_{FC1}. T cells from 2-year vaccinated donors were used in Study 4, and those from 6-year vaccinated donors were used in Study 5 (Table 3). Three of 6 cats in Study 4 and 3 of 6 cats in Study 5 receiving partially-to-completely FLA-matched T-cell population resisted challenge (Table 3). However, when the recipients of partially FLA-matched T-cell transfer were removed, the protection rates increased to 3 of 4 for Study 4 and 3 of 5 for Study 5. In Study 4, donor CD4⁺ and CD8⁺ populations were monitored for CTL-activity specific to FIV through mRNA expression of cytolysin, cytotoxins, and cytokines. High levels of cytolysin (perforin) and cytotoxins (perforin, GrzA, GrzB, and GrzH) and Th1 cytokines were observed in CD8⁺ T cells and/or CD4⁺ T cells in donors (BDM, QVF, QWC) of protected A-T recipients from Study 4 (Fig. 1, top set). In contrast, the T cells from donors (BDA, QVD, QWE) of unprotected recipients produced minimal levels of both IL2 and CTL-associated mRNAs with high IFN α and IFN γ mRNA levels in CD4⁺ T cells (Fig. 1, bottom set). All donors in Study 4 received 4 vaccinations before their T cells were used for A-T. However, T cells from donor QVD at Years 5 and 6 of vaccination conferred A-T protection to recipients VSD and SBC in Studies 3 and 5, respectively. After Study 4 in Year 1 of vaccination, QVD had received prototype FIV vaccinations 2X annually prior to Studies 3 and 5.

Furthermore, the quality of anti-FIV T-cell immunity improved with long-term vaccination (section 3.3 below). Hence, only those cats that received at least 17×10^6 T cells, received donor T cells with strong anti-FIV activities, and received 100% FLA-matched donor T cells were protected against heterologous-subtype pathogenic FIV_{FC1} challenge (Table 3).

3.3. The quality of T-cell immunity induced over 4 years

Proliferation of CD4⁺ and CD8⁺ T cells were observed against multiple regions of FIV p24 using peptide pools (Fig. 2A and 2B). Six vaccinated donors were used in Year 2 and the same five donors with the exception of the replacement of cat QWC by long-term vaccinated littermate QWD due to death of QWC before year 4. These cats were vaccinated annually and their responses to overlapping peptide pools were evaluated in Years 2 and 4 (Fig 2). Notably, more proliferation responses were detected in Year 4 than Year 2 of vaccination (CD4⁺ T-cell responses: 56 vs. 38; CD8⁺ T-cell responses: 50 vs. 31). A slightly higher magnitude and greater numbers of IFN γ responders were observed in the PBMC of vaccinated A-T donors in Year 4 than Year 2 (IFN γ responses: 33 vs. 19) (Fig. 2C). The IL2 assay was unavailable in Year 2 of vaccination and analyzed only for Year 4. High number and magnitude of IL2 responses were observed to peptide-pools Fp10, Fp12, and Fp14 (Fig. 2D).

Since IFN γ responses to the overlapping FIV RT peptide pools in Years 1 and 2 (Fig. 3D, Year 2 shown) of vaccination were minimal, T-cell proliferation assays were not performed until Year 4. Both the CD4⁺ and CD8⁺ T cells had moderate-to-high responses to multiple RT pools (Fig. 3A and 3B). The PBMC from Year 4 had IL2 and IFN γ responses to multiple RT pools (Fig. 3C and 3D). Remarkably, no responses were detected to pools RT8 or RT12 when these peptide pools induced substantial T-cell proliferation.

4. Discussion

In a previous A-T study using parent-to-offspring half-matched FLA, only 36% protection was observed against medium-to-high (20–50 CID₅₀) challenge doses with homologous FIV_{Pet} [18]. However, in a completely FLA-matched system produced by bone marrow transplantation (BMT), adoptive transfer of washed whole blood from vaccinated/BMT recipient to BMT donor or BMT-recipient sibling conferred 100% protection against a high dose (100 CID₅₀) of homologous FIV_{Pet} challenge [18]. Since these studies used either purified PBMC or washed whole blood, the current studies performed with T cells achieved significant protection in 87% (13 of 15, $p < 0.001$; Studies 1–3, Table 2) of recipients receiving partial-to-complete FLA-matched T cells against homologous FIV_{Pet}.

In comparison, only A-T of T cells from 100% FLA-matched, vaccinated donors conferred protection to recipients against vaccine-induced NAb-resistant, heterologous-subtype FIV_{FC1} (Table 3). In Study 4, the lack of FLA class-I and/or class-II compatibility in recipients BDD and BDQ as well as the low number of T cells for adoptive transfer in recipient BDD may have contributed to the lack of protection observed. However, in Study 5 (Table 3), 2 of 3 unprotected cats (WFA and OCE) were completely FLA matched with their donors but received the lowest numbers of T cells which may indicate that the number of T cells transferred was also an important factor in protection against FIV_{FC1}. The other unprotected cat (OCD, Study 5) received a low number of T cells but from a FLA-unmatched donor.

Most importantly, anti-FIV T-cell activities with high cytokine and cytotoxin production induced by the prototype FIV vaccine were vital in conferring protection. Since the commercial FIV vaccine also conferred protection against FIV_{FC1} and other moderately NAb-resistant heterologous FIV strains [21], the anti-FIV T-cell immunity generated by the commercial vaccine was most likely the immunity that conferred protection. For example, although unprotected recipient QVG received the highest amount of completely FLA-matched T cells from donor QVD, this donor's CD4⁺ and CD8⁺ anti-FIV T-cell immunity, as measured by cytolysin and cytotoxin mRNA production to FIV antigen, appeared similar to the profile (Fig. 1, bottom set) of other donors (BDA, QWE) which failed to confer protection.

T-cell immune responses to FIV p24 peptide pools were detected as early as Year 1 of vaccination in previous publications [20,28] and Year 2 of vaccination in the current study (Fig. 2, Year 2) which increased with annual boosts (Fig. 2, Year 4). Although IFN γ responses to p24 peptide pools were detected as early as Year 1, IFN γ responses to RT peptide pools were not detected until Year 3 (Fig. 3D). The reason for the difference in

responses between the p24 and RT peptide pools is most likely due to the major difference in the amount of these proteins expressed by the virus present in the vaccine. Both prototype and commercial FIV vaccines contain a large amount of p24 and a very low amount of RT [30]. Thus, T-cell epitopes on p24 may be involved in protection throughout Years 1–6 of vaccination but those on RT may be involved in protection starting in Years 3–4.

While a large number of recipients of A-T of T cell enriched populations from FIV-vaccinated cats were performed in Studies 1–5 (Tables 2 and 3), recipients of A-T consisting of B cells from FIV vaccinated cats was limited to Study 1 (Table 2). Although the result from Study 1 is not statistically significant, T cells appeared to be involved in conferring A-T protection, but the number of B-cell recipients was too small to determine whether B cells alone can confer A-T protection. In regards to the potential of B cells to afford protection to recipients of A-T, studies by others have shown that the commercial dual-subtype FIV vaccine [31] as well as priming T cells with FIV_{Pet} Env pDNA [32] induced high NAbs to FIV_{Pet}. However, in current studies, all recipients of A-T from vaccinated cats, except for the unprotected recipients, had no NAbs to FIV_{Pet} or FIV_{FC1} (data not shown) and no FIV Abs (Tables 2 and 3, immunoblot). Furthermore, both NAbs and FIV Abs were not detected in the sera from the protected recipients tested on day 3 or 7 after A-T (data not shown). Similarly, protection against FIV challenges in the absence of NAbs has been reported with FIV- RT pDNA vaccine and inactivated FIV-infected cell vaccine [33,34].

Limitations imposed on control group selection were primarily a consequence of resource availability as the number of semi-inbred cats that could be used for vaccination, including control vaccinations, was limited. Due to the timing required to use consistent age-matched recipients for the adoptive transfer studies, these studies were completed in smaller, manageable sized groups. Load limitations of magnetic sorting also limited the number of cats which could be processed for adoptive transfer in a setting of one full day. In our previous vaccine studies, active vaccination of naïve cats with uninfected vaccine cell line (FeT-J) in FD-1 adjuvant, FD-1 adjuvant/HuIL12, and FD-1 adjuvant containing FeIL12 conferred no protection [21]. Therefore, the potential for protection against FIV challenge in a control group, consisting of recipients by A-T of T-cell enriched populations from donors immunized with vaccine formulations identical to the commercial and prototype vaccines but with non-specific antigen (e.g., FeT-J without FIV antigen), was reasonably low. Resource availability was also an issue with blood draws, as 20% blood volume was collected from vaccinated donors solely to support the sorting of PBMC into the largest T-cell enriched population possible for A-T. The subsequent blood draw for analysis of T-cell mediated immunity was delayed 6–8 weeks to allow for an adequate refractory period per University of Florida IACUC protocols.

The recent CMV-vectored SIV vaccine study demonstrated the importance of anti-SIV CD8⁺ T cells [16,17]. In human vaccine trials, the CD4⁺ CTL activities to the V2 region of HIV-1 surface envelope were observed in vaccinated subjects of the most successful human vaccine trial RV144 [35,36]. These studies using HIV-positive subjects and the SIV/ macaque model demonstrate the critical role played by the T cells in lentiviral protection [10–16]. Current A-T Study 2 also demonstrated the importance of both anti-FIV CD4⁺ and CD8⁺ T cells in conferring protection with prototype FIV vaccine and most likely with

commercial FIV vaccine [21]. Thus, efforts should be made in developing a T-cell based vaccine against HIV-1 and FIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- 23/33 recipients of adoptive T-cell transfer from vaccinated donors were protected.
- 81% (17/21) protection was against homologous FIV (vaccine strain).
- 50% (6/12) protection was against neutralizing Ab-resistant, different-subtype FIV.
- Protection required >60% to 100% MHC-matching between each donor-recipient pair.
- T cells mediate protection conferred by prototype and commercial FIV vaccines.

CAT#	CELL TYPE	CTL-ASSOCIATED CYTOLYSIN / CYTOTOXINS				INFLAMMATORY CYTOKINES		TH1 CYTOKINES		TH2 CYTOKINES			
		Perf	GrzA	GrzB	GrzH	TNF α	IFN α	IFN γ	IL-2	IL-4	IL-6	IL-10	
DONORS OF PROTECTED	BDM	CD4+ T	+	±	±	+	±	+	+	+	±	+	
	QVF	CD4+ T	+	+	-	+	+	+	+	+	+	+	
	QWC	CD4+ T	+	+	+	+	+	+	+	+	-	±	
	BDM	CD8+ T	±	+	+	+	-	±	+	+	-	±	+
	QVF	CD8+ T	+	+	+	+	±	+	+	+	±	-	±
	QWC	CD8+ T	±	-	+	-	-	±	±	-	±	-	-
Average Response:		62%	70%	58%	58%	40%	58%	80%	50%	55%	30%	60%	
DONORS OF UNPROTECTED	BDA	CD4+ T	-	-	-	±	±	+	±	-	±	-	+
	QVD	CD4+ T	±	-	-	±	+	+	+	±	+	-	±
	QWE	CD4+ T	-	±	±	+	-	+	+	±	±	-	±
	BDA	CD8+ T	±	±	-	±	-	±	±	±	-	-	-
	QVD	CD8+ T	±	±	±	±	+	±	-	±	-	+	±
	QWE	CD8+ T	-	+	+	+	-	+	±	-	-	±	+
Average Response:		17%	23%	25%	42%	35%	54%	29%	21%	27%	15%	36%	

Fig. 1. Cytokine and cytotoxin mRNA levels of vaccinated A-T donors. The chart (A) consists of three A-T donors of protected recipients from Study 4 (Year 1) (top set) and three A-T donors of unprotected recipients (bottom set). All % relative densitometric values are considered negative (blue box) or positive (red box) when the media control-subtracted values are <3% or >10%, respectively. Those values between 3%-10% are assigned (±) in an orange box. The relative densitometric value of each band is compared to the corresponding β -actin band and the media control for the corresponding target mRNA is subtracted. Abbreviations: cytotoxic T lymphocyte-associated (CTL-assoc.), T helper-1 (Th1), T helper-2 (Th2), media control (M), inactivated dual-subtype FIV viruses (F), staphylococcal enterotoxin A (S), perforin (Perf), granzyme A (GrzA), granzyme B (GrzB), granzyme H (GrzH), tumor necrosis factor- α , interferon- α (IFN α), interferon- γ (IFN γ), interleukin (IL). Average response in percentage refers to the mean of the CD4⁺ and CD8⁺ T-cell response for the two groups of 3 cats each (Donors of Protected and Donors of Unprotected). Note that donors QVD, BDA, and QVF were used in Studies 3–5, whereas donor BDM was used in Studies 3 and 4. The mRNA results of all donors are from Study 4 performed in Year 1 of vaccination.

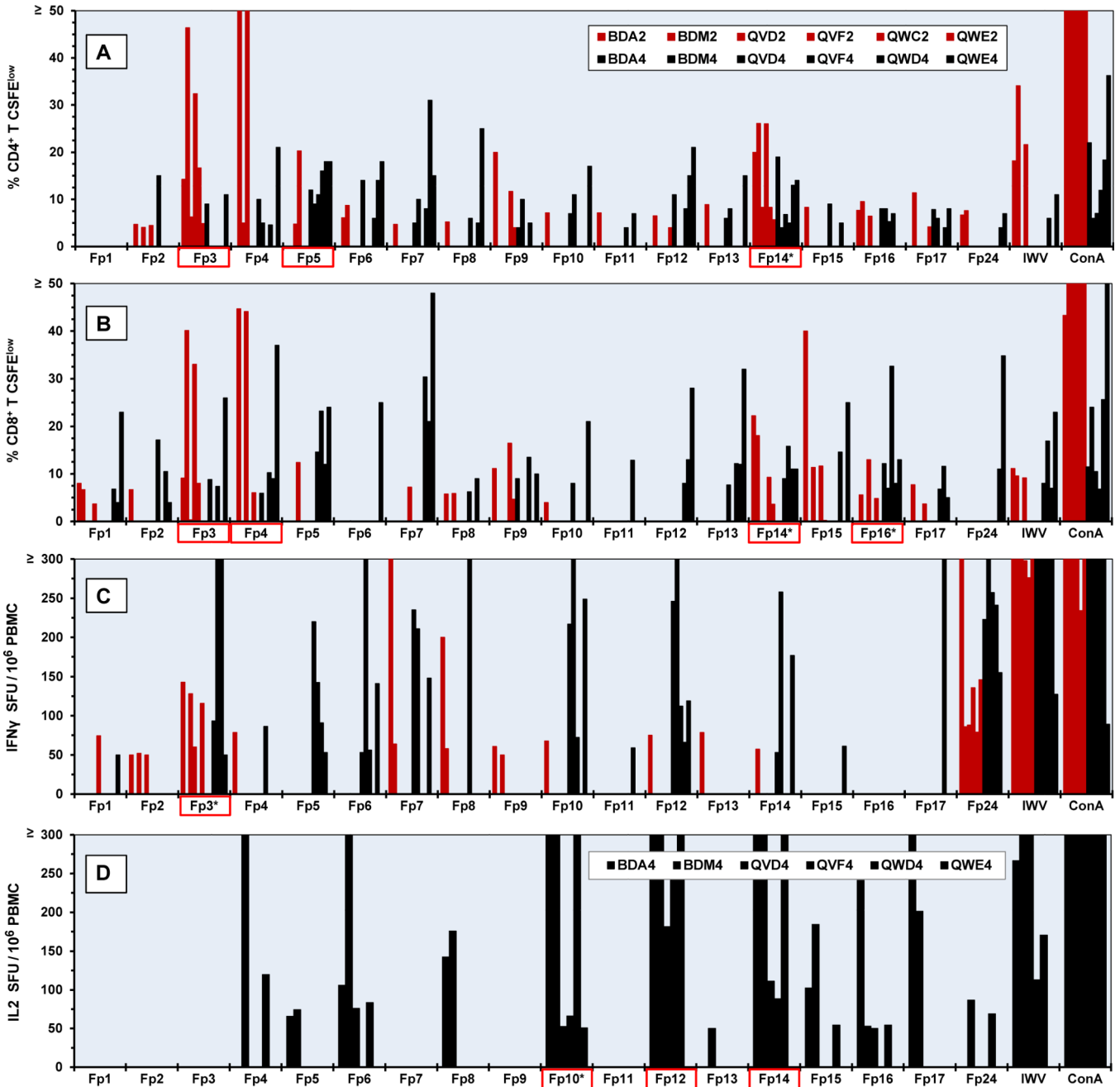


Fig. 2. Cellular immunity to FIV p24 peptide pools on Years 2 and 4 of vaccination. CD4⁺ (A) and CD8⁺ (B) T-cell proliferation in response to overlapping FIV p24 peptide pools (Fp1-Fp17) are shown for six A-T donors in Year 2 (red bar) and Year 4 (black bar). The IFN γ (C) and IL2 (D) production in response to the p24 peptide pools are shown for Years 2 and 4 for IFN γ and Year 4 for IL2. Cat identification codes are depicted in A (inset) for panels A-C. Each donor is designated as either 2 for Year 2 or 4 for Year 4 following the cat identification code (i.e., BDA2 represents BDA’s responses at Year 2 and BDA4 represents BDA’s responses at Year 4). The order of donors in the inset from left to right corresponds

to the order of the bars shown from left to right. Each peptide pool contains 3–4 peptides with an overlap of 8 amino acids. Those peptide pools denoted with an asterisk have the highest number of combined Year-2 and Year-4 responses and those with red-lined box have 80% of the total number of possible responses per peptide pool. The threshold of IL2 and IFN γ production and T-cell proliferation are set at 50 SFU/ 10⁶ PBMC and 4% CFSE^{low}, respectively. Abbreviations: inactivated dual-subtype FIV viruses (IWV), T-cell mitogen concanavalin A (ConA), spot forming unit (SFU), CFSE-based proliferation of CD4⁺ T cells (% CD4⁺ T CFSE^{low}), CFSE-based proliferation of CD8⁺ T cells (% CD8⁺ T CFSE^{low}).

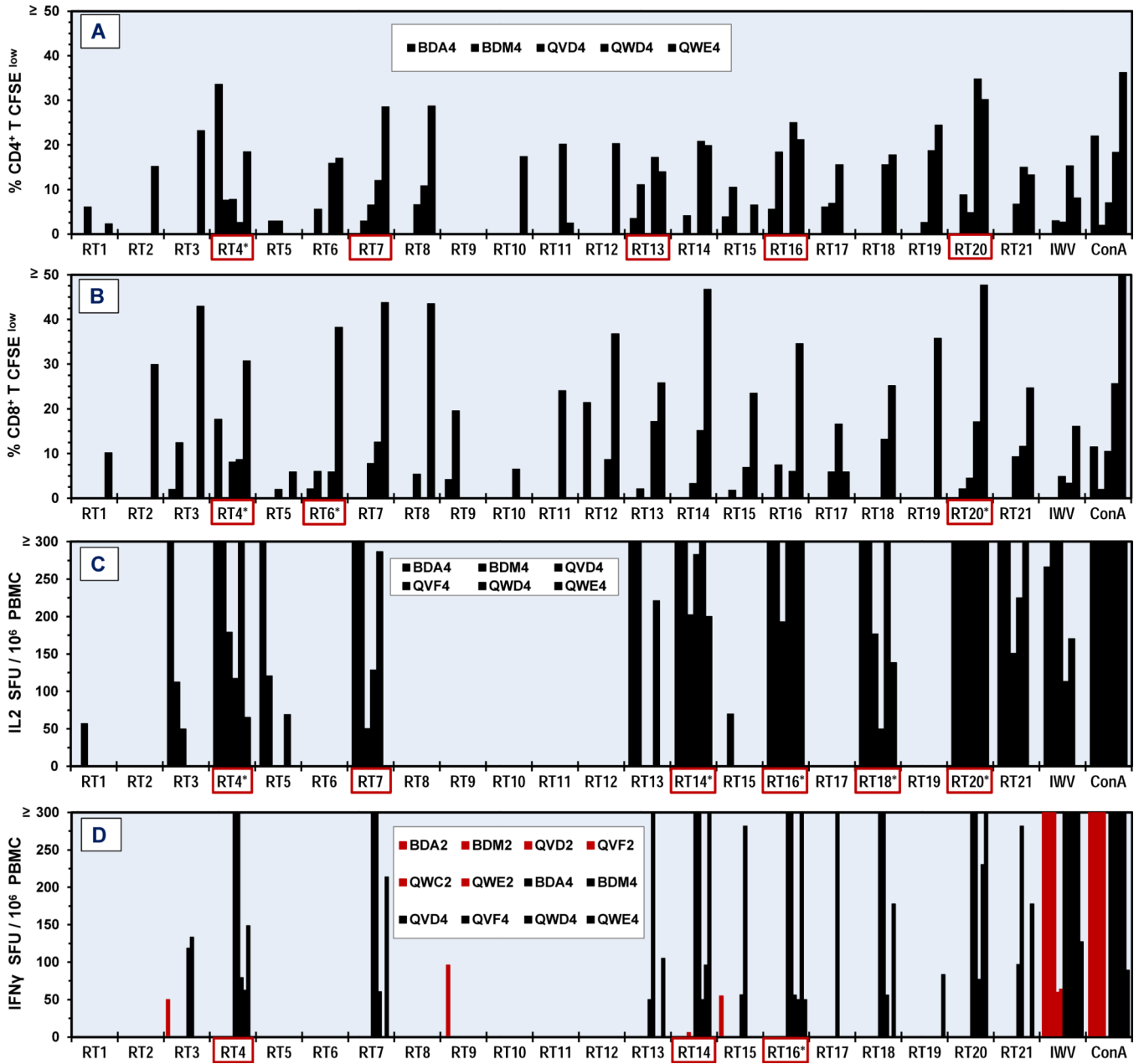


Fig. 3. Cellular immunity to FIV RT peptide pools on Year 4 of vaccination. CD4⁺ (A) and CD8⁺ (B) T-cell proliferation and IL2 (C) and IFN γ (D) production in response to overlapping FIV RT peptide pools (RT1-RT21) are shown for five (A,B) to six (C,D) A-T donors from Year 4 (black bar). The IFN γ production for Year 2 is also shown (D, red bar) but one donor (QWC2) in Year 2 has been replaced with a different donor (QWD4) in Year 4. Each peptide pool contains 4–5 peptides with an 8-amino acid overlap. The insert for A is also for B. Those peptide pools with asterisk have the highest number of Year 4 responses and those with a red box have 80% of the total number of possible responses per peptide pool. The

threshold of IL2 and IFN γ production and T-cell proliferation are 50 SFU/10⁶ PBMC and 2% CFSE^{low}, respectively. Abbreviations are the same as Fig. 2 legend.

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Table 1

Feline MHC class-I and -II allele specific primers for semi-inbred cats.

Allele Specific Primer Set*	Primer Sequence	Product Length
A1 (FLA Class-I)	F: 5'-CGAGGAGACGCGGAACAT-3' R: 5'-CACGTCACAGCCATACATTG-3'	127 bp
A2 (FLA Class-I)	F: 5'-CCCGAATCCCAGGGAAGA-3' R: 5'-GGAGCAACGTGTTTCAGGTT-3'	128 bp
B3 (FLA Class-I)	F: 5'-CTCCCACTCCCTGAGGTATTT-3' R: 5'-GTTGTAGTAGCGGAGGAC-3'	256 bp
B4 (FLA Class-I)	F: 5'-TGTGACATCGGACCGAACA-3' R: 5'-GGTAGTTCCTGTAGTGCTCT-3'	178 bp
B5 (FLA Class-I)	F: 5'-GCCCGAATCCGAGGTAT-3' R: 5'-CCAGGTAGTTCCTGATGTCT-3'	297 bp
B6 (FLA Class-I)	F: 5'-GCGGAAGGTGAAGAACAC-3' R: 5'-CCGTCATAGGAGTCCTGATT-3'	168 bp
D1 (FLA-DRB)	F: 5'-GAGTCCGAGCGGTATCA-3' R: 5'-GAAGTCCAGAGTGCCCTTTCT-3'	556 bp
D2 (FLA-DRB)	F: 5'-GACACCTCATCACATTTCTTAG-3' R: 5'-CTGCTCCAGGTGGTCCTT-3'	207 bp
D3 (FLA-DRB)	F: 5'-GACACCTCACCACATTTCTTGTT-3' R: 5'-CTCGTTCATGTACTTGGCGT-3'	186 bp
D4 (FLA-DRB)	F: 5'-GTGGAAGTTCGAGTGCATTATC-3' R: 5'-CTTCCGCTCCAGGACCT-3'	184 bp
D5 (FLA-DRB)	F: 5'-GACACCTCACCACATTTCTTGTT-3' R: 5'-CTCGTTCATGTACTTGGCAATG-3'	186 bp
D6 (FLA-DRB)	F: 5'-ACATTTCTTAACCATGTGGAAGTTC-3' R: 5'-CCGTAGTTGTGTCTGCAGTA-3'	237 bp
D7 (FLA-DRB)	F: 5'-TGATGGCAGCTCTGATGGTA-3' R: 5'-GCTGTCCAAGCGCAAGTT-3'	182 bp

Table 2
A-T studies using FLA-matched and unmatched cats against homologous FIV_{Pet} challenge.

Study-Group	Immune Status ^a	A-T		FLA Match ^{bc}		A-T Cell Type ^d		A-T Cell (×10 ⁶ /A-T) ^{cde}								FIV Antibodies/Isolation/PCR ^g		B/L/Th	Protection Rate (%)	p-value ^h
		Donor	Recipient	I	II/MLR	Type ^d	CD4	CD8	T ^f	B	3wpc	6wpc	9wpc	12wpc	21wpc	21-27wpc				
1A	Vac	3EC	3EA	N/N/+		T	19	8	30	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	3/4 (75%)	0.114	
(Y-1)	Vac	3HA	3HB	N/N/+		T	27	10	45	20	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	6DB	6DA	N/N/+		T	2	0.2	2	0.4	-/+	+/+	+/+	+/+	+/+	+/+	+/+			
1B	Vac	9QD	9QK	N/N/+		T	22	5	31	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0/4 (0%)		
	Vac	3EC	3EB	N/N/+		B	4	2	11	13	±/-	+/+	+/+	+/+	+/+	+/+	+/+			
	Vac	6DB	6DC	N/N/+		B	13	3	19	13	-/+	+/+	+/+	+/+	+/+	+/+	+/+			
	Non-Vac	901	803	N/N/-		PBMC	33	10	52	N	-/+	+/+	+/+	+/+	+/+	+/+	+/+			
	Non-Vac	807	805	N/N/-		PBMC	23	8	39	42	-/+	+/+	+/+	+/+	+/+	+/+	+/+			
2A	Vac	BDB	BDI	+/+/+		T	22	17	42	4	-/-	-/-	-/-	-/-	-/-	-/-	-/-	8/10 (80%)	0.048ⁱ	
(Y-1)	Vac	BDB	BDK	±/-/+		CD8 ⁺ T	5	14	25	3	-/-	-/+	-/+	-/+	-/+	-/+	-/+			
	Vac	BDF	BDH	+/-/+		CD8 ⁺ T	5	17	33	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	BDF	BDJ	+/+/+		CD4 ⁺ T	24	3	42	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	BDC	BDA	+/+/+		T	42	19	65	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	BDC	BDD	±/-/+		CD4 ⁺ T	38	1	44	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	QVB	QVE	+/+/+		T	56	26	82	7	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	QVB	QVF	+/+/+		CD4 ⁺ T	47	1	68	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	QVC	QVD	+/+/+		T	13	17	36	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
	Vac	QVC	QVG	+/+/+		CD8 ⁺ T	6	24	33	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-			
2B	Non-Vac	BDG	BDE	N/N/+		T	127	28	157	4	-/-	-/+	+/+	+/+	+/+	+/+	+/+	0/4 (0%)		
	Non-Vac	QVA	QWA	N/N/-		PBMC	65	33	98	21	-/-	+/+	+/+	+/+	+/+	+/+	+/+			
	Non-Vac	None	271	N		PBS	N	N	N	N	-/-	-/+	+/+	+/+	+/+	+/+	+/+			
	Non-Vac	None	350	N		PBS	N	N	N	N	-/-	-/+	+/+	+/+	+/+	+/+	+/+			
3A	Vac	BDA ^j	VSF	±/-/+		T	36	16	65	0.6	-/-	-/-	-/-	-/-	-/-	-/-	-/-	6/7 (86%)	0.024	
(Y-5)	Vac	BDM	DVA	±/-/+		T	16	4	28	0.2	-/-	-/+	+/+	+/+	+/+	+/+	+/+			
	Vac	QVD	JVSD	+/+/+		T	44	11	63	1.7	-/-	-/+	-/+	-/+	-/+	-/+	-/+			
	Vac	QVF ^j	VSE	+/+/+		T	57	23	89	6.4	-/-	-/+	-/+	-/+	-/+	-/+	-/+			

Study-Group	Immune Status ^a	A-T		FLA Match ^{bc}		A-T Cell Type ^d		A-T Cell ($\times 10^6/A-T$) ^{ede}		FIV Antibodies/Isolation/PCR ^g						B/L/Th	Protection Rate (%)	p-value ^h
		Donor	Recipient	I / II / MLR	1 / II / MLR	CD4	CD8	T ^f	B	3wpc	6wpc	9wpc	12wpc	21wpc	21-27wpc			
	Vac	QVZ	VVC	+ / + / +	T	12	2	21	0.1	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -		
	Vac	VOG	VON	+ / + / +	T	66	27	106	2.4	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -		
	Vac	QWF	QWH	+ / + / +	T	35	23	65	0.8	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -	- / - / -		
3B	Non-Vac	VWG	VVA	N	T	40	24	68	0.7	- / - / -	- / - / -	+ / + / -	+ / + / -	+ / + / -	+ / + / +	+ / + / +	0/4 (0%)	
	NU	NU	QVO	N	PBS	N	N	N	N	- / - / -	- / - / -	+ / + / +	+ / + / +	+ / + / +	+ / + / +	+ / + / +		
	NU	NU	9HJ	N	PBS	N	N	N	N	- / + / +	+ / + / +	+ / + / +	+ / + / +	+ / + / +	+ / + / +	- / + / +		
	NU	NU	5HE	N	PBS	N	N	N	N	- / - / -	- / - / -	+ / + / -	+ / + / -	+ / + / -	+ / + / -	+ / + / -		

T cells only from Group 1A+2A+3A vs. Control Group 1B+2B+3B:

<0.001

^aVaccinated donor (Vac), non-vaccinated donor (Non-Vac), or donor not used (NU).

^bDonor-recipient FLA compatibility of 90–100% (+), 50% (\pm), and <50% (-) (see Supplemental Table 1). MLR results are shown as either compatible (+) or not compatible (-)

^cNot done (N).

^dEnriched for CD4⁺ T cells (CD4⁺ T), CD8⁺ T cells (CD8⁺ T), T cells (T), and B cells (B). The bolded number multiplied by 10⁶ represents the total number of CD4⁺ T cells, CD8⁺ T cells, T cells, or B cells transferred. The bolded T-cell count plus bolded B-cell count multiplied by 10⁶ approximates the total PBMC transferred. PBS added (PBS) instead of cells.

^eThe non-B and non-T lymphocyte contamination accounted for 0.1%-3.0% (absolute number of 0.4 $\times 10^5$ -1.3 $\times 10^6$ transferred) compared to the total T- and B-cell population of the T-cell preparation transferred with each A-T. The non-T and non-B populations are negative for CD3, CD4, CD8, CD21, IgG, and IgM.

^fT-cell counts were higher than the total of CD4⁺ T-cell plus CD8⁺ T-cell counts due to CD3⁺CD4⁻CD8⁻ and CD3⁺CD4⁺CD8⁺ T-cell counts. In Study 3, T-cell counts are higher than the combined count for CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells mainly due to the CD3⁺CD4⁻CD8⁻ T cells.

^gFIV antibodies, virus isolation (isolation), and proviral PCR (PCR) are shown as either positive (+) or negative (-). Virus isolation on lymph node (L), bone marrow cells (B), and thymocytes (Th) performed at the termination of each study.

^hP-value of FLA matched group vs. control group unless stated otherwise. Bolded values indicate significance.

ⁱTotal population of T cells, CD4⁺ T-cells and CD8⁺ T cells ($p=0.048$); only whole T-cells ($p=0.029$).

^jThe protected cats from Study 2 vaccinated and used as A-T donors in Study 3.

Table 3

A-T protection against heterologous subtype-B FIV_{FC1}.

Study-Group	Immune Status ^a	A-T		FLA Match ^{b,c}		A-T Cell Type ^c	A-T Cells (×10 ⁶) ^{c,d,e}			FIV Antibodies/Isolation/PCR ^{c,g}						B/L/Th		Protection Rate (%)	p-value		
		Donor	Recipient	I	II/MLR		CD4	CD8	T _f	B	2	3wpc	6wpc	9wpc	12wpc	16wpc	25wpc			25-40wpc	
4A (Y-1)	Vac	BDA	BDD	±/-/+		T	5	3	9	2	-/-/-	-/-/-	-/+/-	+/-/+	+/-/+	+/-/+	+/-/+	+/-/+	3/6 (50%)	0.257	
	Vac	BDM	BDI	+ / + / +		T	13	10	23	6	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-			
	Vac	QVF	QVE	+ / + / +		T	52	22	83	5	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-			
	Vac	QVD	QVG	+ / + / +		T	86	30	129	11	-/-/-	-/+/-	+/-/+	+/-/+	N	+/-/+	+/-/+	+/-/+			
	Vac	QWC	BDR	+ / + / +		T	37	7	46	7	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-			
	Vac	QWE	BDQ	± / - / +		T	20	3	24	5	-/-/-	-/-/-	-/-/-	-/+/-	+/-/+	+/-/+	+/-/+	+/-/+			
4B Non-Vac	Non-Vac	228	128	N / N / -		T	82	21	109	15	-/-/-	-/-/-	+/-/+	+/-/+	N	+/-/+	+/-/+	0/4 (0%)			
	NU	NU	130	N		PBS	N	N	N	N	-/-/-	-/-/-	-/+/-	-/+/-	+/-/+	+/-/+	+/-/+				
	NU	NU	228	N		PBS	N	N	N	N	-/-/-	+/-/+	+/-/+	+/-/+	N	+/-/+	+/-/+				
5A (Y-6)	Vac	QVD	SBC	+ / + / +		T	11	6	17	0.3	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	N	-/-/-	-/-/-	3/6 (50%)	0.257	
	Vac	QVF	SBD	+ / + / +		T	34	4	47f	0.1	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	N	-/-/-	-/-/-			
	Vac	QVZ	OCB	+ / + / +		T	18	6	25	0.0	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	N	-/-/-	-/-/-			
	Vac	VOG	WFA	+ / + / +		T	9	3	13	0.0	-/-/+	+/-/+	+/-/+	+/-/+	N	+/-/+	N	+/-/+			
	Vac	QWF	OCE	+ / + / +		T	5	3	8	0.0	-/-/-	+/-/+	+/-/+	+/-/+	N	+/-/+	N	-/-/-			
	Vac	BDA	OCD	± / - / +		T	10	2	13	0.1	-/-/-	+/-/+	+/-/+	+/-/+	N	+/-/+	N	+/-/-			
5B	Non-Vac	QVQ	SBE	N / N / +		T	8	4	13	0.3	-/+/-	+/-/+	+/-/+	+/-/+	N	+/-/+	N	+/-/+	0/4 (0%)		
	NU	NU	OCC	N		N	N	N	N	N	-/+/-	+/-/+	+/-/+	+/-/+	N	+/-/+	N	+/-/-			
	NU	NU	OLA	N		N	N	N	N	N	-/+/-	+/-/+	+/-/+	+/-/+	N	+/-/+	N	+/-/+			
	NU	NU	SBB	N		N	N	N	N	N	+ / + / +	+ / + / +	+ / + / +	+ / + / +	N	+ / + / +	N	+ / + / +			

^aVaccinated donor (Vac), non-vaccinated donor (Non-Vac), or donor not used (NU).

^b Donor-recipient FLA compatibility of 90–100% (+), 50% (±), and <50% (-) (see Supplemental Table 1). MLR results are shown as either compatible (+) or not compatible (-).

^cNot done (N).

^dEnriched for T cells (T). The bolded number multiplied by 10⁶ represents the total number of T cells transferred. PBS added (PBS) instead of cells. Numbers listed for all populations denoted in the column header display actual numbers for each cell phenotype transferred.

Group 4A+5 A vs. Control Group 4B+5B: 0.023

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⁶The non-B and non-T lymphocyte contamination accounted for 0.1%-3.0% (absolute number of 0.4×10^5 - 1.3×10^6 transferred) compared to the total T- and B-cell population of the T-cell preparation transferred with each A-T. The non-T and non-B populations are negative for CD3, CD4, CD8, CD21, IgG, and IgM.

⁷T-cell count also includes $CD3^+CD4^-CD8^-$ and $CD3^+CD4^+CD3^+$ T-cell counts. Total T-cell count for donor QVF in Study 5 is higher than the combined count for $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells mainly due to the presence of $CD3^+CD4^+CD8^+$ T cells.

⁸FIV antibodies, virus isolation (Isolation), and proviral PCR (PCR) shown as either positive (+) or negative (-). Virus isolation on lymph node (L), bone marrow cells (B), and thymocytes (Th) performed at the termination of each study.