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A vector expressing feline mature IL-18 fused to IL-1β antagonist protein signal sequence is an effective adjuvant to a DNA vaccine for feline leukaemia virus

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Abstract

DNA vaccination using vectors expressing the gag/pol and env genes of feline leukaemia virus (FeLV) and plasmids encoding feline interleukin-12 (IL-12) and IL-18 completely protected cats from viraemia following challenge [Hanlon L, Argyle D, Bain D, Nicolson L, Dunham S, Golder MC, et al. Feline leukaemia virus DNA vaccine efficacy is enhanced by coadministration with interleukin-12 (IL-12) and IL-18 expression vectors. J Virol 2001;75:8424–33]. However, the relative contribution of each cytokine gene towards protection is unknown. This study aimed to resolve this issue. IL-12 and IL-18 constructs were modified to ensure effective expression, and bioactivity was demonstrated using specific assays. Kittens were immunised intramuscularly with FeLV DNA and various cytokine constructs. Together with control kittens, these were challenged oronasally with FeLV and monitored for 15 weeks. All six kittens given FeLV, IL-12 and IL-18 were protected from the establishment of persistent viraemia and four from latent infection. Of six kittens immunised with FeLV DNA and IL-18, all were protected from viraemia and five from latent infection. In contrast, three of five kittens given FeLV DNA and IL-12 became persistently viraemic. Therefore, the adjuvant effect on the FeLV DNA vaccine appears to reside in the expression of IL-18.

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1. Introduction

Feline leukaemia virus (FeLV) is a common retrovirus of cats, and a major cause of death. The virus spreads mainly via saliva and nasal secretions [2] and infection results in one of several outcomes. Cats that mount a sufficiently powerful immune response will recover, usually with the development of virus neutralising antibodies (VNAb) [3], and FeLV-specific cytotoxic T cells (CTL) [4]. Infected cats that do not mount an adequate immune response are usually anergic to the virus and become persistently viraemic [5,6]. Most viraemic cats succumb to FeLV-related disease within 2–4 years of infection. Many of the animals that ostensibly recover develop a latent infection in which the virus is repressed by the host immune response to the bone marrow and probably other tissues [7,8]. In the majority of these cases, virus is eliminated from the bone marrow within 30 months of exposure [9,10], but in some cases may persist and may be reactivated in animals that are immunosuppressed or treated with corticosteroids [8].
FedL V infections have been controlled in the field by the detection and isolation of viraemic cats, and more recently also by vaccination. Several types of FedL V vaccines are available for use in pet cats, including inactivated, adjuvanted whole virus [11,12], inactivated, non-adjuvanted whole virus [13], inactivated, adjuvanted virion subunits [14], adjuvanted, recombinant FedL V gp70 subunit [15,16], and a live canarypox-FedL V recombinant expressing FedL V gag and env genes [17]. However, no commercially available FedL V vaccine provides complete protection from both persistent infection and latent bone marrow infection [18], so there is clearly a need for a more effective product.

Studies of the immune response to FedL V have shown that recovery from infection is associated with the initial development of virus-specific CTL followed by the appearance of VNAbs [4]. It follows, therefore, that a vaccine designed to stimulate cell-mediated immunity, such as DNA vaccination, may be particularly useful in blocking the initial stages of FedL V infection. Indeed, a DNA vaccine consisting of plasmid vectors encoding gag/pol and env genes of FedL V/A-Glasgow-1 [19] protected cats against both the establishment of persistent viraemia and latency when administered together with plasmids encoding feline IL-12 and IL-18 [1]. The rationale for using both cytokines was that IL-12 and IL-18 act synergistically on T and natural killer (NK) cells to stimulate the production of interferon-gamma (IFN-\gamma), a mediator of the induction of CTL [20,21]. In the previous vaccination study [1], those animals that were protected by the vaccine had higher virus-specific effector CTL in the peripheral blood and lymphoid organs than cats that became persistently viraemic [4]. However, the relative contribution of each cytokine to the efficacy of the vaccine was not established: the IL-12 construct alone in combination with the FedL V DNA was not tested for efficacy, and the failure of the IL-12 construct to act as an adjuvant might have been due to inappropriate expression of both subunits. In order to resolve this issue, we have now carried out a further study using improved IL-12 and IL-18 cytokine constructs, and have tested the efficacy of each cytokine independently as well as together.

Feline IL-12 is assembled from two protein subunits, p35 and p40 [22,23]. The p40 subunit of IL-12 is able to form a homodimer molecule [24], which can bind the IL-12 receptor without promoting activity, thereby acting as a physiological antagonist [25]. This effect may have influenced the results of the previous study [1]. We have now generated an expression vector containing the two subunits linked by a peptide linker sequence in order to prevent this antagonism [26,27]. Feline IL-12 p40 subunit. Anti-rabbit horseradish peroxidase (HRP) forming units (FFU)/ml. Rabbit anti-feline p40 peptide antibody, kindly provided by Vigil Schijns, Intervet International B.V., was generated using a 17 amino acid peptide sequence from feline IL-12 p40 subunit. Anti-rabbit horseradish peroxidase (HRP) and mouse monoclonal IgG1 anti-protein A antibody was purchased from Sigma. C8.6 IgG1 mouse anti-human IL-12 p40/70 antibody cross-reacted with feline IL-12 (BD PharMingen).

2. Materials and methods

2.1. Cell lines, virus strains and antibodies

All culture media and supplements were purchased from Invitrogen Life Technologies (Paisley, UK) unless otherwise stated. Cells were maintained at 37° C in an atmosphere of 5% CO2. Detection of FedL V and VNAbs was performed using the QN10 cell line [32]. A human renal epithelial cell line, 293T, expressing simian virus 40 (SV40) [33], and LN156 cells recovered from equine lymph nodes were maintained as described previously [34,35].

The FedL V challenge virus was a biological clone of FedL V/A-Glasgow-1 cultured in FEA cells as described previously [36]. The batch of virus used had a titre of 4.2 × 10**5** focus forming units (FFU)/ml.

Rabbit anti-feline p40 peptide antibody, kindly provided by Vigil Schijns, Intervet International B.V., was generated using a 17 amino acid peptide sequence from feline IL-12 p40 subunit. Anti-rabbit horseradish peroxidase (HRP) and mouse monoclonal IgG1 anti-protein A antibody was purchased from Sigma. C8.6 IgG1 mouse anti-human IL-12 p40/70 antibody cross-reacted with feline IL-12 (BD PharMingen).

2.2. Preparation of FedL V antigen and feline cytokine DNA constructs

The FedL V DNA vaccine consisted of a combination of two DNA plasmids, one encoding gag/pol (pUSE1-’CMVT(gag/pol)) and the other encoding env (pUSE1-’CMVT(env)) of FedL V/A-Glasgow-1, as previously described [1].

The cloning of feline signal ILRAP-IL-18 has been described previously [31].

Feline flexi-IL-12 was a single chain fusion construct where subunits p40 and p35 were linked by a peptide linker sequence [26] and maximal bioactivity of murine flexi-IL-12 was found when p40 was encoded at the 5’ end of the construct [27]. Feline p40 and p35 were synthesised by Pfu PCR using the following primers: 5’-ggcggtaacggtgacgaTGCTTCGCTTGTTGCTTGTTGGTTCTCTCTCTGAC-3’, 5’-p40 primer: 5’-ggcggtaacggtgacgaTGCTTCGCTTGTTGCTTGTTGGTTCTCTCTCTGAC-3’, 5’-p35 primer: 5’-ggcggtaacggtgacgaTGCTTCGCTTGTTGCTTGTTGGTTCTCTCTCTGAC-3’. 5’-p40 primer: 5’-ggcggtaacggtgacgaTGCTTCGCTTGTTGCTTGTTGGTTCTCTCTCTGAC-3’. 5’-p35 primer: 5’-ggcggtaacggtgacgaTGCTTCGCTTGTTGCTTGTTGGTTCTCTCTCTGAC-3’. The 3’ p40 and 5’ p35 primers incorporated partial peptide linker sequences which were united at an engineered NarI restriction site to generate full length flexi-IL-12. Feline
flexi-IL-12 was then cloned into the mammalian expression vector pCI-neo (Promega).

2.3. Expression and bioactivity of feline cytokine constructs

The expression and bioactivity of feline ILRAP-IL-18 in vitro was shown by Western blotting and KG-1 bioassay, respectively [31].

In vitro expression of feline flexi-IL-12 was demonstrated by Western blotting. 293T cells were separately transfected with 20 μg DNA of feline flexi-IL-12, feline p40 [1] and pCI-neo using LipofectAMINE and OPTIMEM-1 reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Transfections were harvested after 48 h and feline flexi-IL-12 supernatant was concentrated 10-fold by centrifugation (OmniFuge 2.RS, 15 min at 4 °C using Centripiles cartridges) Samples of supernatant and cell lysate (produced by successive freeze/thaw in dry ice ethanol) were electrophoresed under denaturing conditions on a 12% SDS-PAGE gel and transferred to a PVDF Hybond-P membrane (Amersham Pharmacia Biotech) by immunoblotting. The membrane was exposed to primary antibody (rabbit anti-feline p40 peptide) at a dilution of 1:1500 and a secondary antibody (anti-rabbit HRP) at a concentration of 1:3000.

Bioactivity of feline flexi-IL-12 was shown as previously described for equine IL-12 [35]. Briefly, LN156 equine lymph node cells, stored in liquid nitrogen, were thawed rapidly and supplements were added. Cells were seeded onto a 96-well plate and incubated for 24 h at 37 °C. Ten-fold dilutions of flexi-IL-12 and pCI-neo transfection samples were prepared in triplicate. Also, triplicate dilutions of flexi-IL-12 with 10 ng/ml mouse anti-human IL-12 p40/p70 feline cross-reactive antibody and flexi-IL-12 with 10 ng/ml mouse anti-protein A were prepared. All samples were incubated for 37 °C for 2 h and 100 μl of each dilution were added to LN156 cells and mixed gently. Cells were incubated at 37 °C for 72 h.

Equine IFN-γ was measured using a sandwich ELISA prepared from sheep and rabbit polyclonal antibody raised to recombinant equine IFN-γ expressed in bacteria [35]. A standard curve of amount of recombinant equine IFN-γ against optical density was used to establish levels of equine IFN-γ induced by each dilution series.

2.4. Vaccination protocol

DNA plasmids pUSE1-’CMVT( gag/pol), pUSE1-’CMVT( env), pCI-neo, ILRAP-IL-18 and flexi-IL-12 were purified as described previously [1]. Endotoxin levels in each batch were estimated using the Limulus Amebocyte Lysate technique (BioWhittaker Europe) and the threshold level for endotoxin was taken as <50 EU/mg. DNA was diluted with endotoxin-free PBS (Sigma) to a total volume of 0.2 ml for each inoculation.

Fig. 1. Schedule of the experiment, showing vaccination, viral challenge, blood sampling, virus isolation and antibody detection. Black arrow: immunisation of 100 μg of each DNA construct, intramuscularly to quadriceps femoris; grey arrow: viral challenge of 10⁶ FFU delivered to each nostril; white arrow: blood sampling, at position (1) pre-trial, (2) 48 h post-immunisation, (3) pre-challenge; black diamond: virus isolation; black triangle: virus neutralizing antibody detection; black circle: anti-FeLV antibody; black square: bone marrow sample used for virus isolation.

Thirty-five specific-pathogen free 13–15-week-old kittens, purchased from a commercial source, were arranged randomly into six groups of six, apart from group B which consisted of five animals. Groups were housed in separate rooms, with 9.36 m² of floor area and a height of 2.36 m. Studies were conducted within UK Home Office Inspectorate guidelines and animals were fed a commercial food source and water.

Animals were immunised and sampled as shown in Fig. 1. Blood samples were collected in heparin tubes, and plasma separated and removed for storage at −70 °C. Animals were challenged according to the method used by Harbour et al. [37] (Fig. 1). Each viral challenge was diluted to a volume of 1 ml with RPMI 1640 and administered oronasally, 0.25 ml into each nostril and 0.5 ml orally. Animals were euthanased 15 weeks post-challenge, blood was collected under anaesthesia by intracardiac withdrawal and bone marrow was extracted from the femur by aseptic techniques. All blood and bone marrow samples were tested for FeLV and some blood samples for the presence of VNAb and anti-FeLV antibody.

2.5. Detection of infectious FeLV

The presence of FeLV was established using VNAb and anti-FeLV antibody [32]. Bone marrow cells were cultured for 14–17 days and tested for the presence of FeLV [38]. After the initial culture of cells for 14 days, cells were subcultured 1:2 and cultured for a further 3 days. Culture supernatants were then examined for the presence of FeLV [32].

2.6. Detection of FeLV antibodies

At certain points in the trial (Fig. 1), the presence of VNAb was established in plasma samples by the focus reduction pro-
tocol [32]. Plasma samples taken on day of challenge and 15 weeks later were also tested for antibodies to FeLV proteins by Western blot analysis [39].

2.7. Statistical analyses

Statistical analysis was performed on the virus isolation results. The sum of total positive virus isolation results and sum of total negative virus isolation results for each group was compared using Fisher’s exact test (F.E. test). This test calculates an exact probability value for the relationship between two dichotomous variables, as found in a two by two cross table. Comparison of the VNAb titres in vaccination groups was performed using the Student’s t-test.

3. Results

3.1. Feline IL-12 and IL-18 are expressed and are bioactive in vitro

To ensure that the cytokine gene adjuvants to be included in the vaccines were effective, the constructs used by Hanlon et al. [1], were modified. The expression and bioactivity of feline IL-12 has been described previously [31]. In the present study, the expression of flexi-IL-12 was demonstrated by Western blot analysis of transfection products. Proteins in the supernatant fluid or cell lysate were electrophoresed in a 12% SDS-PAGE gel, transferred to PVDF membranes and incubated with rabbit anti-feline p40 peptide antibody followed by anti-rabbit HRP antibody (Fig. 2). The flexi-IL-12 supernatant contained a 75 kDa protein corresponding to flexi-IL-12 protein, which was not evident in the cell lysate. There was no evidence of IL-12 p40 subunit in the flexi-IL-12 supernatant, but this protein was demonstrated in the p40 transfection supernatant, indicated by a 40 kDa band.

3.2. Inoculation with FeLV DNA vaccine alone, or cytokine genes alone, does not induce protection against FeLV

The modified cytokine constructs were then used as adjuvants for the FeLV DNA vaccine. The level of protection induced by a vaccine was assessed by its ability to prevent infection after viral challenge. A persistently infected cat was defined as one from which infectious virus could be isolated from the plasma 15 weeks after challenge at the end of the trial. Transient infection can occur when a cat is viraemic at some point post-challenge but subsequently becomes negative and remains free of virus for the remainder of the experiment. Latent infection was detected by the isolation of virus from cultured bone marrow cells from cats that were not viraemic at the end of the experiment.

The groups in the study are shown in Table 1. Virus isolation was carried out on blood samples taken at 3-week intervals post-challenge, and on the culture fluids harvested from cells grown from bone marrow samples (Table 1). Thus, the incidence of persistent, latent and total FeLV infection in the vaccination groups was assessed (Table 2). In this study, there were no instances of transient infection.
Table 1

| Group | Vaccine | Adjuvant | Cat | Viraemia at weeks post infection | Marrow virus |
|-------|---------|----------|-----|---------------------------------|-------------|
|       |         |          |     | 3     | 6     | 9     | 12    | 15    |          |
| A     | FeLV    | IL-12 + IL-18 | 1   | −     | −     | −     | −     | −     | +        |
|       |         |          | 2   | −     | −     | −     | −     | −     | +        |
|       |         |          | 3   | −     | −     | −     | −     | −     | −        |
|       |         |          | 4   | −     | −     | −     | −     | −     | −        |
|       |         |          | 5   | −     | −     | −     | −     | −     | −        |
|       |         |          | 6   | −     | −     | −     | −     | −     | −        |
| B     | FeLV    | IL-12    | 8   | −     | +     | +     | +     | +     | +        |
|       |         |          | 9   | −     | −     | −     | −     | −     | −        |
|       |         |          | 10  | −     | +     | +     | +     | +     | +        |
|       |         |          | 11  | −     | +     | +     | +     | +     | +        |
|       |         |          | 12  | −     | −     | −     | −     | −     | −        |
| C     | FeLV    | IL-18    | 13  | −     | −     | −     | −     | −     | −        |
|       |         |          | 14  | −     | −     | −     | −     | −     | −        |
|       |         |          | 15  | −     | −     | −     | −     | −     | −        |
|       |         |          | 16  | −     | −     | −     | −     | −     | −        |
|       |         |          | 17  | −     | −     | −     | −     | −     | −        |
|       |         |          | 18  | −     | −     | −     | −     | −     | −        |
| D     | FeLV    | None     | 19  | +     | +     | +     | +     | +     | +        |
|       |         |          | 20  | −     | −     | −     | −     | −     | +        |
|       |         |          | 21  | −     | −     | −     | −     | −     | −        |
|       |         |          | 22  | −     | +     | +     | +     | +     | +        |
|       |         |          | 23  | +     | +     | +     | +     | +     | +        |
|       |         |          | 24  | −     | +     | +     | +     | +     | +        |
| E     | None    | IL-12 + IL-18 | 25  | −     | −     | −     | −     | −     | +        |
|       |         |          | 26  | +     | +     | +     | +     | +     | +        |
|       |         |          | 27  | −     | −     | −     | −     | −     | −        |
|       |         |          | 28  | +     | +     | +     | +     | +     | +        |
|       |         |          | 29  | +     | +     | +     | +     | +     | +        |
|       |         |          | 30  | −     | −     | −     | −     | −     | −        |
| F     | None    | None     | 31  | +     | +     | +     | +     | +     | +        |
|       |         |          | 32  | −     | −     | −     | −     | −     | +        |
|       |         |          | 33  | −     | +     | +     | +     | +     | +        |
|       |         |          | 34  | +     | −     | +     | +     | +     | +        |
|       |         |          | 35  | +     | +     | +     | +     | +     | +        |
|       |         |          | 36  | −     | −     | −     | −     | −     | −        |

Three weeks after challenge, three of the six unvaccinated control cats (group F) were viraemic, and a further cat, number 33, became viraemic 6 weeks after challenge. These four animals were persistently infected at the termination of the study. Latent infection was detected in the remaining two non-viraemic unvaccinated cats at the end of the study.

In animals inoculated with FeLV DNA vaccine alone (group D), four cats were viraemic 6 weeks after challenge and remained so. Latent infection was detected in one additional non-viraemic animal in this group. Therefore, the FeLV DNA vaccine alone (group D) did not provide any protection against persistent infection when compared with unvaccinated control animals (group F). However, the DNA vaccine reduced the level of latent infection in this group in comparison to unvaccinated animals, although given the small number of animals involved in this experi-

Table 2

| Group | DNA inoculum | Total no. of animals | No. of persistently infected | No. of latently infected | Persistent + latent infection/total no. of animals |
|-------|--------------|----------------------|------------------------------|--------------------------|-----------------------------------------------|
|       | FeLV | IL-12 + IL-18 |                        |                            |                                             |
| A     | +    | +               | 6                         | 0                         | 2                                             |
| B     | +    | +               | 5                         | 3                         | 0                                             |
| C     | +    | −               | 6                         | 0                         | 1                                             |
| D     | +    | −               | 6                         | 4                         | 1                                             |
| E     | +    | +               | 6                         | 3                         | 2                                             |
| F     | −    | −               | 6                         | 4                         | 2                                             |

LH O’Donovan et al. / Vaccine 23 (2005) 3814–3823
In control animals inoculated with flexi-IL-12 and ILRAP-IL-18 (group E), three cats were persistently infected post-challenge. Of the three remaining animals, two were found to have a latent infection. Although fewer cats were infected, these results suggest that the cytokines alone did not provide any significant protection from challenge.

In conclusion, neither DNA vaccine nor cytokine gene adjuvants alone provided significant protection from persistent or latent infection.

3.3. Administration of IL-12 and IL-18 cytokine genes with FeLV DNA provides complete protection against FeLV viraemia

Kittens immunised with both gene adjuvants, flexi-IL-12 and ILRAP-IL-18 (group A), were consistently free of viraemia throughout the experiment. Two of these six cats, cats 1 and 2, were latently infected with virus. This adjuvant combination, therefore, elicited a high level of protection compared to the combined level of viraemia and latent infection in unvaccinated animals (F.E. test, \( p = 0.030 \)).

3.4. The adjuvant effect resides in IL-18 and not IL-12

Group B comprised kittens given FeLV DNA and flexi-IL-12. Of the five vaccinated cats, three were persistently infected at 15 weeks post-challenge. When the bone marrow of this group was analysed, virus was isolated from only the viraemic cats, but not from the two non-viraemic animals. When these results are compared to the levels of infection in unvaccinated animals and those injected with DNA vaccine alone, this indicates that flexi-IL-12 DNA does not enhance the efficacy of the FeLV DNA vaccine.

| Group | Vaccine | Cat | Virus isolated from | Antibody |
|-------|---------|-----|---------------------|----------|
|       |         |     | Plasma | Marrow | VNAb | gp30 | p15E | p27 |
| A     | FeLV    | 1   | –      | +      | 16   | –    | +    | +    |
|       | IL-12 + IL-18 | 2  | –      | –      | 64   | +    | +    | +    |
|       |         | 3   | –      | –      | 64   | (s)  | +    | (s)  |
|       |         | 4   | –      | –      | 256  | +    | +    | (s)  |
|       |         | 5   | –      | –      | 16   | –    | +    | (s)  |
|       |         | 6   | –      | –      | 16   | –    | (s)  | –    |
| B     | FeLV    | 8   | +      | +      | 0    | –    | –    | +    |
|       | IL-12   | 9   | –      | +      | 64   | +    | (s)  | –    |
|       |         | 10  | +      | +      | 0    | –    | (s)  | (+)  |
|       |         | 11  | +      | +      | 16   | +    | +    | (+)  |
|       |         | 12  | –      | –      | 1024 | +    | +    | (+)  |
| C     | FeLV    | 13  | –      | –      | 256  | +    | +    | (s)  |
|       | IL-18   | 14  | –      | –      | 0    | +    | –    | –    |
|       |         | 15  | –      | –      | 16   | (s)  | +    | (s)  |
|       |         | 16  | –      | –      | 16   | +    | (s)  | –    |
|       |         | 17  | –      | –      | 4    | (s)  | +    | –    |
|       |         | 18  | –      | +      | 256  | +    | +    | –    |
| D     | FeLV    | 19  | +      | +      | 0    | +    | +    | +    |
|       | None    | 20  | –      | +      | 0    | +    | (s)  | –    |
|       |         | 21  | –      | –      | 64   | (s)  | +    | (s)  |
|       |         | 22  | +      | +      | 0    | –    | –    | –    |
|       |         | 23  | +      | +      | 0    | –    | –    | (s)  |
|       |         | 24  | +      | +      | 0    | +    | +    | (+)  |
| E     | None    | 25  | –      | +      | 256  | +    | +    | –    |
|       | IL-12 + IL-18 | 26 | +      | +      | 0    | +    | +    | (+)  |
|       |         | 27  | –      | +      | 64   | (+)  | +    | –    |
|       |         | 28  | +      | +      | 0    | –    | (s)  | +    |
|       |         | 29  | +      | +      | 0    | +    | +    | +    |
|       |         | 30  | –      | –      | 0    | +    | –    | –    |
| F     | None    | 31  | +      | +      | 0    | –    | –    | (s)  |
|       | None    | 32  | –      | +      | 1024 | +    | +    | (+)  |
|       |         | 33  | +      | +      | 0    | +    | +    | (+)  |
|       |         | 34  | +      | +      | 0    | (s)  | +    | –    |
|       |         | 35  | +      | +      | 0    | +    | +    | (+)  |
|       |         | 36  | –      | +      | 16   | +    | +    | (+)  |
The kittens of group C were inoculated with ILRAP-IL-18 as a gene adjuvant to the FeLV DNA vaccine. Virus was not detectable in the blood of any of the six animals post-challenge, compared to four animals in the control group F (p = 0.030). Only one cat, number 18, was found to be harbouring FeLV in the bone marrow.

When the total of persistent and latent infection in vaccine groups was assessed, one animal from group C was infected compared to six unvaccinated cats (group F) (F.E. test, p = 0.008), and five animals inoculated with vaccine alone (group D) (F.E. test, p = 0.040). These results strongly suggest that ILRAP-IL-18 DNA enhances the efficacy of the DNA vaccine, and that the DNA vaccine in combination with ILRAP-IL-18 is an extremely effective vaccine against FeLV infection. This result was only statistically significant when the combination of persistent and latent infection was combined.

3.5. Antibody production after challenge, but not following vaccination, indicates that vaccinal immunity was not sterilising

The level of protection afforded by the two vaccines that contained IL-18 in addition to FeLV DNA was of a very high order. To determine if any of these vaccines had induced sterilising immunity, assays of anti-FeLV antibodies were carried out on the plasma of the kittens after their course of vaccination, on the day of challenge, and 15 weeks later at the end of the trial. From our experience of other similar DNA vaccines in the cat [1,40], it was anticipated that none of the DNA vaccines would induce antibodies. Consequently, the induction of sterilising immunity by any of the vaccines should result in blocking the replication of the challenge virus and might not be followed by the appearance of antiviral antibodies in the plasma. The results of assays for VNAb and antibodies to individual FeLV proteins detected by Western blotting are shown in Table 3.

None of the kittens had antibodies following vaccination. In contrast, 15 weeks after challenge nearly all of the kittens had antibodies in at least one of the assays, indicating that they had experienced virus growth. As expected, VNAb were present in almost all (18 of 21) of the non-viraemic cats and in very few (1 of 14) of the persistently infected cats. The median VNAb titre of the 12 protected cats in group A and C that had received FeLV DNA and IL-18 DNA was 16 compared to 64 in the 7 non-viraemic cats in groups D, E and F that received control preparations. However, this difference was not significant (t-test, p = 0.128). By immunoblotting, antibodies to gp70 (SU), p27 (CA) or p15E (TM) could be identified. Again there were no clear differences in the responses of cats in each group.

4. Discussion

We have confirmed the findings of Hanlon et al. [1], that a vaccine containing FeLV gag/pol and em genes together with the cytokine gene adjuvants IL-12 and IL-18 provides complete protection against FeLV viraemia in cats. The rationale for using these particular cytokines was that they induce cell-mediated immune responses, which at that time were believed to be responsible for protection against FeLV infection, and are now known to be involved [6]. While each cytokine promotes a Th1 type of immune response, IL-12 and IL-18 act synergistically by enhancing the expression of IFN-γ [20], a pleotropic cytokine that is crucial to the normal functioning of cell-mediated immunity [41]. In the previous FeLV DNA vaccine study, the relative role of each cytokine gene was not established; a group of cats vaccinated with FeLV DNA and IL-18 alone was not included in the vaccine trial and there were doubts about the efficacy of the IL-12 construct. In particular, since the subunits of IL-12 were expressed from separate plasmids, it was possible that p40 homodimer formation antagonised active IL-12 [25], inhibiting the adjuvant effect of the cytokine. Therefore, in the present study a primary aim was to modify the cytokine constructs of feline IL-12 and IL-18 and to show that each cytokine was expressed at high levels in vitro and was bioactive, prior to their use in the vaccine experiment.

The cloning of feline flexi-IL-12 originated from the design used to clone human and murine IL-12 constructs [26,27]. The two subunits, p35 and p40 were linked by a peptide linker sequence (Gly-Gly-Gly-Gly-Ser), minus the leader sequence of the downstream subunit [26]. Several other IL-12 constructs have been reported, including subunits encoded on separate plasmids [1], subunits encoded in tandem under a variety of promoter systems [42,43], the use of an internal ribosome entry site sequence between subunits [44], and a self-cleaving peptide 2A of foot-and-mouth disease virus [45]. However, only the structure of flexi-IL-12 ensures that there is production of equimolar amounts of each subunit so that p40 homodimer formation is prevented. Studies on murine IL-12 constructs found that flexi-IL-12 with the p40 subunit situated upstream, elicited the highest level of bioactivity of all constructs. Accordingly, feline flexi-IL-12 was designed in this way.

The flexi-IL-12 was synthesised and released from transfected cells with no degradation or release of p40 protein subunit. Further, the bioactivity of the product was verified using an assay of IFN-γ production from equine lymph node cells [35]. Ideally, an assay measuring IFN-γ production from feline cells should have been used. However, these cells were in short supply and consequently, attempts to develop a feline specific assay were unsuccessful. The use of a neutralising and non-neutralising antibody in this assay provided evidence that the IFN-γ stimulation was specific to the feline IL-12 protein present in the sample.

The ILRAP-IL-18 that was used in the vaccine trial was believed to be an improvement on the construct used previously. The modified plasmid was shown to be expressed at high level, and the IL-18 to be secreted from the transfected cells and to have bioactivity by Western blot analysis and bioassay in vitro [31,46].
The FeLV DNA component of the vaccine consisted of a combination of two plasmid vectors, pUSE1 CMVT (gag/pol) and pUSE1' CMVT(e20) of FeLV/A/Glasgow-1. This vaccine was identical to that used in the previous trial and contains all the viral genes of FeLV. It has been shown that these genes are expressed in vitro [1], and that cotransfection of the plasmids produced virus-like particles that were capable of infecting susceptible cells [23,1].

A further modification to the preceding vaccination study was the use of oronasal route rather than intraperitoneal route for administration of the challenge virus. Oronasal administration is the preferred method as it more closely simulates natural infection than intraperitoneal inoculation that was used previously [1]. We considered that it was important to establish whether a vaccine administered by parental inoculation could protect against challenge by the natural mucosal route. In order to overcome the natural resistance to oronasal FeLV infection that develops in kittens of the age used in the trial, a protocol was employed in which fractionated doses of virus were given over a period of 8 days. Previously, 83% of 15–17-week-old cats developed persistent viremia using this schedule [37]. In the present study, only 4/6 of the non-vaccinated control kittens became persistently viremic, which per se made comparison of the efficacy of each vaccine difficult because of the small numbers of animals in each group. However, the remaining two cats had latent infections and comparisons based on the total number of overt and covert infections allowed more meaningful comparisons to be made.

Our major aim was to establish whether immune protection afforded by a FeLV DNA-vaccine with IL-12 and IL-18 was due to synergism between the two cytokines, or due to a single cytokine acting alone. Clearly, the combination of cytokine genes together with FeLV DNA provided excellent protection, as before. We also found that ILRAP-IL-18 alone was sufficiently potent as an adjuvant to protect animals from persistent viremia and latent infection, whereas the flexi-IL-12 adjuvant did not produce significant protection. This result is in line with studies of other viruses that failed to demonstrate a positive effect of IL-12 on DNA vaccination, including FIV [47], feline coronavirus [48] or Japanese encephalitis virus (JEV) [49]. Indeed, in some of these studies, inhibition of the anti-viral immune response was found. In certain cases, immunosuppression has been related to the dose of IL-12 DNA and the timing of IL-12 administration. Thus, high doses of recombinant murine IL-12 increased the susceptibility of mice to lymphocytic choriomeningitis virus whereas lower doses enhanced the immune response [50]; and an adenovirus vector expressing IL-12 enhanced the cellular immune response to a hepatitis C vaccine, but higher doses of IL-12 abrogated this effect [51]. In addition, immunosuppression was induced when IL-12 was administered before or together with JEV, but not if given after the immunisation [49].

In the present study, there was no evidence that delivery of the flexi-IL-12 construct had an effect on vaccination or infection, or inhibited the adjuvant effect of IL-18. Thus, it had no effect on the outcome of the challenge when administered alone with FeLV-DNA, or with FeLV DNA and IL-RAP IL-18. Although the IL-12 construct was expressed in vitro, we cannot be sure that there was high level expression in vivo.

It is most likely that the IL-18 component in the vaccine was the effective adjuvant. Many other studies have shown the positive effect of IL-18 DNA as a gene adjuvant to DNA vaccination [52,53]. The predominant function of IL-18 in the immune system is the stimulation of the Th1-type immune response through production of IFN-γ [20,21]. IL-18 also enhances the proliferation of T cells in a dose-dependent manner, an effect that is not IFN-γ dependent [20]. In addition, this cytokine selectively enhances Fas ligand (FasL)-mediated cytotoxicity of murine Th1 cells, and stimulates perforin-dependent cytotoxicity of NK cells [54,55]. Therefore, the functions of IL-18 explain the impressive protective effect of ILRAP-IL-18 that was observed in this study. As a gene adjuvant, ILRAP-IL-18 enhanced the cell-mediated immune response that has been shown to be vital in resistance to FeLV, particularly in the early stages of viral infection [6].

A cellular immune response, probably mediated through CTL, is alone sufficient to protect cats from FeLV infection [6]. As in the previous trial, none of the vaccines induced VNAb before challenge. In fact no commercial FeLV vaccine consistently induces VNAb, although non-neutralising antibodies to the antigens in the vaccine, such as gp70, are elicited [32,56]. Analysis of the few feline DNA vaccination experiments that have been documented, show varying humoral responses to the antigen concerned. In one case, a minimalistic immunogenic defined gene expression vector, which expressed FIV gp140 and feline IL-12, elicited protection without producing a specific humoral response [57]. However, a DNA plasmid encoding FIV gp120 was found to induce a virus-specific antibody response [58]. Evidence suggests that the particular viral antigen used, as well as the vaccine schedule and route of administration have a strong influence over the presence or type of immune response produced, including the development of specific antibodies. While the level of protection achieved in this trial was very high, it was not due to sterilising immunity since anti-FeLV antibodies were produced by all of the cats following challenge. However, the median VNAb titre of the kittens vaccinated with FeLV DNA and IL-18 DNA that resisted the challenge was lower than that of the few cats in the control groups that did not become viraemic. Although this difference in titre was not significantly different, the result gives an indication that virus growth might have been inhibited in the vaccinated kittens immediately following challenge.

Overall, this study shows that of the two cytokine adjuvants used in combination with this FeLV DNA vaccine, ILRAP-IL-18 is the dominant active factor responsible for eliciting protection from viral challenge.
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