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# Reporter gene expression in dendritic cells after gene gun administration of plasmid DNA

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**Abstract**

Dendritic cells (DC) play an integral role in plasmid DNA vaccination. However, the interaction between plasmid DNA and DC in vivo is incompletely understood. In this report, we utilise the sheep pseudoafferent cannulation model to examine the interaction between plasmid DNA encoding enhanced green fluorescent protein (*p*EGFP) and afferent lymph DC (ALDC) following gene gun administration. The results show that peaks of fluorescent ALDC tended to appear around days 1–4 and 9–13, then erratically thereafter for up to 2 months. Phenotypic analysis showed that EGFP+ ALDC expressed MHC class II, WC6, CD1b, and SIRPα markers. Plasmid, detected by PCR, was found in lymph cells and cell-free plasma on a daily basis, and was present variably for up to 2 months. Plasmid was also detected in purified CD1b+ ALDC, but the presence of plasmid did not correlate with EGFP expression by ALDC. Free EGFP in afferent lymph plasma was detectable by luminometry only after three administrations of the plasmid. The results show that gene gun administered *p*EGFP persisted for extended periods after a single administration, leeching out of skin on a daily basis. The plasmid was associated with both the cellular and fluid components of afferent lymph. EGFP protein appeared in afferent lymph in a pulsatile manner, but associated only with ALDC.


*Keywords:* Enhanced green fluorescent protein; Sheep; Gene gun; Dendritic cells

## 1. Introduction

Plasmid DNA vaccination is a novel strategy for inducing protective immune responses against a wide variety of pathogens [1]. A major advantage of DNA vaccination is that both humoral and cytotoxic immune responses are generated. However, immune responses to DNA vaccines are often weak and/or short lived particularly in humans or livestock [2]. For these obstacles to be overcome, a greater understanding of the mechanisms involved is required. Several studies have demonstrated an obligate role for bone marrow-derived cells in presenting the encoded antigen to T cells [3–5]. Other studies have demonstrated that induction of primary and memory responses were due to rapidly migrating cells from skin following gene gun administration or injection of plasmid [6,7]. In the case of particle bombardment, this was the case irrespective of whether the antigen was membrane-bound, secreted, or intracellular [6]. Raz et al. [8] showed that after intradermal injection of plasmid, cells resembling macrophages or dendritic cells (DC) as well as keratinocytes and fibroblasts were transfected. Other studies showed that direct transfection of skin DC occurred following gene gun administration [9,10]. In contrast, Corr et al. [11] provided evidence that in vivo priming of immune responses following injection of plasmid occurred predominantly by antigen transfer. Thus, after DNA vaccination, DC appear to acquire antigen either by direct transfection or by cross priming.

Condon et al. [9] showed that gene gun immunization results in the localisation of transfected skin-derived DC in the draining lymph nodes. Recently, it has been shown that relatively high numbers of skin DC transfected by gene gun lodge in the draining lymph nodes [12]. However, the role of afferent lymph DC (ALDC) in DNA vaccination

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has not been examined. ALDC can be accessed in sheep via the technique of pseudoafferent lymphatic cannulation [13,14]. Afferent lymph normally contains 3–10% ALDC, <1–3% neutrophils and 87–96% lymphocytes [15]. The ALDC are characterised by their high forward and side scatter properties, expression of MHC class II and DEC205 (designated WC6 in ruminants) [13,16,17], and lack of expression of macrophage markers, such as CD14 and CD11b [17,18]. Usually, around 60–70% of ALDC express CD1b [13] and between 60 and 90% express SIRPα [17,19]. In the present study, we have used the pseudoafferent cannulation model in sheep and a reporter plasmid encoding enhanced green fluorescent protein (*p*EGFP) to examine reporter gene expression following gene gun administration to skin. We show that substantial numbers of ALDC exhibit EGFP fluorescence and that both EGFP-positive DC and free plasmid continue to drain the vaccination site for up to 2 months.

## 2. Materials and methods

### 2.1. Pseudoafferent lymphatic cannulation

The prefemoral lymph node of 18-month-old Suffolk or Finnish-landrace sheep ($n = 11$) was excised, and 6 weeks later the pseudoafferent lymphatic duct cannulated as described previously [13]. Cannulations were allowed to stabilise for at least 7 days prior to the start of the experiments. Afferent lymph was collected using heparinized cannula and bottles. Total volumes of lymph collected were recorded on a daily basis. The total number of ALDC was determined by flow cytometry.

### 2.2. Plasmid

The plasmid *p*EGFP-C1 encoding a red shifted variant of green fluorescent protein (EGFP) under the control of a CMV immediate early promoter was obtained from Clontech (Basingstoke, Hants, UK). The plasmid was transformed into competent *Escherichia coli* DH5α cells and purified using a Megaprep kit from Qiagen Ltd. (Crawley, West Sussex, UK) according to the manufacturer's instructions. A control plasmid DNA encoding β-galactosidase (β-gal), obtained from Clontech, was produced in a similar manner. Plasmid function was assessed in ovine skin fibroblasts in vitro by flow cytometry and/or RT-PCR, following either electroporation or gene gun administration. The β-gal plasmid was assessed by staining for β-gal on cytosmears of the fibroblasts. The fibroblasts were cultured in DMEM supplemented with 2 mM final concentration of L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5% fetal calf serum at 37 °C, 5% $CO_2$.

A Helios (BioRad), helium powered, gene gun was used to propel *p*DNA-coated gold beads that were prepared according to the manufacturer's instructions. The gene gun parameters (pressure, particle size/number and PVP concentration) were optimised before use. One hundred micrograms *p*EGFP (1 mg/ml stock in 1 × TE pH 8.0) and 100 μl 0.05 M spermidine were mixed with 20 mg 1.0 μ gold beads. One hundred microlitres of 1 M $CaCl_2$ was slowly added to precipitate the *p*EGFP on to the beads, which were then washed five times in 100% ethanol (spinning at 1800 × *g*, 15 s between washes). The beads were resuspended in 2.4 ml ethanol containing 0.05 mg/ml PVP, and bound to the inner surface of plastic tubing. Plasmid function of the *p*EGFP-coated gold beads was assessed in ovine skin fibroblasts in vitro following gene gun administration by flow cytometry and RT-PCR.

### 2.3. In vivo administration of pEGFP

The *p*EGFP-coated particles were targeted towards the dermal–epidermal junction at a pressure of 500 psi using a pressurised helium source. Each vaccination consisted of five shots around the prefemoral afferent lymphatic drainage area, resulting in a total of around 15 μg of *p*EGFP coated on 0.5 mg of gold beads being introduced into the skin. A template was used to standardise the distance between administration site and cannulation site and thus minimising variation between sheep. To demonstrate that *p*EGFP coated onto gold beads was functional in vivo, the plasmid was administered via the gene gun to two sheep three times at weekly intervals. The animals were primed with about 15 μg of *p*EGFP in the flank, and boosted with similar amounts 1 and 2 weeks later. Blood samples were taken before immunisation and 1 week after the last boost. Anti-EGFP antibodies in titres up to 1/4000 were detected in serum by Western blotting using recombinant EGFP (rEGFP) (Clontech) as antigen.

### 2.4. Flow cytometry

Flow cytometry analyses were performed using a Becton Dickson FACScan machine and the CELLQUEST software. Afferent lymph cells were harvested (350 × *g*/5 min) from 10 ml of afferent lymph, washed three times in sterile PBS containing 2% bovine serum albumin and resuspended in 5 ml sterile PBS/2% BSA. Live gates based on high side scatter and forward scatter were used in the flow cytometric analysis of ALDC [13]. EGFP fluorescence by ALDC was determined using the same excitation and emission wavelengths as fluorescein isothiocyanate (FITC). Immunostaining of ALDC was performed using monoclonal antibodies to MHC class II DRB (SW73.2 [13]), CD11b (IAH-CC125 [18]), WC6/DEC205 (IL-A53 [16]), CD1b (VPM5 [16]), and SIRPα (IL-A24 [17]). Isotype-matched irrelevant monoclonal antibodies or normal mouse or rat serum diluted 1/1000 were used as negative controls as appropriate. Cells from afferent lymph (10 ml) were prepared essentially as previously described [13]. Briefly, cells were pelleted by centrifugation (350 × *g*/5 min), washed twice in sterile phosphate-buffered saline containing 1% bovine serum albumin (PBS-1% BSA) and resuspended at a concentration of $1.0 \times 10^7$ cells/ml in PBS-1% BSA. Optimised dilutions of monoclonal antibodies

were incubated with $5.0 \times 10^6$ cells in 0.5 ml PBS-1% BSA for 20 min at 4 °C. The cells were then washed three times in 1 ml PBS-1% BSA. The cells were incubated in a 1:250 dilution of anti-mouse-biotin (Sigma Chemical Company), for 20 min at 4 °C, washed and incubated in a 1:250 dilution of phycoerythrin–streptavidin (Sigma Chemical Company) for a further 20 min, washed and then analysed by flow cytometry (approximately 10,000 cells counted per tube).

### 2.5. Purification of CD1+ ALDC

CD1b+ ALDC were purified by magnetic activated cell sorting (MACS). One hundred millilitres of afferent lymph were centrifuged at $1750 \times g$ for 15 min at 4 °C. The cells were resuspended in 5 ml of cold PBS/1% BSA, 5 mM EDTA, 0.1% sodium azide, and layered onto a 14.5% metrizamide cushion in the same buffer. The interface cells were removed and washed in 10 ml buffer then resuspended in buffer to concentration of $1 \times 10^8$ cells per ml. One millilitre was centrifuged at 1500 rpm for 5 min, and the cells resuspended in 0.5 ml buffer. The cells were incubated with a 1:50 dilution of VPM5-biotin for 30 mins at 4 °C. The cells were washed three times, resuspended in 0.5 ml buffer, then incubated with MACS streptavidin beads for 30 mins at 4 °C and loaded onto a MACS RS+ column (Miltenyi Biotech). The bound cells were washed and eluted in 1 ml buffer. The eluted cells were spun at $350 \times g$ at 4 °C and resuspended in 0.2 ml buffer. Immunostaining performed with VPM5 showed that >95% of the purified cells were CD1b positive.

### 2.6. Luminometry

Cells were removed from afferent lymph by centrifugation at $350 \times g$, for 5 min, at 4 °C and EGFP fluorescence in the supernatant (2 ml) was measured by luminometry using a Perkin-Elmer Luminescence LS 50B Spectrophotometer. The excitation and emission wavelengths were set at 488 nm and 509 nm, respectively. A standard curve of fluorescence versus rEGFP concentration (0–100 pg/ml) was established using afferent lymph plasma spiked with rEGFP.

### 2.7. PCR

Cellular DNA was isolated from 5 ml of afferent lymph plasma. The afferent lymph cells were pelleted by centrifugation ($350 \times g$/5 min), washed three times in sterile PBS and lysed in 200 μl of cell lysis buffer (1.28 M sucrose, 40 mM Tris–Cl, pH 7.5, 20 mM $MgCl_2$, 4% Triton X-100) precooled to 4 °C and 600 μl precooled $dH_2O$ added. The lysing cells were mixed by inversion and incubated on ice for 10 min before being pelleted by centrifugation at $1300 \times g$, for 15 min, at 4 °C, to remove nuclei. The supernatant was passed through a DNA miniprep column (Qiagen) following the manufacturer's instructions and the plasmid eluted in 50 μl of 10 mM Tris pH 8.5 and used in the PCR reaction. Plasmid DNA from CD1b+ ALDC purified cells was similarly extracted. Plasmid DNA was also extracted from 2 ml of cell free afferent lymph. Afferent lymph was centrifuged at $10000 \times g$, for 5 min, at room temperature. The supernatant was passed through a DNA miniprep column (Qiagen). Following the manufacturer's instructions, *p*DNA was eluted with 50 μl of 10 mM Tris pH 8.5 and used in the PCR reaction.

DNA containing *p*EGFP-C1 was amplified by PCR using forward and reverse primers. Forward primer: 5′-AAA-AAC-CTC-CCA-CAC-CTC-C-3′; Reverse primer: 5′-TCC-ACA-CCC-TAA-CTG-ACA-CAC-3′ The reaction mixture contained 2 μl of 10× reaction buffer (New England Biolabs, Hitchen, Herts, UK), 0.2 μl 100 mM dNTPs (final concentration of 5 pMol), 1 ng of the phenol extracted DNA, 2.0 μl Vent polymerase at 2000 units/ml (2 units/20 μl reaction) and was made up to 20 μl with water. The mix was heated to 95 °C for 5 min to separate the double-stranded DNA and cooled to 58 °C, before the Vent polymerase was added. The PCR programme was as follows: 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min, for 25 cycles. A final 5 min, 72 °C elongation phase completed the reaction (Hybaid Omnigene). The reaction products were run on a 1.2% agarose gel, stained with ethidium bromide and photographed under UV light. A positive control used *p*EGFP instead of the extracted DNA, whilst a negative control contained no DNA. The sensitivity of the PCR was as follows: *p*EGFP diluted in water, 3.25 pg; *p*EGFP spiked afferent lymph cell extract, 100 pg; *p*EGFP spiked cell free afferent lymph extracts, 10 pg.

### 2.8. Statistical analyses

Statistical analyses were performed using the Mann-Whitney rank test.

## 3. Results

### 3.1. Phenotypic characterisation of ALDC

A live gate was placed around cells exhibiting high side and forward scatter by flow cytometry as described previously [13]. An example of the gate and the results of immunostaining are shown in Fig. 1. The gated cells were almost all positive for MHC class DR and showed high intensity staining. The gated afferent lymph cells were also almost all positive for the pan DC marker WC6 and negative for the macrophage marker CD11b. About 75% of ALDC were SIRPα+ in this example. These staining patterns are consistent with previous studies of ALDC in sheep, cattle, mice and humans [15–21].

### 3.2. EGFP fluorescence by ALDC following pEGFP administration

To investigate whether ALDC expressed EGFP fluorescence following administration of *p*EGFP, eight sheep received approximately 10–15 mg of plasmid DNA distributed

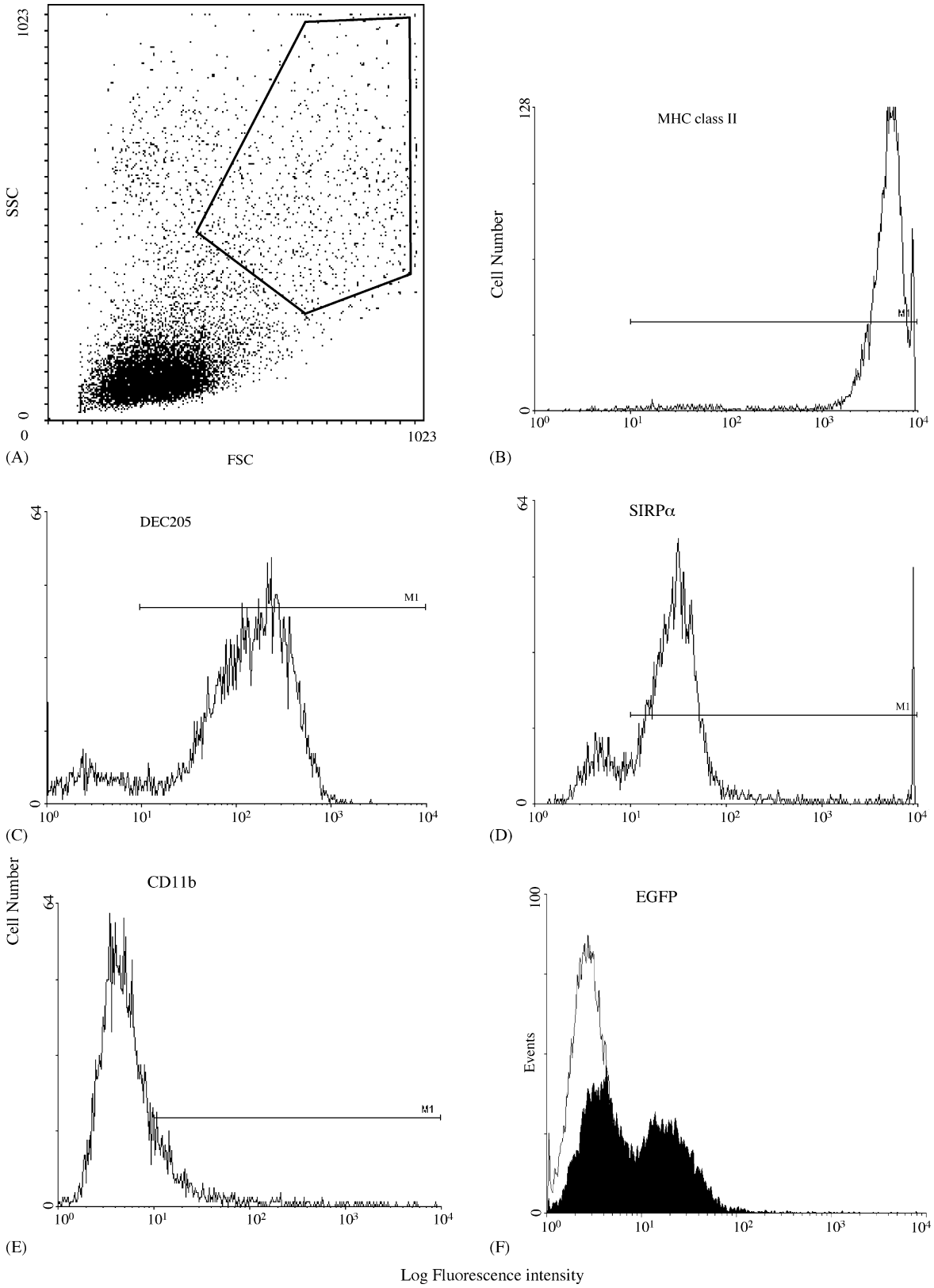


Fig. 1. ALDC characterization. ALDC were characterized using a live gate based on high forward and side scatter (boxed area, panel A). The gated cells were stained for MHC class II DRB (panel B), WC6/DEC-205 (panel C), SIRPα (panel D), and CD11b (panel E). Positive cells were determined by reference to cells treated with normal mouse or rat serum diluted 1/1000. EGFP fluorescence was determined by comparing fluorescence levels exhibited before and after administration of *p*EGFP by gene gun. Panel F shows a typical flow cytometry histogram of EGFP+ ALDC: pre-gene gun administration, unshaded histogram; post-gene gun administration, shaded histogram.

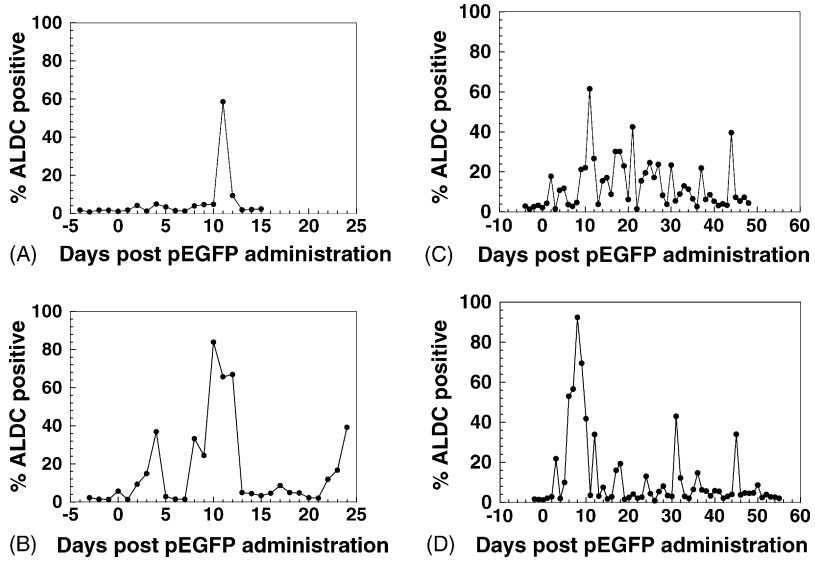


Fig. 2. Flow cytometry analysis of ALDC following administration of *p*EGFP by gene gun in four sheep observed longitudinally (A–D). ALDC were analysed for EGFP fluorescence using the live gate shown in Fig. 1. EGFP fluorescence was determined by comparing fluorescence levels exhibited before and after administration of *p*EGFP by gene gun. Panel A, sheep wt155; panel B, sheep 1345D; panel C, sheep 1766; panel D, sheep 722D.

between five sites in the drainage area of the cannulated pseudoafferent lymphatic duct. EGFP fluorescence in afferent lymph cells was monitored daily by flow cytometry for as long as the cannulated duct flowed (8, 15, 24, 35, 48, 56, 60 and 85 days, respectively). Fluorescent cells were observed at various time points during these periods. When forward and side scatter gates were used to differentiate the distinct cell populations it was seen that only ALDC carried EGFP; lymphocytes were always negative, while neutrophils occasionally showed weak fluorescence. A typical flow cytometry histogram obtained for ALDC is shown in Fig. 1. The intensity of fluorescence observed in the eight sheep varied from low to over one log above the negative control (pre-gene gun administration).

Typical longitudinal data of EGFP+ ALDC obtained are shown for four sheep in Fig. 2A–D. No fluorescence was discernible on ALDC in the immediate period (0–6 h) after gene gun administration. After this lag period, small peaks of fluorescent DC were evident around days 1–4. Larger peaks of fluorescence were usually observed from around day 9 post administration onwards for several days, and in some cases up to 60 days post administration. There were slight variations in this pattern in other sheep with the main peaks appearing a day or two earlier or later. The proportion of ALDC showing fluorescence varied from a few percentage up to 90% (Fig. 2). The peaks were sometimes restricted to single days, while in other cases the peaks spanned over several days. In control sheep receiving plasmid DNA encoding β-gal by gene gun or receiving no gene gun treatment, ALDC fluorescence remained low over extended periods (not shown).

Analysis of the numbers of EGFP+ ALDC draining into the afferent lymphatics is shown for six sheep in Table 1. The numbers of EGFP+ ALDC detected over the whole collection period ranged between $3.3 \times 10^6$ and $9.6 \times 10^6$, with a

Table 1
Number of EGFP+ ALDC draining into the afferent lymphatics after *p*EGFP administration

| Number of EGFP + ALDC ($\times 10^6$) | | | |
|---|---|---|---|
| Sheep ID (days observed)[a] | Total (%)[b] | Major peak (%)[c] [days PI][d] | Average/day[e] |
| 1345D (24) | 3.9 (18.6) | 2.9 (53.9) [8–12] | 0.16 |
| 722D (56) | 8.7 (13.2) | 4.9 (65.2) [6–10] | 0.16 |
| WT155 (15) | 3.3 (8.0) | 2.4 (38.7) [11–12] | 0.22 |
| 741D (18) | 3.7 (3.7) | 3.0 (26.2) [13–15] | 0.21 |
| 1766D (48) | 4.7 (10.0) | 1.1 (30.7) [9–12] | 0.10 |
| 062 (62) | 9.6 (7.4) | 3.7 (26.1) [22–28] | 0.15 |
| Median | 4.3 (11.6) | 3.0 (40.1) | 0.16 |

[a] Number of days analysed.
[b] Percentage of total EGFP+ALDC relative to the total number of unstained ALDC.
[c] Percentage of total EGFP+ ALDC relative to the total number of unstained ALDC in the major peak.
[d] Days post-inoculation constituting the major peak of fluorescence.
[e] Number of EGFP+ ALDC/day averaged over whole observation period.

Table 2
Relationship between ALDC phenotypic markers and EGFP fluorescence following administration of *p*EGFP by gene gun

| Sheep ID | Day[b] | EGFP[c] fluorescence | % DC positive[a] | | | |
|---|---|---|---|---|---|---|
| | | | CD1 | SERPα | WC6 | MHCclass 2 |
| 734D | 3 | + | 25.7 | 15.2 | 19.4 | nd |
| | | – | 0.2 | 5.2 | 3.3 | |
| | 5 | + | 17.0 | 22.0 | 23.5 | 17.9 |
| | | – | 1.3 | 0.6 | 0.4 | 2.1 |
| | 7 | + | nd | 60.3 | 60.9 | nd |
| | | – | | 4.0 | 2.9 | |
| Y158 | 3 | + | 25.7 | 15.0 | 19.4 | nd |
| | | – | 0.2 | 5.2 | 0.3 | |
| | 9 | + | 55.5 | 52.9 | 63.1 | nd |
| | | – | 0.0 | 13.6 | 0.0 | |
| 741D | 9 | + | 10.7 | 8.0 | 8.2 | 8.9 |
| | | – | 0.2 | 0.4 | 0.4 | 0.0 |
| Median | | + | 25.7 | 18.6 | 21.5 | nd |
| | | – | 0.2 | 4.6 | 0.4 | |
| *P* value[d] | | | 0.006 | 0.004 | 0.003 | |

[a] ALDC were stained for CD1, SIRPα, MHC class II DRB and WC6/DEC205 using monoclonal antibodies VPM5, IL-A24, SW73.2 and IL-A53, respectively. Untreated cells and cells treated with normal mouse or rat serum diluted 1/1000 were used as negative controls.
[b] Day post-gene gun administration of *p*EGFP.
[c] EGFP+ ALDC were determined by reference to ALDC prior to administration of *p*EGFP by gene gun; nd, not done.
[d] Statistical analysis of surface marker expression in EGFP+ and EGFP− ALDC subsets was performed using the Mann-Whitney rank test.

median value of $4.3 \times 10^6$. These numbers represented between 3.7 and 18.6% of total ALDC collected over the whole period, with a median value of $0.16 \times 10^6$ EGFP+ ALDC per day. The major peak of fluorescent cells gave a median value of $3 \times 10^6$ EGFP+ ALDC, and constituted a high proportion of the total ALDC present over the peak days.

### 3.3. Two colour analyses of ALDC

Staining was performed in three sheep following *p*EGFP administration for pan DC and subset markers. Staining performed for WC6 or MHC class DR showed that almost all of the gated ALDC were positive for these markers (usually in excess of 95%, data not shown). In relation to EGFP+ ALDC, it can be seen in Table 2 that the great majority of EGFP+ ALDC were WC6+. In two sheep, similar results were obtained for MHC class II DR staining. EGFP+ staining resided mainly in CD1b+ and in SIRPα+ subsets irrespective of the day of sampling post *p*EGFP administration. The results show that EGFP fluorescence was associated mainly with cells displaying typical DC surface markers.

### 3.4. Estimation of free EGFP in afferent lymph plasma following pEGFP administration

Afferent lymph plasma was monitored by luminometry for free EGFP on a daily basis after *p*EGFP administration by gene gun. In eight sheep receiving a single *p*EGFP administration, no lymph sample gave a reading that was significantly above background levels. To determine the effect of more than one administration, *p*EGFP was administered to one sheep twice (Sh2558) and a second sheep three times (Sh062). The second administration was given on day 11 and the third on day 22. In sheep 2558, the profiles of EGFP fluorescence by ALDC showed a similar pattern as in Fig. 2 after

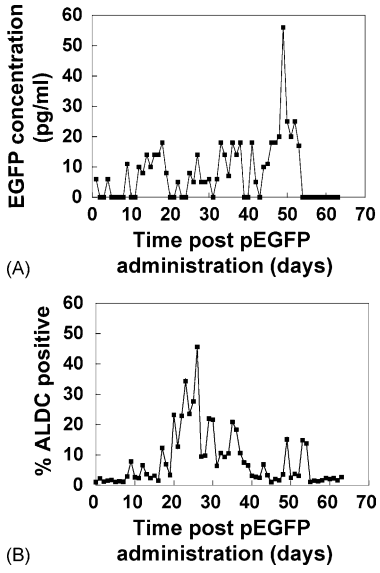


Fig. 3. Longitudinal analysis of EGFP in afferent lymph following three administrations of *p*EGFP by gene gun. Afferent lymph plasma was clarified by centrifugation and analysed for EGFP fluorescence using a fluorimeter. The amounts of fluorescence detected were calculated with reference to a standard curve generated by adding known amounts of recombinant EGFP to afferent lymph plasma from control sheep. EGFP fluorescence associated with ALDC was detected as described in Fig. 2. (A) luminometry analysis of lymph plasma from sheep 062 for EGFP fluorescence; (B) flow cytometry analysis of ALDC for EGFP fluorescence.

each administration, but no free EGFP was detected in plasma (not shown). However, elevated readings for free EGFP were obtained from afferent lymph plasma after *p*EGFP was given three times in sheep 062 (Fig. 3A). The equivalent EGFP fluorescence detected on ALDC by flow cytometry is shown in Fig. 3B. A large number of peaks of fluorescent ALDC were observed from day 9 (equivalent to day 31 from first administration) onwards until day 53 (equivalent to day 75) post administration. By reference to the standard curve generated with rEGFP, the concentration of free EGFP ranged between 10 and 50 pg/ml of afferent lymph plasma (Fig. 3A). Using the known total daily volumes of afferent lymph collected and the EGFP concentrations determined by luminometry, the total amount of EGFP detected in lymph plasma over the whole collection period was estimated to be ~50 ng.

### 3.5. Detection of pEGFP in afferent lymph following gene gun administration

To determine if *p*EGFP was detectable in afferent lymph and, if so, if it was associated with afferent lymph cells or plasma or both following gene gun administration, the cell and plasma fractions of afferent lymph were subjected to PCR. Typical results obtained are shown in Figs. 4 and 5. PCR-positive results were obtained from both plasma and the washed cell pellets for substantial proportions of the time period observed in each sheep. Longitudinal analysis revealed the presence of plasmid almost on a daily basis. In one sheep, *p*EGFP was found in afferent lymph plasma for at least 50 days (Fig. 5). Purified CD1b+ ALDC were analysed in three

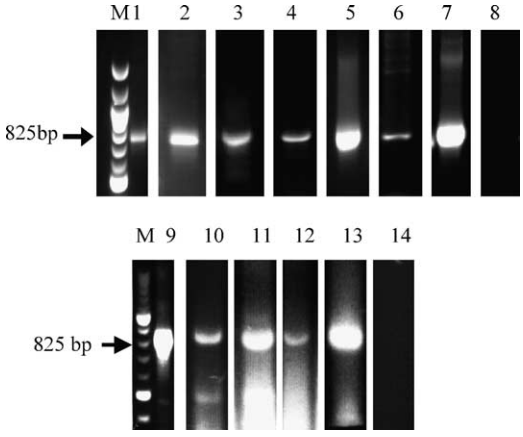


Fig. 4. PCR analysis of afferent lymph cell pellets, CD1+ ALDC and lymph plasma for EGFP sequences following administration of *p*EGFP by gene gun. (A) Ethidium bromide stained gels showing EGFP PCR products generated from afferent lymph cell pellets from sheep 1766 (lanes 1–3), sheep 722D (lane 4), sheep 1345D (lane 5), and from clarified afferent lymph plasma from sheep 1345D (lane 6). The positive (plasmid) and negative (water) controls are shown in lanes 7 and 8, respectively. M, molecular weight marker ladder. (B) Ethidium bromide stained gel showing EGFP PCR products generated from purified CD1+ ALDC from sheep 1766 (lanes 10–11), sheep 722D (lane 12), and sheep 1345D (lane 13). The positive (plasmid) and negative (water) controls are shown in lanes 9 and 14, respectively. M, molecular weight marker ladder.

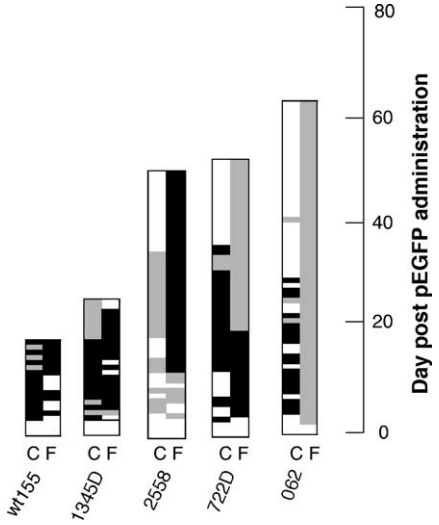


Fig. 5. Longitudinal PCR analysis of afferent lymph cell pellets (C) and cell free lymph (F) for EGFP sequences following administration of *p*EGFP by gene gun to five sheep (wt155, 1345D, 2558, 722D and 062). Five millilitres of afferent lymph were centrifuged to obtain cell pellets and cell free lymph. The cell pellets were washed three times in PBS before extraction of DNA. Plasmid DNA was extracted from 2 ml of cell free plasma. PCR was performed using primers specific for *p*EGFP for 25 cycles. The reaction products were run on an agarose gel and stained with ethidium bromide. Solid bars, positive PCR results; open bars, negative PCR results; shaded bars, not tested.

sheep, and shown to contain plasmid by PCR (Fig. 4). The results thus showed that the plasmid persisted in skin for extended periods and drained into the afferent lymphatics in free and cell-bound form almost on a daily basis. There was no obvious correlation between the presence of plasmid in afferent lymph and fluorescence by ALDC.

## 4. Discussion

In this study, we show for the first time that ALDC carry antigen encoded by a plasmid DNA immunogen administered to skin by gene gun. The ALDC were defined by their expression of MHC class II, WC6, CD1b and SIRPα markers. The results make the connection between observing antigen in skin DC [8] and antigen in DC in draining lymph nodes [9]. In addition, our longitudinal studies show the magnitude of antigen carriage by ALDC and the pattern of migration of antigen-positive cells after gene gun administration. In contrast to the low numbers of fluorescent ALDC observed following intradermal injection of *p*EGFP [22], high proportions of ALDC were fluorescent after gene gun administration. In some instances, over 60% of ALDC were EGFP positive. The results are consistent with the view that gene gun administration is very efficient process for engaging DC [23,24].

It is thought that following DNA vaccination, DC acquire antigen either through direct transfection with plasmid or through cross-priming mechanisms [11,25–27]. In our experiments, the gene gun pressure was adjusted to deliver gold particles maximally to the ovine dermal–epidermal junction.

Thus, some Langerhans cells (LC) in the epidermis and interstitial DC in the upper dermis may have been transfected directly with plasmid-coated gold particles immediately after gene gun administration.

Previous studies have demonstrated that keratinocytes are the main skin cells transfected by the gene gun method [28,29]. Thus, EGFP produced by these cells could also have cross-primed LC, interstitial DC or ALDC with antigen. However, EGFP is a non-secreted protein and may not have been released as free protein in large amounts. Indeed, free EGFP could not be detected by luminometry at levels above 5 pg/ml in lymph plasma, except when priming was followed by two boosts. It is possible that EGFP synthesised and released in the skin may have been taken up by DC in skin, leaving little to detect in lymph plasma. Overall, the results suggest that either free EGFP is produced in very low quantities or that most of it is taken up by cells in skin following gene gun administration, and then transported to the draining lymph node mainly in a cell-bound form.

We observed that anti-EGFP antibody was produced following priming and boosting with *p*EGFP in non-cannulated sheep. In previous studies, we showed that uptake of antigen by ALDC in vitro and in vivo is greatly enhanced by the presence of antibody [30], converting a sub-stimulating dose of antigen into a stimulating dose for T cell proliferation [31]. Indeed, the proportions of fluorescent ALDC observed here are similar to those observed following intradermal injection of FITC-labelled antigen into primed sheep. However, antibody-mediated uptake of EGFP by ALDC is unlikely in the present study since the animals were not primed to EGFP prior to cannulation. Also, the cannulated animals' immune system is likely to have remained naïve following plasmid administration, since all plasmids and cells draining the site of administration would have been removed into the collection bottle.

The patterns of migration of fluorescent ALDC found in this study were unexpected. These consisted of a small peak around days 1–4, a large peak around days 9–13, and irregular peaks at varying intervals thereafter for up to 2 months. The results indicate that plasmid and/or EGFP remained in the skin over this time period. Other studies have found plasmid persisting at the injection site for variable periods. Udvardi et al. [32] detected plasmid in skin up to 1 week after gene gun administration. Similarly, both plasmid and specific RNA were found in skin 11 days after intradermal injection by Hengge et al. [33]. In other experiments, plasmid was shown to remain at the skin injection site for 3–5 months [8] and 6–8 weeks after intramuscular injection [34]. Thus, our results showing plasmid in afferent lymph plasma for up to 50 days are consistent with these observations.

Protein antigen production and retention in skin, after DNA vaccination, has been examined in several studies. Yu et al. [35] showed that antigen was detectable at 4 h, peaked between 16 and 72 h, decreased at 7 days and absent at 14 days following topical application of plasmid. Raz et al. [8], using intradermal injection, found that antigen was detectable by 3 days, maximal at 10 days, and greatly reduced by 30 days. Our results show that antigen traffics from the site of administration to the draining lymph node over a period of several weeks. However, the mechanisms underlying the observed migration patterns are unknown.

It is known that LC in skin migrate rapidly to draining lymph nodes after skin irritation [36], infection [37], or painting with antigen [38–40]. Recent reports have shown that plasmids containing CpG motifs also induce LC migration in skin sheets, with 50% depletion occurring within 2 h [41]. This effect may be mediated through CpG motif recognition by toll-like receptor 9 (TLR9) [42]. These observations suggest that injection of free plasmid into skin would result in LC uptake of plasmid via TLR9 and their rapid migration into the afferent lymphatics. Indeed, we showed previously that a peak of fluorescent ALDC appeared 2 h after intradermal injection of *p*EGFP [22]. Given that most of the plasmid delivered by gene gun is likely to be deposited intracellularly, the TLR9-activation mechanism could be triggered by plasmid-TLR9 interaction in the endosome [43]. However, plasmid also appears in free form in lymph plasma when there are no EGFP+ DCs in lymph. The role of plasmid-induced DC migration is therefore uncertain. The mechanism of plasmid release from transfected cells is unknown as is the fate of the plasmid when it reaches the lymph node.

A recent study [12] found approximately $5 \times 10^3$ antigen-positive DC in draining lymph nodes in mice following gene gun administration. This represented ~100-fold greater numbers than had been shown in previous studies [9]. We showed here that between 100,000 and 220,000 EGFP+ ALDC per day drained into the afferent lymphatics. On average, about 10% of total ALDC were EGFP+ over the whole course of collection. In individual peaks of fluorescence, between 2 and 5 million EGFP+ ALDC representing between 26 and 65% of ALDC drained into the lymphatics over a period of around 4 days. These results indicate that large numbers of antigen-positive ALDC drain into the afferent lymphatics after gene gun administration of plasmid. These results suggest that previous estimates of the numbers of antigen-positive DC draining to lymph nodes [9,12] are significant underestimates.

It was also shown by Garg et al. [12] that ablation of the injection site had no effect on the numbers of transfected DC in the lymph nodes. It was concluded that DC migration from skin after gene gun administration was not continuous and peaked after 5 days. Our results differ from these. We found that ALDC containing *p*EGFP and/or EGFP drained into afferent lymph for extended periods up to 2 months. The reasons for this difference are unclear, though may reflect differences in the amount of plasmid administered, i.e., 15 μg versus 1.5 μg, or in movement of transfected cells away from the site of administration.

The pulsitile nature of the appearance of fluorescent ALDC also remains to be explained. *p*EGFP does not contain specific secretory sequences and it is likely that EGFP would remain largely intracellular. It might be speculated that build up of EGFP inside transfected skin cells could result in

apoptosis. Indeed, while most studies show that EGFP generally has low toxicity, some cell lines undergo apoptosis or necrosis following EGFP expression [44,45]. It is conceivable that some cell types in vivo may thus undergo apoptosis due to EGFP expression. It might be envisaged that successive rounds of apoptosis in transfected skin cells in vivo may result in the generation of apoptotic bodies containing plasmid and/or EGFP. Since DC can take up apoptotic bodies efficiently [46], LC and other skin DC could acquire such apoptotic bodies and appear as peaks of fluorescent ALDC. However, Garg et al. [12] used a mutated caspase-2 gene to induce slow apoptosis of gene gun transfected cells and concluded that antigen positivity of DC in draining lymph nodes was not due to transfer from dead cells to live cells. Recently, DCs have been shown to capture antigen from live cells by a 'nibbling' process [47]. Continual nibbling of EGFP-containing cells could provide a mechanism for EGFP acquisition by skin DC without involving apoptosis or necrosis, but does not in itself explain the pulsitile appearance of antigen-laden DC in afferent lymph. Further work is required to elucidate the mechanisms underlying the observed migration patterns of fluorescent ALDC following gene gun administration of EGFP plasmid.

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