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Systemic DNA immunization against ovine lentivirus using particle-mediated epidermal delivery and modified vaccinia Ankara encoding the gag and/or env genes

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Abstract

To determine whether systemic immunization with plasmid DNA and virus vector against visna/maedi virus (VMV) would induce protective immune responses, sheep were immunized with VMV gag and/or env sequences using particle-mediated epidermal bombardment and injection of recombinant modified vaccinia Ankara. The results showed that immunization induced both humoral and cell-mediated responses prior to and after virus challenge. The vaccination protocol did not prevent infection, but immunization with the gag gene or a combination of gag and env genes resulted in significantly reduced provirus loads in blood and mediastinal lymph node, respectively. Provirus loads in lung and draining lymph node were unaffected, but p25 expression was undetectable in lungs of animals immunized with a combination of gag and env genes. Analysis of target tissues for lesions at post-mortem showed that immunization with the env gene caused a significant increase in lesion score, while the gag gene or a combination of gag and env genes had no effect. Inclusion of the ovine interferon-γ gene in the initial priming mixture had minimal effect on immune responses, provirus load, or lesion development, although it resulted in a decreased p25 expression in the lung. The results thus show that systemic immunization with gag or a combination of gag and env genes reduces provirus load in blood and lymphoid tissue, respectively whereas env immunization has no effect on provirus load but increased lesion development.

Keywords: Sheep; Lentivirus; DNA vaccination; Particle-mediated epidermal bombardment; Gene gun; Immune response

1. Introduction

Visna/maedi virus (VMV) and caprine arthritis encephalitis virus (CAEV) are members of the Lentivirus genus and are collectively known as small ruminant lentiviruses (SRLV). These viruses cause slow progressive infection of sheep and goats which results in the development of a multisystem disease variably involving the lungs, brain, joints and mammary glands [1]. VMV and CAEV target cells of the

monocyte macrophage lineage and do not infect T cells, in contrast to the related immunodeficiency viruses of man (HIV), monkeys (SIV), and cats (FIV) [2], [3] and [4]. Transmission of VMV and CAEV occurs from infected mothers to offspring by ingestion of virus-containing colostrum and milk and between adults through contact with infected material such as respiratory secretions. Both humoral and cell-mediated immune responses are generated in the infected animals during the asymptomatic period, though these fail to clear the virus and animals remain infected for life [1].

Several reports have addressed the issue of whether vaccination, especially with viral structural proteins, could control SRLV. Inactivated virus was utilized in early studies. However, these resulted in the appearance of exacerbated disease with more rapid onset [5], [6] and [7]. Similarly, immunization of sheep with an immunodominant T-helper peptide derived from the CAEV GAG protein in adjuvant resulted in increased virus load after intravenous challenge [8]. The lesions in SRLV infections are thought to be immune mediated, since immunosuppression reduces tissue inflammation [9], and depletion of CD4+ T cells causes a reduction in the number of virus-infected macrophages in vivo [10]. The results of the vaccine studies were thus interpreted to mean that immunization simply resulted in immune responses that stimulated virus replication and caused tissue damage. However, recently it has been shown that immunization of goats with recombinant gp135 glycoprotein of CAEV induces neutralizing antibodies [11], suggesting that protective effects may be possible. Indeed, the use of plasmid DNA vaccination or live viral vectors encoding CAEV env gene has demonstrated that reductions in virus load and disease can be achieved [12].

Particle-mediated epidermal delivery (PMED) has been used in many vaccine studies with increasing success not only in rodent models but also in humans, non-human primates and several veterinary species [13]. Comparisons of PMED with needle-based approaches to DNA vaccine delivery show that PMED often induces higher antibody and CD8+ T cell responses [14] and [15]. PMED has also been shown to induce protective responses against SIV and herpesviruses at mucosal sites [16], [17] and [18]. This feature would be important for pathogens such as VMV and CAEV that use the mucosal route. In a recent study by Gonzalez et al. [19], particle-mediated bombardment of mucosal tissues was used to immunize sheep with the VMV gene (env) encoding the two envelope proteins. The results showed reduced virus load over a period of 18 months before the challenge virus re-appeared. The results suggested that mucosal immunization with the VMV env gene could dampen virus replication for an extended period.

In recent years, heterologous prime-boost regimes have been assessed in vaccination development whereby priming is achieved by plasmid DNA constructs and boosting by a recombinant (viral or bacterial), recombinant proteins or synthetic peptides [20], [21] and [22]. In addition, immune modulators such as cytokines and chemokines have been included in the immunization protocol. In rodent models, molecular adjuvants such as IFN-γ, IL-12, GM-CSF often have profound effects on immune responses generated [23]. Recently, we investigated the effect of a prime-boost protocol whereby sheep were immunized with VMV gag gene (encoding the virus core proteins) and env gene via the respiratory tract. Plasmid DNA/polyethylenimine complexes were used to prime and boost, then a recombinant modified vaccinia Ankara vector encoding the same genes was given as a heterologous boost [24]. The results showed that env gene immunization induced reduced provirus load in blood, while the gag gene immunization was associated with reduced lesion formation. Inclusion of IFN-γ plasmid in the immunization mixture did not enhance either humoral or cell-mediated immune responses or protective effects, suggesting that this cytokine was not a good mucosal molecular adjuvant for SRLV.

In the present study, we examined the effect of immunizing with VMV genes encoding the p55gag precursor, gp150env precursor or a combination of these using PMED to prime and boost, and recombinant modified vaccinia Ankara (MVA) to provide a protein boost. We also investigated whether including the IFN-γ gene (ifn) would enhance any protective effect when delivered via the skin.

2. Materials and methods

2.1. Sheep for vaccination experiments

One- to six-year old Lleyn sheep (females and castrated males) were obtained from a VMV-free accredited flock. Prior to immunization, absence of VMV was confirmed by commercially available ELISA (Elitest MVV/CAEV, Hyphen Biomed) following the manufacturer's instructions and by real-time PCR as described below. The animal experiments were performed at three European centres in accordance with national regulations and institutional guidelines.

2.2. Viruses and plasmids

VMV strain EV1 [25] was grown and titred as described previously [26]. The same stock of virus was used to challenge sheep in all three centres as described previously [24]. Recombinant modified vaccinia virus Ankara (MVA) expressing either the gag p55 or the env gp150 genes of VMV EV1 or beta-galactosidase (for control immunization, MVA-pSC11) were described previously [27] and [24]. Recombinant MVAs were named MVA-gag and MVA-env, respectively. MVA-gag and MVA-env were shown to express GAG and ENV, respectively by Western blot using anti-GAG and anti-SU ENV specific rabbit sera (data not shown and [27]). For injection, recombinant MVAs were purified over a sucrose cushion and then a sucrose gradient before they were pelleted and re-suspended in 1 mM Tris–HCl pH 9.0. Virus was stored in aliquots at −80 °C and an aliquot used to determine titre. Virus was diluted in PBS for administration to animals, and 108 pfu was given per animal subcutaneously.

Plasmids used for immunizations in this study were derivates of pN3-EGFP (Clontech Laboratories, Inc.) from which the EGFP gene was removed to generate plasmid pN3 as described [24]. This was used as the control empty plasmid with no expressed gene in immunizations as well as the parental plasmid to make pN3-gag, pN3-env and pN3-ifn with the VMV gagp55 and envgp150 genes and the ovine ifn-γ gene, respectively. Expression of p55 GAG and gp150 ENV was determined by RT-PCR and Western blotting (data not shown) and [27]. Expression of IFN-γ in vitro was determined by RT-PCR and ELISA [24].

2.3. Preparation of DNA-coated gold particles for gene gun immunization

Gene gun parameters used were as previously described [28]. In brief, 22–25 mg of 1 μm gold particles (BioRad Laboratories Ltd.) were coated with DNA by addition of 100 μg plasmid DNA (1 mg/ml in water), 100 μl 0.05 M spermidine (Sigma–Aldrich Company Ltd.) and 100 μl 1 M CaCl2 (Sigma–Aldrich Company Ltd.), resulting in a DNA loading ratio of 4 μg/mg gold. The mixture was precipitated for 10 min at room temperature and subsequently washed six times in 1 ml dehydrated 99.9% (v/v) ethanol (VWR International Ltd.). The DNA/gold particles were then re-suspended in 2.4 ml of ethanol containing 0.05 mg/ml polyvinylpyrrolidone. Each cartridge, containing approximately 1 μg of plasmid DNA, was delivered using the Helios Gene gun system (BioRad Laboratories Ltd.). The gene gun discharge pressure used to propel the DNA-coated gold particles into the dermal–epidermal junction was 500 psi.

2.4. Expression and purification of recombinant GAG proteins

Plasmid clones of VMV strain EV1 gag p25 and p17 (in pRSET plasmids) have been described previously [29]. Recombinant p25 and p17 and mock preparations were produced as previously described [24].

2.5. Experimental design

2.5.1. Immunization

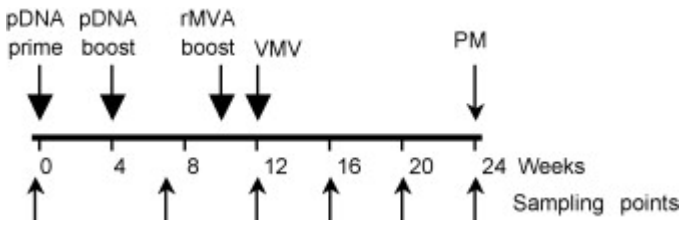
The immunization groups and immunogens used are shown in Table 1 and the experimental design time points are given in Fig. 1. Groups and the plasmids that were administered were as follows: gag (n = 8) – pN3-gag and pN3; gag-ifn (n = 8) – pN3-gag and pN3-ifn; env (n = 8) – pN3-env and pN3; env-ifn (n = 8) – pN3-env and pN3-ifn; gag-env (n = 10) – pN3-gag, pN3-env and pN3; gag-env-ifn (n = 10) – pN3-gag, pN3-env and pN3-ifn; control (n = 22) – pN3. All experimental groups received a total of 30 μg plasmid at weeks 0 and 4, made up of 10 μg of each VMV or IFN-γ gene plasmid and pN3. The control group received 30 μg pN3. Sheep were boosted with 108 pfu recombinant MVA subcutaneously at week 10. Groups gag and gag-ifn were given MVA-gag and MVA-pSC11; env and env-ifn were given MVA-env and MVA-pSC11; gag-env and gag-env-ifn were given MVA-gag and MVA-env (108 pfu each virus). The control group received MVA-pSC11.

Table 1.

Immunization groups and immunogens used in the study.

Sheep group	Number of animals	Immunogen	
		Plasmid	MVA
Control	22	pN3	MVA-pSC11
gag	8	pN3-gag + pN3	MVA-gag + MVA-pSC11
gag-ifn	8	pN3-gag + ifn	MVA-gag + MVA-pSC11
env	8	pN3-env + pN3	MVA-env + MVA-pSC11
env-ifn	8	pN3-env + pN3-ifn	MVA-env + MVA-pSC11
gag-env	10	pN3-gag + pN3-env + pN3	MVA-gag + MVA-env
gag-env-ifn	10	pN3-gag + pN3-env + pN3-ifn	MVA-gag + MVA-env

Fig. 1.



Experimental design. The times of epidermal immunization with plasmid DNA (pDNA), subcutaneous injection with recombinant MVA (rMVA), and intra-tracheal challenge with VMV are shown. Animals were bled before immunization (priming at week 0); after plasmid DNA immunization (week 7); after recombinant MVA boost and before challenge (week 12); and after challenge (weeks 16, 20 and 24). Animals were slaughtered at week 24–25 and tissue samples taken.

The immunizations were performed at three different laboratories as follows: Laboratory 1 – control, gag and gag-ifn; Laboratory 2 – control, env and env-ifn; and Laboratory 3 – control, gag-env and gag-env-ifn.

2.5.2. VMV challenge

Challenge was performed 12 weeks post-priming by injection of 1 × 103 TCID50 VMV EV1 in 1 ml of PBS via the intra-tracheal route as described previously [30].

2.5.3. Sampling

Blood samples were taken before the start of immunization (up to 2 weeks before priming, which was done at week 0), after DNA priming and boosting (week 7), after recombinant MVA boosting but before challenge (week 12), and after VMV challenge (weeks 16, 20 and 24 i.e. weeks 4, 8 and 12 post-challenge). Sheep were euthanased and necropsied 24–25 weeks after the start of the experiment (12–13 weeks post-challenge) and lung and mediastinal lymph node samples taken.

2.6. Measurement of anti-VMV antibodies in serum

Serum was taken from clotted blood, stored at −20 °C and used to measure anti-VMV antibodies in an indirect ELISA using whole VMV antigen as previously described [24]. The reciprocal of the last positive serum dilution was taken as the titre of the antibody and the titre was expressed as log2(titre/50) i.e. negative sera were given a value of 0 and titres of 100, 200, etc., were assigned values of 1, 2 and so on. Untransformed titres were analysed statistically.

For Western blotting, 100 µl of virus antigen prepared as described above were boiled with 100 µl 2 × Laemmli sample buffer (125 mM Tris buffer with 5% mercaptoethanol, 2% SDS, pH 6.8), applied to a 4.5% stacking gel and a 10–16% gradient separation gel and blotted onto a transfer membrane following electrophoresis. Transfer was carried out at 4 °C for 120 min in 25 mM Tris–glycine buffer, pH 8.8, containing 20% methanol, at 100 V and 250 mA. After the transfer, the membrane was blocked for 1 h at room temperature with 0.1 M Tris–HCl-buffered saline, pH 7.8, containing 0.5% Tween 20 and 2% low fat milk powder. The serum and conjugate was diluted in 0.1 M Tris–HCl-buffered saline, pH 7.8, containing 0.1% Tween 20 (TBS-T) and 1% low fat milk powder. After blocking, the membrane was cut into strips and incubated with serum samples diluted 1:500 overnight at 4 °C on a roller. Rabbit anti-goat IgG conjugated to alkaline phosphatase was used at a dilution of 1:5000 for 1 h at room temperature. The blots were washed extensively in TBS-T between each step. The blots were developed with NBT/BCIP diluted 1:50 in AP buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl2 pH 9.5). Prestained molecular markers were used in the immunoblotting.

Neutralization tests were performed as described previously [24]. Neutralization titres of the sera were calculated as the reciprocal of the serum dilution that caused complete neutralization in 50% of inoculated cultures.

2.7. Measurement of anti-VMV T cell reactivity

Blood was collected into heparin (10 U/ml final) and peripheral blood mononuclear cells prepared by buffy coat and Ficoll-Metrizoate gradients, then suspended in RPMI-1640 medium with 25 mM HEPES (Sigma–Aldrich Company Ltd.), supplemented with 2 mM l-glutamine, 50 μM beta-mercaptoethanol, antibiotics (100 U penicillin and 100 μg streptomycin/ml), 2.5 μg amphotericin B/ml and 10% foetal calf serum (FCS) (10% RPMI) [31]. T cell proliferation, cytotoxicity, and IFN-γ production were measured as described previously [31] and [24]. The T cell proliferation stimulation index (SI) for each antigen was calculated using the formula SI = cpm with antigen/cpm with mock antigen. Results were analysed using the median stimulation index of the antigen dilutions. For the CTL assay, animals were deemed to show the presence of precursor CTL if the percentage specific 51Cr release from VMV infected minus mock infected autologous cells was >10% above that from heterologous infected minus mock infected cells [31], in which the killing of mock infected and heterologous cells was less than 10%. The frequency of CTL positive animals was used for analysis of results.

The results of the real-time RT-PCR assays for IFN-γ RNA expression were taken as Ct values where Ct is defined as the threshold cycle of the PCR at which amplified product was first detected. For analysis, cytokine levels were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Analysis was performed using the mathematical model described by Pfaffl [32]. Relative quantification of IFN-γ target gene transcript was made in comparison to GAPDH reference gene transcript using the equation: ratio = (Etarget)Ct target(control − sample)/(Eref)Ct ref(control – sample) where Etarget and Eref are the real-time PCR efficiencies of IFN-γ and GAPDH gene PCRs, respectively. Due to technical limitations samples from sheep immunized with gag-env or gag-env-ifn that were stimulated with whole virus were not analysed.

2.8. Measurement of proviral load

Provirus DNA loads in blood and lung and mediastinal lymph node tissue were measured by real-time PCR as described previously [24]. The results are expressed as mean copy number per microgram of template DNA.

2.9. Determination of pathological changes

Lungs were removed aseptically and samples taken from four different lung lobes [the right accessory (cranial) lobe, the right apical (medial) lobe, the right caudal lobe and the left cardiac (cranial) lung lobe)] and mediastinal lymph node, and subjected to histological analysis as described previously [24]. Pathological changes typical of VMV infection in the lung were scored as follows: in the lung, lymphoid follicle hyperplasia and interstitial pneumonia as well as bronchiole-associated lymphoid tissue hyperplasia,

perivascular infiltrates, congestion and oedema were scored on a scale of 0–4; and in the mediastinal lymph node, lymphoid follicle reactivity and cortical hyperplasia were scored on a scale of 0–2. The mean of all scores was calculated for each animal.

2.10. Immunochemistry

Immunostaining for VMV p25 was performed on tissues obtained from the right caudal lung lobe. Tissue samples were collected and fixed in Bouin solution for 6–8 h, followed by several 70% alcohol washes. Samples were paraffin-embedded and 4 µm sections were stained by immunohistochemical labelling using Envision PlusTM peroxidase® method (Dako Cytomation). Antigen retrieval was carried out in citrate buffer pH 6, for 3 min in a pressure cooker and cooling in distilled water at room temperature prior to blocking endogenous peroxidase with 3% hydrogen peroxide. Protein blocking (Dako Cytomation) was performed for 15 min at room temperature followed by incubation for 1 h at 37 °C with VPM70 (primary monoclonal antibody against VMV p25 protein). After washing in TBS buffer, the sections were incubated with Envision™ mouse peroxidase for 30 min at room temperature, washed in TBS buffer and incubated with 3-3-diaminobenzidine (DAB; Dako Cytomation) for 10 min as substrate for the peroxidase reaction. The slides were rinsed, counterstained with hematoxylin and mounted under DPX. Replacement of primary antibody by TBS was used for the negative control sections.

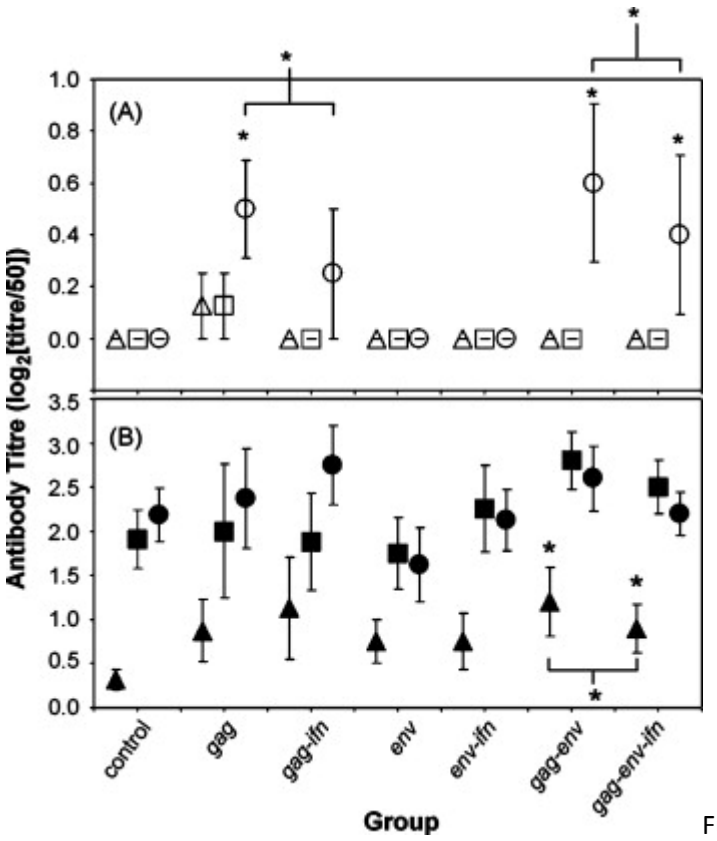
2.11. Statistical analysis

Data analysis consisted of comparing specific pairwise groups at particular time points. If possible, data were transformed to give a normal distribution then analysed with a standard 2-sample Student's t-test. Where data could not be normalised through transformation the non-parametric equivalent – the Mann Whitney test – was used. Frequency data were analysed using Fisher exact tests. In all cases a $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Induction of antibody responses

The presence of antibody was tested using a whole virus ELISA with an anti-sheep IgG detection step (Fig. 2). There was no evidence of antibody induction by the plasmid immunization (week 7) in any of the groups. However, after the MVA boost the gag, gag-env and gag-env-ifn groups all showed significantly increased antibody levels compared to the control group ($p < 0.001$; $p = 0.002$; $p = 0.033$, respectively) (Fig. 2A). There was no statistically significant effect of immunization with ifn-γ. When the gag and gag-ifn, and gag-env and gag-env-ifn groups were pooled induction of antibody was seen in both pooled groups at week 12 ($p = 0.006$). The antibody response compared to controls was increased after challenge in the gag-env and gag-env-ifn groups at week 4 post-infection only (week 16 after immunization) ($p = 0.032$; $p = 0.047$, respectively, pooled groups $p = 0.013$) (Fig. 2B). Following this time point all groups showed similar titres of antibody. The antibodies were of the IgG1 subclass, with no IgG2 antibody responses being observed (data not shown).



Full-size image (35 K)

Fig. 2.

Antibody titre determined using whole virus ELISA. Serial two-fold dilutions of sera from sheep were tested on a whole VMV ELISA before and after challenge with VMV. The results are given as the mean titre (log2[titre/50]) ± standard error of the group. Time post immunization: (A) – ▵ week 0; □ week 7; ○ week 12; (B) – ▲ week 16; ■ week 20; ● week 24. (*) Titre greater than the control group ($p < 0.05$, groups gag, gag-env and gag-env-ifn week 12, and gag-env and gag-env-ifn week 16) using Mann–Whitney tests.

Anti-ENV antibodies were detectable by Western blotting and the number of positive animals scored. These antibodies were found in all animals receiving the env immunogen (env, env-ifn, gag-env, gag-env-ifn) at week 16 but not in the animals that received gag or gag-ifn until week 20 (data not shown). The majority of animals were anti-ENV antibody positive by week 12 after challenge (week 24, data not shown). No neutralizing antibody was detectable in any sample (data not shown).

3.2. T lymphocyte responses

3.2.1. Cytotoxic T lymphocytes

At week 7, following plasmid immunization, only the gag-env-ifn group showed statistically significantly increased numbers of CTL positive sheep compared to controls ($p = 0.007$) (Fig. 3B), though the env group showed a similar trend ($p = 0.052$). However, this was not maintained after MVA immunization (week 12) ($p = 0.648$ and $p = 0.645$, respectively). After challenge, more sheep became CTL positive but again few groups were significantly different to the control group: the gag-env group at week 20 ($p = 0.005$) and env group at week 24 ($p = 0.032$) (Fig. 3E and F). Immunization with the ifn gene did not increase the number of CTL positive animals after challenge, so groups were pooled for further analysis. The CTL frequencies in the gag

and gag-ifn combined groups were not statistically significantly different from controls. The combined env and env-ifn groups gave significantly higher frequencies compared to controls at week 24 ($p = 0.020$). The combined gag-env and gag-env-ifn groups were significantly higher than controls at week 20 ($p = 0.012$). When animals that received the gag immunogen (gag, gag-ifn, gag-env, gag-env-ifn) were compared to controls, the CTL frequencies were not significantly different. In contrast, the frequencies observed in animals receiving the env immunogen (env, env-ifn, gag-env, gag-env-ifn) were significantly higher than controls at week 7 ($p = 0.041$), week 20 ($p = 0.028$) and week 24 ($p = 0.047$).

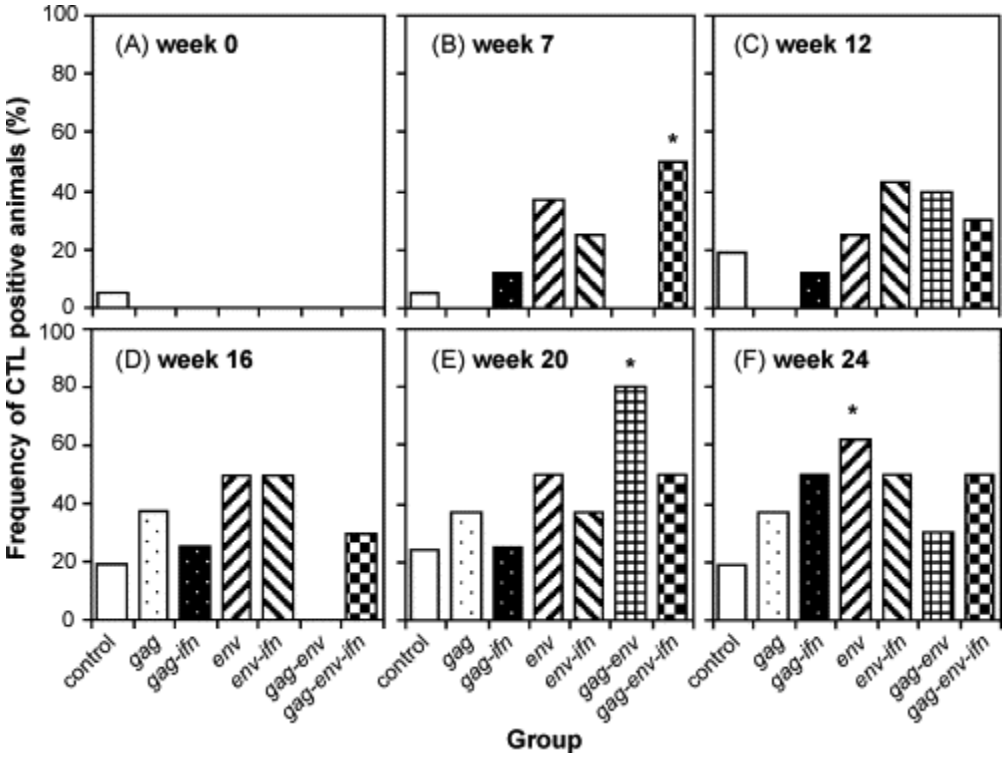


Fig. 3.

CTL responses. The proportion of CTL positive animals in each immunization group is shown at the different time points. (*) Proportion of CTL positive animals greater than the control group (before challenge, gag-env-ifn group week 7, $p = 0.007$; after challenge, gag-env week 20, $p = 0.005$ and env week 24, $p = 0.032$) using a Fisher's Exact test.

Within groups, the control group did not differ from time 0 at any time point. The pooled env with env-ifn and gag-env with gag-env-ifn groups showed statistically significant increases in the number of CTL positive animals after plasmid vaccination ($p = 0.043$ and 0.047, respectively) with increasing statistical significance after MVA boost compared to week 0 ($p = 0.042$ and 0.008, respectively). After challenge, there was no further increase in the number of CTL positive sheep compared to week 12 (after MVA immunization) in groups immunized with either gag or env. The results show that immunization had been successful at inducing CTL responses, although these responses were not strong.

3.2.2. T lymphocyte proliferative responses

Immunization with plasmid expressing ovine IFN-γ had no effect on the magnitude of the T cell proliferative response of PBMC to any of the three antigens used (EV1, p25 gag and p17 gag). The immunogen and the

respective immunogen + ifn groups were therefore pooled for analysis. No increased proliferative response was seen with whole virus EV1 antigen after plasmid immunization (week 7, Fig. 4A). Following the MVA boost at week 10, transiently increased proliferative responses were seen at weeks 12 and 16 (after challenge) in the gag and gag-ifn groups combined and the gag-env and gag-env-ifn groups combined ($p = 0.022$ and 0.009, respectively for gag and $p = 0.032$ and 0002, respectively for gag-env) and only at week 12 in the env and env-ifn groups combined ($p = 0.003$) (Fig. 4A).

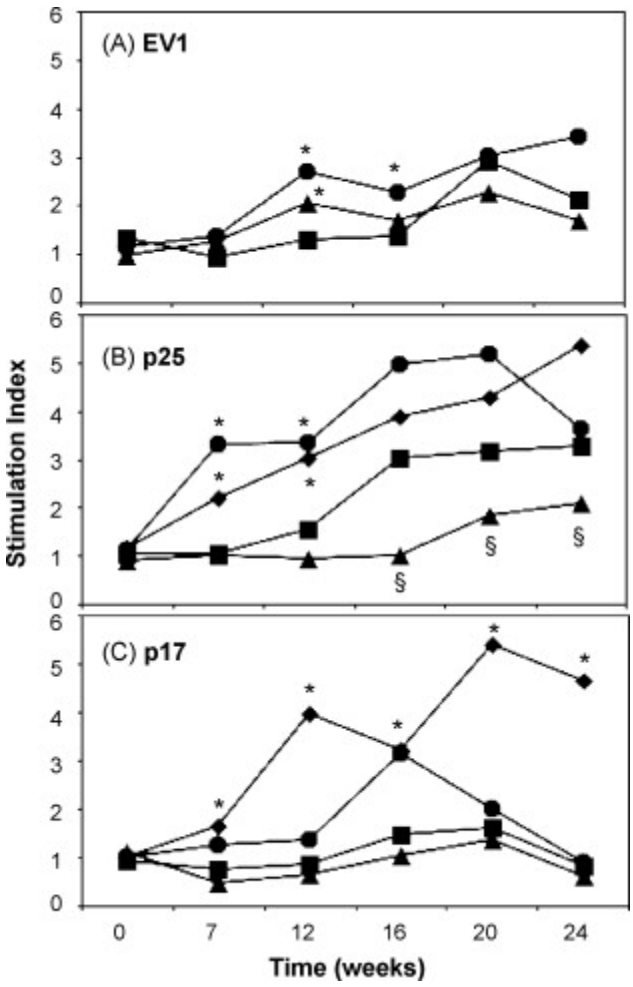


Fig. 4.

T cell proliferation responses. The degree of T cell proliferative responses is shown. Results are expressed as the median of the group's median stimulation index for each antigen against time. Antigen used: (A) EV1; (B) p25; (C) p17. Groups: ■ control; ● gag + gag-ifn; ▲ env + env-ifn; ♦ gag-env + gag-env-ifn. Due to technical limitations, the gag-env and gag-env-ifn groups do not have data for EV1 antigen. (*) Significantly greater than controls; (§) Significantly lower than controls $p < 0.05$. Analysis by Mann–Whitney test of median stimulation indices.

Stimulation with p25 or p17 GAG antigens, did not induce significantly greater proliferative responses in env and env-ifn groups compared to controls before challenge as expected (week 7 and 12, Fig. 4B), and also after challenge (weeks 16, 20 and 24). Indeed, depressed responses were seen to p25 GAG after challenge in the pooled env and env-ifn groups ($p = 0.001$, 0.005 and 0.011 at week 16, 20 and 24, respectively) (Fig. 4B) and to p17 GAG immediately after challenge in the single env group ($p = 0.031$) (week 16, data not shown). In contrast, statistically significant increases in T cell proliferation to the p25 antigen were found with the combined gag and gag-ifn groups and the combined gag-env and gag-env-ifn groups before challenge at week 7 and week 12 ($p < 0.000$ and $p = 0.05$ for gag and $p = 0.008$ and 0.014 for

gag-env at week 7 and 12, respectively, Fig. 4B). However, these increased proliferative responses compared with controls were not maintained after challenge. When p17 was used as the antigen, the individual (data not shown) and combined gag-env and gag-env-ifn groups showed significantly elevated responses from week 7 onwards ($p < 0.02$ for all time points) (Fig. 4C). In contrast, the combined gag and gag-ifn groups and combined env and env-ifn groups did not show greater proliferative responses to p17 compared with controls at any time point.

3.3. IFN-γ responses

IFN-γ expression by PBMCs following stimulation with EV1, p25, or p17 antigen was assessed during the course of immunization and challenge. In general, very few animals showed significant IFN-γ expression using the EV1 antigen (data not shown). The expression levels of IFN-γ in PBMC in response to stimulation with p25 and p17 prior to challenge were not significantly different from controls (data not shown). Following challenge, there was a trend towards increased expression levels of IFN-γ RNA in response to p25 in the combined env and env-ifn groups at week 16 ($p = 0.072$) and this was significantly increased at week 20 ($p = 0.007$, Fig. 5A) but not week 24 (data not shown). An increase in IFN-γ expression in response to p25 was seen in PBMC from gag-env and gag-env-ifn immunized animals ($p = 0.034$) at week 16 but this was not seen at later time points. However, when all the groups which had been immunized with env were analysed together there was a significant increase in IFN-γ expression by PBMC in response to p25 at both week 16 ($p = 0.019$) and week 20 ($p = 0.012$) (Fig. 5A) but not week 24 (data not shown). When p17 was used as the stimulating antigen the combined gag and gag-ifn groups, env and env-ifn and gag-env and gag-env-ifn groups were all similar to controls at week 16 ($p = 0.290$; $p = 0.211$; $p = 0.091$, respectively) (Fig. 5B). At week 20 the env and env-ifn groups combined showed significantly lower IFN-γ expression levels than controls ($p = 0.036$). At week 24, the env and env-ifn groups combined were not significant different to controls (data not shown).

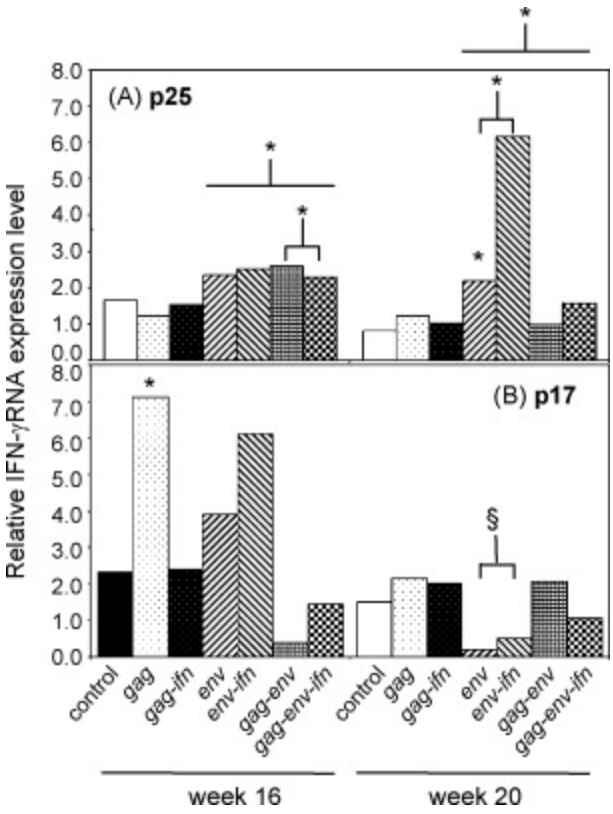


Fig. 5.

IFN-γ RNA expression levels in response to stimulation with GAG p25 and p17. PBMC were cultured for 4 h with 25 μg antigen (A. p25; B. p17) or mock antigen and cellular RNA prepared. IFN-γ expression levels were normalised to GAPDH RNA expression and the normalised levels in different groups compared to controls by Mann–Whitney test. (*) Significantly greater than controls; (§) significantly lower than controls, $p < 0.05$. The median expression ratios (E) of the groups are shown at weeks 16 and 20 (weeks 4 and 8 post-challenge).

3.4. Virus load

Provirus DNA load in blood and tissues (lung and mediastinal lymph node) was measured by real-time PCR. All PBMC samples were negative before challenge (data not shown). There was no effect of immunization with the ifn gene. When the gag and gag-ifn groups were pooled the blood proviral DNA load was reduced in comparison to controls ($p = 0.048$) (Fig. 6A). The provirus load tended to be higher than controls in the other immunized groups, but the values were not statistically different from controls either as single groups or combined with the respective ifn groups ($p > 0.139$).

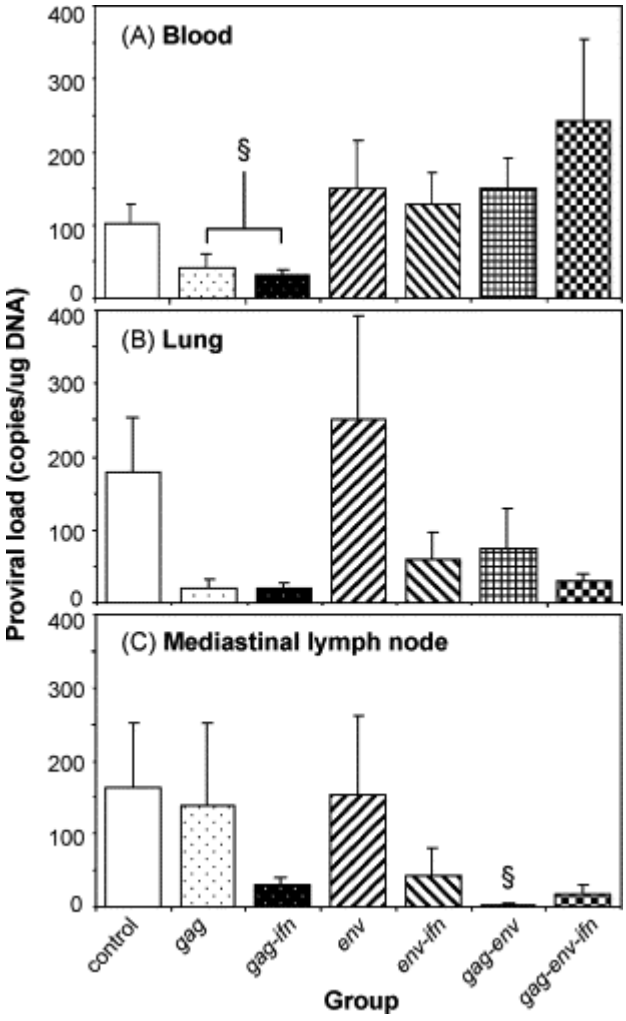


Fig. 6.

Proviral load after challenge in blood, lung and mediastinal lymph node. The proviral DNA, measured using a VMV p25 specific real-time PCR assay, is shown. The results are expressed as mean copy number per μg extracted DNA ± standard error of the group. Blood samples were taken at week 16, 20 and 24 and the maximum copy number of the three measurements used as the value for the sheep. Blood data were transformed to

achieve normality (KS normality test) and equal variance (Levene's test) and groups compared by a Student's t-test. The proviral DNA load in lung sections and mediastinal lymph node was measured at week 12–13 post-challenge (week 24–25 post immunization). The maximum copy number of the four lung segments was used as the value for the animal. Analysis of the lung and mediastinal lymph node data was by Mann–Whitney test since normality could not be achieved through data transformation. (A) Mean of the maximum blood viral load; (B) mean of the maximum lung section load; (C) mean of the mediastinal lymph node proviral load. (§) Proviral load less than the control group ($p < 0.05$, blood: pooled gag and gag-ifn groups; mediastinal lymph node: gag-env).

In the lung, no statistically significant differences in provirus DNA load were observed between controls and immunized groups by PCR ($p > 0.396$) (Fig. 6B). Nearly all lung sections tested were positive for provirus DNA by in situ hybridization (not shown). To obtain a measure of virus expression in lung tissues after challenge, immunohistochemistry for p25 was performed on the right caudal lung lobe taken at post-mortem. The frequency of positive p25 staining reactions in the right caudal lung lobe in immunized and control groups is shown in Table 2. A significantly reduced p25 expression was noted in the animals receiving gag-env and gag-env-ifn compared to controls when the two immunized groups were analysed together ($p = 0.012$). The frequency of samples positive for provirus DNA in this lung lobe also tended to be lower in the gag-env and gag-env-ifn groups compared to controls, though this difference was not statistically significant. The frequency of positive p25 staining observed in the groups receiving ifn combined (9.5%) was significantly lower than that of the other groups combined (39.1%; $p = 0.040$), though the provirus load was not significantly different.

Table 2.

Frequency of positive p25 staining and provirus load in right caudal lung lobe of control and immunized sheep.

Sheep group	p25 staining			Provirus load		
	Number	Frequency (%)[a]	*p*-value [b]	Number	Frequency (%)[c]	*p*-value [d]
Control	21	38.1	–	20	35.0	–
gag	8	50.0	0.683	8	25.0	1.00
gag-ifn	7	14.3	0.372	8	62.0	0.23
env	8	75.0	0.109	8	37.5	1.00
env-ifn	7	14.3	0.372	8	25.0	1.00
gag-env	7	0	0.075	10	20.0	0.68
gag-env-ifn	7	0	0.075	10	10.0	0.21
gag and gag-ifn	15	33.3	1.000	16	43.8	0.73
env and env-ifn	15	46.7	0.736	16	31.3	1.00
gag-env and gag-env-ifn	14	0	0.012	20	15.0	0.27

a Right caudal lung samples taken at post-mortem stained for p25 GAG expression.

b Fisher's exact test; compared to control group.

c Right caudal lung samples taken at post-mortem analysed for the presence of provirus. Samples showing >3 copies/μg DNA were regarded as being positive.

d Fisher's exact test; compared to control group.

The provirus load in the mediastinal lymph node was significantly reduced compared to controls in the gag-env group ($p = 0.046$), but not in the other groups ($p > 0.278$) (Fig. 6C).

3.5. Pathology in tissues

We determined the early (week 12–13 post-infection) pathology scores after infection in control and immunized animals. The scores of the groups are shown in Fig. 7. No group showed protection from the development of disease. However, the env-ifn group had developed worse pathological lesions than the control group by week 12–13 post-infection ($p = 0.011$). When the env and env-ifn groups were combined the pathology scores were significantly higher than controls ($p = 0.009$). The gag and gag-ifn combination and gag-env and gag-env-ifn pooled groups were not different from controls ($p = 0.307$ and $p = 0.537$, respectively). When the lesion scores were assigned into mild/absent (mean score ≤ 1.0) or severe categories (mean score > 1.0) the combined env and env-ifn groups had a higher frequency of severe lesions (10/16) compared to controls (5/22, $p = 0.020$ using Fisher's exact test). The gag and gag-ifn combination and gag-env and gag-env-ifn combination had frequencies of severe lesions that were not different to controls (4/16, $p = 1.000$ and 1/20, $p = 0.187$, respectively).

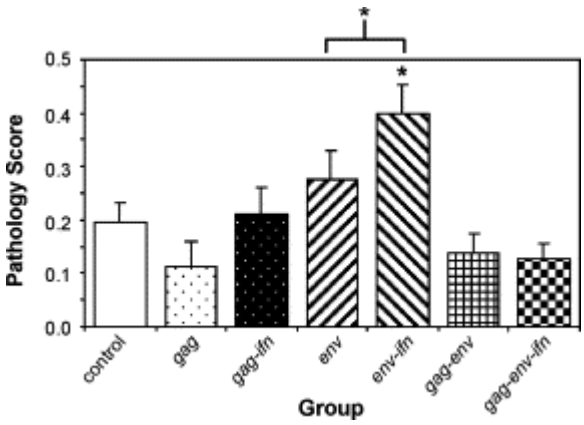


Fig. 7.

Tissue pathology. Pathological changes in the lung and mediastinal lymph node were scored and the results expressed as the mean of the pathology score ± standard error of the group. (*) pathological score greater than the control group ($p = 0.009$, env-ifn group). Analysis by Mann–Whitney test.

4. Discussion

In the present study PMED was used in conjunction with MVA to prime and boost sheep against VMV gag and env genes. Gag and gag-env (but not single env) genes induced elevated antibody responses by ELISA, but these were transitory as by week 24 the levels were not different to controls. However, the response to a combination of gag and env genes gave a more sustained response from week 12 to week 16, compared to the responses observed with single gag gene immunization. There was a delay in the anti-ENV antibody production in the pooled gag and gag-ifn group compared to env immunized animals 4 weeks after challenge. It is possible that gag immunization resulted in the antibody responses being focused on GAG antigens, such that when virus was introduced antibody responses to ENV were competitively inhibited. No delay in the appearance of anti-env antibody responses was seen in animals that received the gag-env immunogen, arguably because the presence of env in the immunization mixture balanced that of gag to prevent a predominant antibody response to gag. Alternatively, the lower provirus load observed in the gag

immunized animals may have caused delayed anti-ENV antibody production. However, anti-whole VMV antibody responses were not delayed or diminished.

The antibody levels obtained following PMED immunization were significantly lower than those observed following immunization with the same immunogens administered via the respiratory tract [24]. However, since the antibody level of controls were similarly lower the effect is likely to be non-specific. Neutralizing antibodies were not induced by the PMED immunization protocol used here, as was the case following mucosal immunization [24].

T cell proliferative responses to VMV antigens following PMED immunization were examined to assess activation of cell-mediated immune responses. The relatively poor proliferative responses observed with EV1 antigen may have been due to the presence of low amounts of GAG and ENV antigens in the virus preparation. As expected, the highest responses using p25 or p17 antigens were found in the gag and gag-env groups. The results indicate that DNA immunization with the full length p55 gag precursor gene induced not only expression of the mature GAG proteins in vivo but that the proteins were processed and presented via an MHC class 2 pathway to T cells.

The proliferative response to p25 or p17 antigens in animals immunized with env or env-ifn was expected to be similar to controls. The observation that both env and env-ifn groups showed significantly lowered responses compared to controls suggests that env immunization interferes with the normal T cell responses to GAG expected after challenge. This was not observed when the same genes were used to immunize sheep mucosally [24]. However, recent studies on immune responses to HIV gag and env genes have revealed an antigen-specific suppressive effect of env on GAG-specific T cell responses in mice [33]. The mechanism(s) underlying this effect of systemic env immunization is unknown.

Increased frequencies of CTLs were found over the course of priming, boosting, and challenge with all immunogens, but the responses were weak and transitory. Immunization using PMED has been shown to induce CTLs in other virus infections, including lentiviruses [34], [35] and [36]. Significant induction has usually required the use of viral boosting vectors, numerous plasmid boosts or high plasmid doses. MVA is often used as a viral vector to provide an efficient protein boost [37] and [38]. However, in our study we could not discern a significant boosting effect of MVA for CTLs. In contrast, MVA provided a boost for antibody production in animals receiving gag or gag-env genes, indicating that MVA does deliver a protein boost in sheep.

Despite the generally low level of immune priming and boosting achieved using PMED and MVA, provirus DNA levels in animals that received the single gag immunogen were significantly decreased in blood though similar to controls in the lungs. These results contrast with those obtained previously in animals immunized mucosally with gag where the provirus loads in blood were not different to controls 12 weeks post-

challenge [24]. This may reflect compartmentalization of immune responses following mucosal and systemic immunization.

The provirus load in lung tissue in animals immunized with gag using PMED was not different to controls. In contrast, mucosal immunization with gag resulted in significantly elevated provirus load in the lungs [24], with values approximately 20–200 fold higher than those observed in lung following PMED immunization. This effect was noted for both gag and gag-env immunized animals. It may be that the quality and/or magnitude of the immune response induced mucosally to gag or gag-env caused enhanced virus replication in the lung compared to immune responses induced systemically.

In the present study, the relationship between p25 staining and provirus load was discrepant in that the frequency of positive p25 sections was significantly lower than controls in gag-env + gag-env-ifn groups combined while the values obtained for provirus load in gag + gag-ifn and env + env-ifn were not significantly different from controls. However, the frequency of provirus positive samples in the gag-env and gag-env-ifn groups also tended to be lower than controls using the right caudal lung lobe, and it is possible that the discrepancy is simply due to efficiency differences between immunostaining and real-time PCR methods or the use of only one lung section for immunostaining.

Evidence for virus replication was also sought previously following mucosal immunization [24] by staining sections from the right caudal lung lobe for the p25 core protein. No significant differences in the frequency of p25 positive staining were found for gag or env immunization after respiratory immunization compared to controls [24]. However, the frequency of p25 observed in animals immunized with gag-env was zero in the present study and low (6.7%; $p = 0.081$) in the mucosal study [24]. Since the provirus loads in lung tissue were similar to controls following PMED immunization and very high following mucosal immunization, it follows that there is a disconnection between provirus load levels and p25 staining. It is conceivable that two types of immune response are operating concomitantly: one that stimulates virus replication, and one that recognises and eliminates productively infected cells. VMV-specific CD4+ T cells have been shown to enhance virus replication in target cells [10] and such cells could have played a role in enhancing provirus loads in the PMED and mucosal [24] studies. As noted above, CTL precursors were observed in blood the present study, and it is possible that they could have reduced the levels of productively infected cells in the lung. However, the CTL response was weak and therefore this effector mechanism appears to have a limited role in controlling virus levels.

To determine whether IFN-γ would enhance immune responses to VMV immunogens, the ifn-γ gene was included in the immunizing mixture. However, the presence of ifn in the immunizing mixture did not alter the provirus DNA load but it did result in decreased p25 expression in the lungs. Previously, we also could not find a significant effect of ifn when immunizing sheep mucosally against VMV [24]. So, the lowering of the blood provirus load observed here is likely to be due mainly to gag immunization with little contribution from the ifn gene. However, the tendency towards reduced p25 staining found in animals that received the ifn gene suggests an anti-viral effect that operates in the later stages of virus replication.

To assess whether IFN-γ production following immunization might play a role in control virus levels, we measured the levels of IFN-γ transcript in PBMCs. The results showed that IFN-γ expression was not elevated in animals immunized with the gag gene. Elevated IFN-γ expression was observed in animals receiving env immunogens in response to p25, and this potentially could have had an anti-viral effect. However, the provirus load in blood was not different from controls in env immunized animals, suggesting that IFN-γ expression in blood is poorly associated with viral load in the studied tissues. The results suggest that IFN-γ induced by immunization may not have contributed to the anti-viral effect observed, though a direct monitoring of IFN-γ mRNA expression in the lung tissue may clarify this point.

PMED immunization with VMV gag or env genes did not prevent lesions developing in the lung and draining lymph node. This is in contrast to the results obtained following mucosal immunization [24] where gag immunization induced a significant reduction in lesion scores. The results suggest again that the quality and/or quantity of the immune response in terms of controlling virus replication and expression may differ significantly following PMED and mucosal immunization.

Indeed, in the present study env immunization with ifn resulted in increased frequency and severity of the lesions. When the env gene was combined with the gag gene for immunization, the lesion-enhancing effect of env was abrogated. It is well recognized that anti-ENV antibody levels correlate with the development of arthritis in goats infected with CAEV [39]. Since the target organs affected by SRLV develop similar lesions consisting of the presence of inflammatory cells, inflammation, and thickening of tissue, the mechanisms operating may be similar. Thus, the lesions in the lungs could have been due to immune complex formation or to binding of these antibodies to virus-infected cells and activation of antibody-dependent cell-mediated cytotoxicity. Another possibility is that anti-ENV T cell responses were responsible for the lesion enhancement [10].

However, recent vaccination studies in goats and sheep have shown that env immunization can induce lower virus loads or disease [12] and [19]. Significant differences between these studies and the present work exist. In the study by Gonzalez et al. [19], PMED immunization was carried out via vulva rather than epidermis. More efficient transfection of dendritic cells with the env gene by particle bombardment due to the absence of a keratin layer in mucosal tissue may have enhanced the env expression and induced local anti-ENV antibody responses, resulting in decreased virus load in blood.

In conclusion, PMED immunization with VMV gag or gag-env genes provides a degree of protection against provirus DNA load, whereas immunization with the env gene results in enhanced lesion development. The findings that systemic immunization with the gag gene results in a reduced blood provirus load and that mucosal immunization with gag results in reduced lesion formation [24] are consistent with observations in HIV vaccination whereby gag-specific responses are protective [40]. The PMED immunization strategy applied using the single gag gene as immunogen may limit spread of infection via the blood to other tissues and warrants further study for the development of SRLV vaccines.

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