

Feline Leukemia Virus (FeLV)-Specific IFN γ + T-Cell Responses Are Induced in Cats Following Transdermal Vaccination With a Recombinant FeLV Vaccine

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KEY WORDS: feline leukemia virus, cell-mediated immunity, transdermal route, recombinant vaccine

ABSTRACT

Induction of feline leukemia virus (FeLV)-specific T cell responses in cats following either transdermal vaccination with a new recombinant FeLV vaccine (r-FeLV) or a killed whole virus vaccine (KV-FeLV) was investigated. Kittens were vaccinated either with the r-FeLV, delivered transdermally via a needle-free delivery device, or the KV-FeLV, delivered in an adjuvant, or were sham-vaccinated with either a recombinant rabies vaccine (r-rabies) or saline as controls. The cats were injected twice at 3-week intervals and challenged with FeLV-A challenge 2 weeks following the last vaccine injection. Blood was collected and peripheral blood mononuclear cells (PBMCs) isolated. T cell response was quantified either by using an IFN γ -ELISpot assay or by flow

cytometry following intracellular staining of the cytokine coupled to surface CD4 and CD8 T cell markers expression. A significant frequency of FeLV-specific T cells was measured in cat PBMCs vaccinated with the transdermally delivered r-FeLV. FeLV-specific cellular activity was not detected in KV-FeLV vaccinated cats and was barely detectable following challenge of both KV-FeLV vaccinated and control cats. Overall, the study presented here demonstrated that needle-free delivered r-FeLV vaccines, in contrast to KV-FeLV, induce FeLV-specific T cell responses and open new research areas regarding antigen-specific T cell monitoring in cats.

INTRODUCTION

Feline leukemia virus (FeLV) is a gammaretrovirus naturally occurring in the domestic cat. FeLV disease outcomes are variable. Cats that mount a sufficient immune response will recover while cats

that do not mount an appropriate virus-specific immunity will become persistently infected.¹⁻³ Among these persistently infected cats, the majority will die from degenerative diseases while a minority will develop neoplastic and proliferative diseases.⁴ In general, viremic cats succumb to FeLV-related diseases within 2-4 years of infection.

An improved understanding of the contribution of the different immune responses to the prevention and control of infectious diseases is an important step toward rational design of new vaccines. Cytotoxic T cells (CTLs) have been well established as being one of the major early defenses associated with viral infections and a positive correlation between FeLV recovery and the level of FeLV-specific circulating CTL has already been shown.⁵ Although several studies have shown that anti-FeLV neutralizing antibodies correlate with immunity to the virus,^{6,7} the majority of cats that develop a transient infection recover before FeLV neutralizing antibodies are detected in the circulation while virus-specific CTL occur within one week post-infection.⁵ Therefore, the development of FeLV-specific cellular responses seems to be critical for a rapid clearance of the virus.

It is now well documented that CTL responses are induced in vivo with vaccines such as DNA plasmids or recombinant viral vectors in a variety of animal models as well as in humans.⁸ One of the most significant changes in the field of veterinary vaccines over the past few years has been the introduction of several recombinant vaccines based on the canarypox vector.⁹⁻¹³ Canarypox vectors undergo an abortive replication cycle in mammalian cells during which the vectors express the inserted gene product in sufficient levels to induce antigen-specific B and T cell immunity.¹⁴⁻¹⁶ Although not fully explained, canarypox virus vectors allow for an increase immunogenicity of the recombinant antigen¹⁷ and do not induce anti-vector inhibiting immunity.¹⁸ In that way, canarypox viruses successfully combine the safety of an inactivated vaccine

with the efficacy of a modified live vaccine.

In addition, the route of antigen delivery can largely influence both the quality and the duration of the immune response. Dendritic cells are suited to deliver antigens to T lymphocytes which is a key step for the induction of an efficient priming of cell-mediated immunity. For these reasons, transdermal vaccination appears to be particularly of interest because it provides direct access to the different types of dendritic cells present in both epidermis (Langerhans cells) and dermis (dermal dendritic cells). Combining a viral-vectored vaccine known to orientate the immune response toward a type 1 response with dendritic cell targeting should provide a high quality vaccine without having to add any adjuvant. This has been demonstrated recently in a study showing that vaccination with a recently licensed transdermal r-FeLV vaccine provided protection against persistent FeLV antigenemia following challenge.¹⁹

In the present study, we compared the transdermal r-FeLV vaccine to a conventional killed and adjuvanted FeLV vaccine based on their ability to induce FeLV-specific cell-mediated immunity in cats. Taken together, we demonstrated that the transdermal r-FeLV vaccine represents a new and straightforward vaccination method that effectively induces primary and virus-recalled FeLV-specific T cell responses without the need of adjuvant.

MATERIAL AND METHODS

Animals, Vaccination, and Challenge

Specific-pathogen-free (SPF) European bred kittens (age 11-12 weeks) of both sexes were purchased from Liberty (Waverly, NY, USA) and housed in our animal facility according to European Community guidelines. The cats were clinically examined once per week and bled under general anesthesia. Cats were anesthetized and vaccinated either with 0.25 mL of the vCP97 ALVAC[®] vector administered using the Vitajet[™] device or with a commercially

available killed whole FeLV vaccine in the right quadriceps according to recommendations. Vaccinated and control anesthetized cats were challenged by oral (0.5 mL) and nasal (0.25 mL per nostril) administration of the FeLV-A Glasgow-1 strain ($4.94 \log_{10}$ CCID₅₀/mL). This animal experiment and the associated procedures were reviewed and approved by Merial's Ethical Committee.

Dendritic Cell Differentiation and Cytokines

Monocyte-derived dendritic cells were generated for each individual kitten. Blood samples were taken 3 days before the immunization day and peripheral blood mononuclear cells (PBMCs) were isolated and frozen. Dendritic cell cultures were initiated in complete RPMI medium containing feline granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 six days before each time point of the study and used when fully differentiated. Recombinant feline GM-CSF and IL-4 were prepared in our laboratory from CHO-K1 cells (ECACC no. 85051005, European Collection of Cell Culture, Salisbury, UK) transiently transfected with GM-CSF- or IL-4-coding plasmids. The supernatant from transfected cells was harvested after 24 hour, centrifuged ($4800 \times g$), and stored at -20°C . The functionality of these 2 cytokines had been tested previously (data not shown): IL-4-containing supernatant was shown to induce feline lymphoblast proliferation up to a dilution of 1:2000, and GM-CSF-containing supernatant was shown to induce the differentiation of granulomacrophagic colonies (CFU-GM) from porcine bone marrow cell culture *in vitro*.

Cell Culture and Ex Vivo Antigen-Specific Stimulation

PBMCs were isolated by Pancoll, d=1.077 (D. Dutcher, France) density-gradient centrifugation ($1100 \times g$ for 30 minutes). PBMCs were washed twice in PBS and subsequently counted. Cell numeration was per-

formed using an adapted version of the robotized ABX Pentra® 120 cell counter (ABX Diagnostics, France). For antigen-specific stimulation, either purified PBMCs or dendritic cells were infected either with a canarypox based vector (ALVAC) expressing env and gag/pol genes of FeLV (vCP97) or with the parental vector as control (CPpp). Infections were performed at an M.O.I. of 5 pfu/cells in 50 μL of RPMI 2% FCS for 1.5 hours. The infected cells were washed twice and incubated to allow *ex vivo* re-stimulation of FeLV-specific T cells. The dendritic cell:PBMC ratio is 1:5.

ELISpot Assay

The 96-well plates MultiScreen® HA (Millipore, Bedford, MA) were coated overnight at 4°C with 100 μL per well of 4 $\mu\text{g}/\text{mL}$ primary antibody (AF781, R&D Systems, USA) in carbonate/bicarbonate buffer (0.05 M, pH 9.6). The antibody coated plates were washed 3 times with PBS and unoccupied sites blocked with complete RPMI medium 10% FCS for 2 hours at room temperature. ALVAC-infected/washed PBMCs were added to the wells (0.5 and 0.25×10^6 cells per well) in 200 μL complete medium and incubated 20-24 hours at $37^{\circ}\text{C} + 5\% \text{CO}_2$. PBMCs were stimulated with PMA/ionomycin (PMA 20 ng/mL; ionomycin 10 μM) as positive control. The cells were then removed and cold distilled water was added to each well for 5 minutes. The wells were then washed 3 times in washing buffer (PBS, 0.05% v/v Tween® 20) and incubated overnight with 100 μL of biotinylated antibody (BAF 764, R&D Systems, USA) at 0.5 $\mu\text{g}/\text{mL}$ at 4°C . The plates were washed 3 times in washing buffer, and 100 μL of a 1/200 streptavidin-peroxydase solution (SA-5004, Vector, USA) was added to each well for 1 hour at 37°C . Plates were then washed 3 times in washing buffer and incubated for 15 minutes at room temperature in the dark with the AEC substrate solution (1 mg of AEC is re-suspended in 1 mL of DMF and dissolved in 30 mL of

acetate buffer pH=4.8, 0.2- μ m filtrated 150 μ L of H₂O₂ were added just before use). The plates were extensively washed with tap water and dried. The spots were counted with a CCD camera system (Microvision Instruments, Evry, France). The number of IFN γ -producing cells per million of PBMCs was calculated based on the cell input.

Antibodies, Staining, and Flow Cytometry Analysis

Anti-feline R-PE-conjugated CD4 (clone 3-4F4) and FITC-conjugated CD8 β (clone FT2) were obtained from Southern Biotechnology Associates, Inc., USA. Anti-human APC-conjugated tumor necrosis factor (TNF) α (clone Mab11) was obtained from Becton Dickinson (New Jersey, USA). Cell staining was performed on an average of 5×10^5 cells in ice using 1 μ g of monoclonal antibodies/ 10^6 cells. Anti-TNF α intracellular staining was performed after fixation and permeabilization of the cells using the Cytotfix/Cytoperm™ kit, according to the manufacturer recommendations (Becton Dickinson, NJ, USA). Stained lymphocytes were analyzed with a fluorescence-activated flow cytometer (BD FACScalibur™, Becton Dickinson) calibrated with Calibrite beads (Becton Dickinson). The distribution of debris was assessed on the basis of forward and right-angle scatter before proceeding with the analysis. A total of 105 events in the lymphocyte gate were examined using a 488 nm wavelength excitation. Acquired events were analyzed using Cell Quest Pro™ software (Becton Dickinson).

Statistical Analysis

Variance analyses were performed using STATGRAPHICS® Plus program for Windows (Manugistics, Rockville, MD) to determine statistically significant differences in the generated data. Principal component analysis (PCA) analysis was realized using the Convex software.

RESULTS

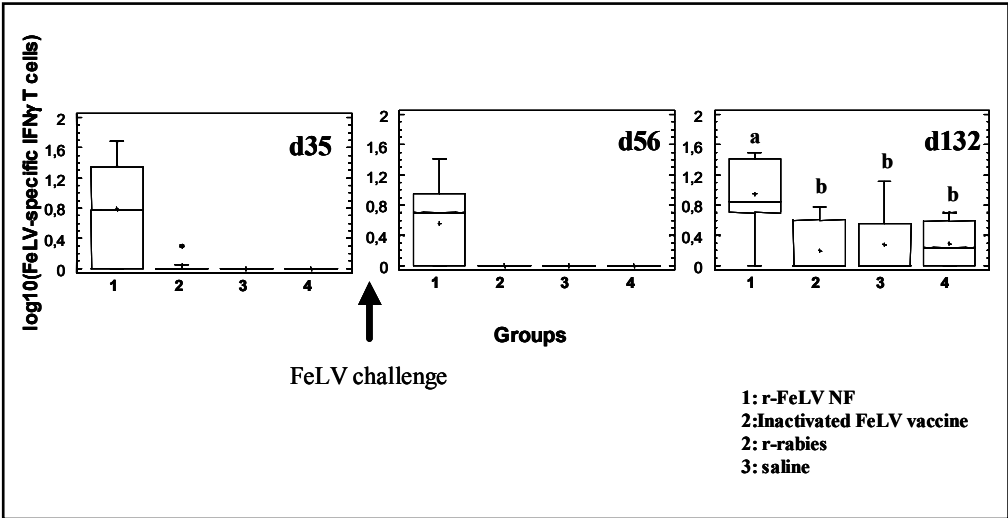
Cats vaccinated with the r-FeLV vaccine delivered by the Vitajet™ device developed a FeLV-specific IFN γ + T cell response

Twelve-week-old kittens were vaccinated either with the ALVAC recombinant vector (107.8 CCID50) expressing the env and gag FeLV antigens by needle-free administration (Vitajet®) or with a conventional KV-FeLV vaccine at Day 0 and Day 21. At Day 35, all of the cats were challenged with a virulent FeLV strain. Fourteen days following the last vaccine administration (Day 35) and 21 and 111 days following the challenge (Day 56 and 132, respectively), blood samples were collected and PBMCs isolated. The fresh PBMCs were ex vivo re-stimulated with the vCP97 ALVAC vector or the parental vector as control. A feline IFN γ ELISpot assay was carried out (Figure 1). Upon ex vivo stimulation, FeLV-specific IFN γ + T cells were detected at each time point analyzed in most of the cats vaccinated with the r-FeLV vaccine. In contrast, this response was not detected in KV-FeLV vaccinated cats at Days 35 and 56. At Day 132, a very weak response was detected in KV-FeLV vaccinated and control groups, while cats from the vaccinated groups consistently showed a FeLV-specific IFN γ + T cell response.

The use of dendritic cells as antigen-presenting cells allows for a more sensitive measurement of the FeLV-specific IFN γ + T cell frequency

In order to increase the sensitivity of our assay, we generated cat autologous monocyte-derived dendritic cells. The fully differentiated dendritic cells were first loaded with FeLV antigens using the vCP97 ALVAC vector and then mixed with in vivo primed PBMCs. The frequency of FeLV-specific IFN γ + T cells after such a stimulation was then compared to the direct stimulation method described above. The frequencies of IFN γ + FeLV-specific T cells at Day 56, comparing both approaches are

Figure 1. r-FeLV-vaccinated cats develop virus-specific IFN γ T cells. Both IFN γ -ELISpot assays were carried out at Days 35 (post-vaccination) and 56 and 132 (post-vaccination and challenge) as described. FeLV-specific responses were determined by subtracting the number of IFN γ events recorded following ex vivo stimulation with the control CPpp construct to the number of IFN γ events recorded following ex vivo stimulation with the FeLV antigen-expressing vCP97 construct. Statistical analysis: a \neq b, $P \leq 0.05$.



illustrated in Figure 2. The results showed that a higher frequency of FeLV-specific IFN γ ⁺ T cells was recovered using the dendritic cells stimulation approach, suggesting that these cells efficiently presented FeLV antigens to in vivo primed specific T cells. Even when using the sensitive dendritic cell approach, only cats from r-FeLV vaccinated group showed a significant cell-mediated immune response.

Cats vaccinated with the r-FeLV vaccine delivered by the Vitajet[®] device developed both CD4 and CD8 TNF α ⁺ FeLV-specific IFN γ ⁺ T cell responses

At Day 132 post-vaccination, PBMCs from all cats were ex vivo re-stimulated with FeLV antigens and subjected to both TNF α intracellular and CD4 and CD8 surface staining. The percentages of both FeLV-specific CD4⁺ TNF α ⁺ and CD8⁺ TNF α ⁺ cells were determined by flow cytometry. Both cell types clearly tended to be higher in the r-FeLV vaccinated group. Moreover, cats presenting a high level of FeLV-specific CD4⁺ TNF α ⁺ T cells were found only in the r-FeLV vaccinated group (Figure 3).

Statistical Comparison of the 4 Groups of Cats: PCA Analysis

All the measured variables (Day 35 IFN γ , Day 56 IFN γ direct and dendritic cell approaches, and Day 132 IFN γ and TNF α) were analyzed in a PCA. The graphical summary of this analysis is shown in Figure 4A. This multivariate analysis revealed that the highest variability (41%) occurred on IFN γ -associated factors (axis 1) on Days 56 and 132 IFN γ post-challenge. The variance analysis on this same axis (Figure 4B) indicates that variation of post-challenge IFN γ secretion is statistically higher in the r-FeLV vaccinated group compared to the other groups. The second component, which accounts for 30% of the variability, mainly expressed both the variations of the IFN γ response measured at Day 35 (pre-challenge) and TNF α responses measured at Day 132 (post-challenge). These responses are shown to be related and cats that responded well at Day 35 tended to show higher TNF α responses in response to the challenge.

Figure 2. Using autologous FeLV antigen-loaded dendritic cells enables recovery of a higher frequency of virus-specific IFN γ + T cells. Monocyte-derived dendritic cell cultures were initiated for all the cats included in the study. After 7 days in culture, fully differentiated dendritic cells were infected either with the vCP97 or the control CPpp constructs. Antigen-loaded dendritic cells were mixed together with PBMCs from the same cat to allow FeLV antigen presentation. FeLV-specific responses were determined by subtracting the number of IFN γ events recorded following ex vivo stimulation with the control CPpp-infected dendritic cells to the number of IFN γ events recorded following ex vivo stimulation with FeLV antigen-expressing vCP97-loaded dendritic cells. Statistical analysis: $a \neq b$, $P \leq 0.05$.

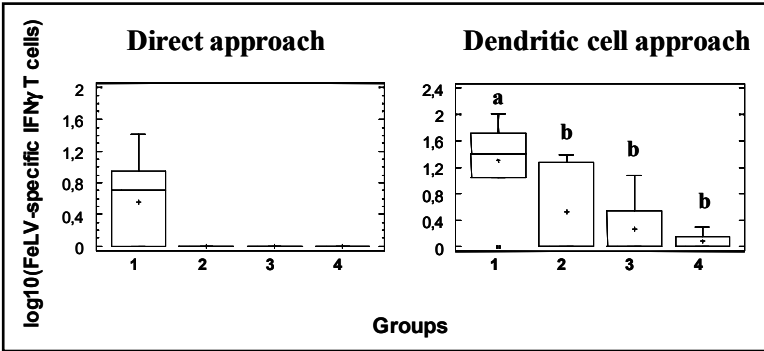
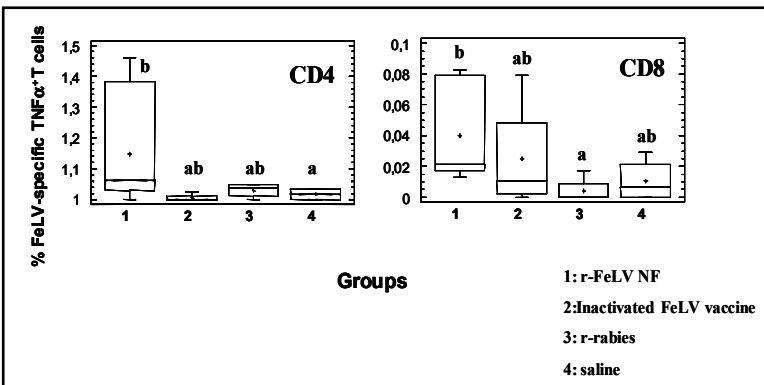


Figure 3. r-FeLV vaccinated and challenged cats present both CD4+ and CD8+ FeLV-specific T cells secreting TNF α . Fresh Day-132 PBMCs were isolated and re-stimulated with FeLV antigens as described. Antigen-stimulated T cells were stained for both CD4 and CD8 and intracellular TNF α . FeLV-specific responses were determined by subtracting the number of IFN γ events recorded following ex vivo stimulation with the control CPpp construct to the number of IFN γ events recorded following ex vivo stimulation with the FeLV antigen-expressing vCP97 construct. Statistical analysis: $a \neq b$, $P \leq 0.05$.



DISCUSSION

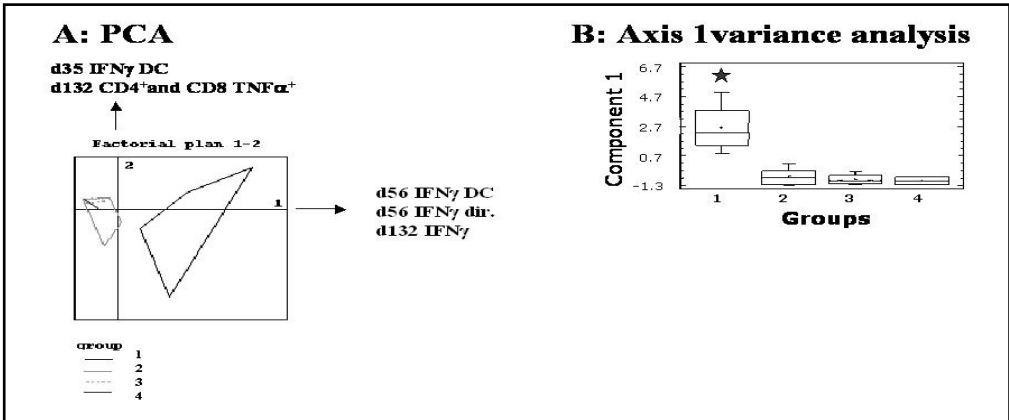
We showed in this study that kittens vaccinated by a recombinant canarypox vector expressing FeLV antigens and delivered by the transdermal route induced post-vaccinal FeLV-specific IFN γ + T cell responses. In contrast, this T cell-mediated immunity could not be detected in response to the

KV-FeLV vaccination. IFN γ is a key component of type I cell-mediated immunity, which has been recognized as a critical actor of anti-viral immunity. Both viral replication inhibition and up-regulation of major histocompatibility complex molecules and activation of antigen-presenting cells have been attributed to IFN γ . Moreover, antigen-specific CD8+ T cells secreting IFN γ in response to their cognate antigen have been shown to present cytotoxic activity, and it is now well recognized that the efficacy of an immune response can be determined by the enumeration of antigen-specific T cells secreting IFN γ .

In response to the challenge, only r-FeLV vaccinated cats showed a significant FeLV IFN γ + T cell response. In con-

trast, both KV-vaccinated and control cats did not develop a consistent IFN γ + T cell-mediated immunity at the time points analyzed which means that these responses were probably not high enough to efficiently fight the virus. We also investigated whether CD4+ and CD8+ T cells from vaccinated and naïve challenge cats would

Figure 4. Principal Component Analysis (PCA). **A.** The PCA provided a global and synthetic view of the associations between the analyzed parameters. The first component (41 % of the global variance) mainly expressed the variations of IFN γ recorded at Day 56 (both stimulation approaches) and Day 132. These 3 parameters are positively correlated amongst themselves and with the first principal component and their variations are represented on this axis. An animal on the right of this axis presented high values for all these parameters. The second component (30 % of the global variance) mainly expressed the variations of Day 35 (DC) and CD4 and CD8 TNF α + T cell response at Day 132. These 3 parameters are positively correlated amongst themselves and with the second principal component, and their variations are represented on this axis. An animal at the bottom of the axis 2 presents high values for all these parameters. **B.** Comparison of the 4 groups was carried out on the first component resulting from the PCA which denotes a statistically significantly higher frequency of FeLV-specific IFN γ + T cell responses for the r-FeLV group.



secrete TNF α in response to FeLV antigens. We found that cats from the r-FeLV-vaccinated group tended to have a higher CD8+ TNF α + FeLV-specific T cell response, although some cats from both the control and KV-FeLV vaccinated groups presented this response, most probably in response to the viral challenge. More surprisingly, we found that the CD4+ TNF α + FeLV-specific T cell response was very low in both challenged control and KV-FeLV vaccinated groups compared to challenged r-FeLV-vaccinated cats. This low CD4+ T cell response may have been induced by the viral infection because FeLV has been shown to reduce T helper functions in infected cats.²⁰ Antigen-specific CD4+ T cell responses have been widely shown to be involved in both quality and duration of virus-specific CTLs²¹⁻²³ and may play a key role in immune defenses against FeLV.

In this study, we compared 2 different approaches of ex vivo antigen-specific T-cell re-stimulation. The direct approach consisted of adding the recombinant

ALVAC vector to the PBMCs from in vivo FeLV-vaccinated or control cats relying on antigen-presenting cells contained in the PBMC population. The second approach consisted of using differentiated autologous dendritic cells derived in vitro from circulating monocytes. We observed that the use of dendritic cells allowed for recovery of a higher level of FeLV-specific T cells. We hypothesized that dendritic cells better processed and presented the FeLV antigens to primed T cells. Analyses of antigen-specific T cell responses in cats have been hampered mainly because of the lack of specific tools and practical methods for re-stimulation of T cells. The antigen-specific ex vivo T cell re-stimulation approaches presented here should open new areas of research regarding diseases and the rational for vaccine development for cats.

All the time points analyzed were combined together in a PCA. The purpose is to obtain a small number of linear combinations of initial parameters, which account for most of the variability in the data. This

exploratory analysis gives a global and synthetic view of the different associations between the parameters and shows the group effect on an overall figure of the observed phenomenon. Thanks to this analysis, the inference is usually carried out on the principal component resulting from the PCA, on which the discrimination seems to be the best. The comparison of the group made on a global criterion, which is a linear combination of correlated and principal parameters, can emphasize the slight differences observed for each parameter separately. The graphical summary presented in Figure 4 showed that the post-challenge IFN γ responses from cats of the r-FeLV-vaccinated group are significantly higher as compared to the other groups. The individual differences observed in terms of frequency of FeLV-specific IFN γ + T cells in this vaccinated group could be explained by the genetic heterogeneity of the cats included in the study (outbred cats). Moreover, this analysis indicated that post-vaccinal IFN γ responses and post-challenge CD4+ and CD8+ TNF α + T-cell responses are associated and positively correlated factors suggesting that pre-existing r-FeLV-induced T cell-mediated immunity is efficiently recalled after the challenge. This finding is of particular importance regarding FeLV protection because it has been reported that recovery from infection is associated with the initial development of virus-specific cytotoxic T cells. In this regard, a recently published study comparing the canarypox-based vaccine and a conventional inactivated vaccine, both given subcutaneously, showed that protection against FeLV viremia was 80% and 50%, respectively.²⁴

CONCLUSION

Altogether, we demonstrated that a recombinant FeLV vaccine given transdermally induced a broad FeLV-specific T cell response including both IFN γ and TNF α secreting cells. Based on previous reports regarding the role of the cell-mediated immune responses for the control of FeLV

infection, the induction of virus-specific cell-mediated immunity described here should greatly help to explain the good performances of that type of vaccine.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge D Jas, PM Guigal, and the animal caretakers for their outstanding management of the cat experiment.

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