DNA vaccines in sheep: CTLA-4 mediated targeting and CpG motifs enhance immunogenicity in a DNA prime/protein boost strategy

Nicholas J. Kennedy a,b,c,*, Terry W. Spithill c, Jan Tennent d, Paul R. Wood d, David Piedrafita a,b,c

a Department of Biochemistry and Molecular Biology, Monash University, Clayton 3800, Australia
b The Cooperative Research Centre for Vaccine Technology, The Bancroft Centre, PO Royal Brisbane Hospital, Qld 4029, Australia
c Institute of Parasitology, McGill University, Inc. Anne de Boviere, H3K3V9 Canada
d Pfizer Australia, 45 Poplar Road, Parkville, Australia
e Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne, Vic. 3010, Australia

Received 3 June 2005; received in revised form 20 July 2005; accepted 7 August 2005
Available online 6 September 2005

Abstract

DNA vaccines have proven to be an efficient means of inducing immune responses in small laboratory animals; however, their efficacy in large out-bred animal models has been much less promising. In addressing this issue, we have investigated the ability of ovine cytotoxic lymphocyte antigen 4 (CTLA-4) mediated targeting and ruminant specific CpG optimised plasmids, both alone and in combination, to enhance immune responses in sheep to the pro cathepsin B (FhCatB) antigen from Fasciola hepatica. In this study, CTLA-4 mediated targeting enhanced the speed and magnitude of the primary antibody response and effectively primed for a potent memory response compared to conventional DNA vaccination alone, which failed to induce a detectable immune response. While the CpG-augmentation of the CTLA-4 targeted construct did not further enhance the magnitude or isotype profile of the CTLA-4 induced antibody titres, it did result in the induction of significant antigen-specific, lymphocyte-proliferative responses that were not observed in any other treatment group, showing for the first time that significant cellular responses can be induced in sheep following DNA vaccination. In contrast, CpG-augmentation in the absence of CTLA-4 mediated targeting failed to induce a detectable immune response. This is the first study to explore the potential adjuvant effects of ruminant specific CpG motifs on DNA vaccine induced immune responses in sheep. The ability of CpG-augmented CTLA-4 mediated targeting to induce both humoral and cellular immune responses in this study suggests that this may be an effective approach for enhancing the efficacy of DNA vaccines in large out-bred animal models.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: DNA vaccine; Immunogenicity; CTLA-4; CpG; Fasciola hepatica

1. Introduction

Over the past decade, DNA vaccination has evolved as a very efficient means of inducing humoral and cell mediated immune responses to a variety of viral, bacterial and parasitic antigens in small animal models [1,2]. However, the efficiency of DNA vaccination in large out-bred animals, including humans, has not been as encouraging and continues to remain a significant immunological hurdle to be overcome. As such, a diverse range of different strategies have been developed in an attempt to enhance the potency of DNA vaccines (reviewed in [3]).

One such strategy is to target antigen directly to immune cells or particular cellular locations using antigen-ligand fusions [4]. CTLA-4 present on activated T-cells binds with high affinity to the B7 (CD80/86) molecules [5] on the
Fasciola hepatica
sites [25,26]. Several studies have suggested that vaccincides in many countries, new alternatives to chemotherapy [28,29]. Due to the development of resistance to fasciolosis, with an estimated 2.4 million people infected worldwide [27], it is also an emerging pathogenic disease of humans around US$ 3 billion annually to the global agricultural sector. The disease primarily affects ruminants and results in an estimated economic loss of gigantica (tropical liver fluke). While this disease primarily involves cattle [22,23], non-human primates [24] and humans [25,26].

Fasciolosis is a disease caused by the trematode parasites Fasciola hepatica (temperate liver fluke) and Fasciola gigantica (tropical liver fluke). While this disease primarily affects ruminants and results in an estimated economic loss of around US$ 3 billion annually to the global agricultural sector [27], it is also an emerging pathogenic disease of humans with an estimated 2.4 million people infected worldwide [28,29]. Due to the development of resistance to fasciolicides in many countries, new alternatives to chemotherapy are needed [30]. Several studies have suggested that vaccination may be an efficacious means of controlling fasciolosis [31–35].

A cathepsin B-like cysteine protease has been identified in the excretory/secretory (E/S) material of F. hepatica newly excysted juvenile liver fluke [36]. Although the exact functions of cathepsin B-like proteases in liver flukes are unknown, the observation that they are expressed during the early stage of infection suggests that they may play a role in parasite excystment and migration into host tissues [36,37] and thus may be a potential vaccine candidate for the prevention of fasciolosis. A number of recent reports have highlighted the importance of cathepsin B type molecules in parasite survival and host protection. For example, a cysteine protease B from L. mexicana has been shown to be at least partially responsible for suppressing host protective immune responses in a mouse model [38]. Further, it was recently observed that vaccinating sheep with a pool of cathepsin B-like molecules resulted in partial protection against H. contortus infection [39].

While targeting, CpG augmentation and heterologous prime/boosting have independently shown some efficacy when tested in isolation, there are no reports exploring the potential synergistic effects of using a combination of these strategies in large animal models. In the present study, we investigated the ability of CTLA-4 mediated targeting and CpG-optimised vectors, both alone and in combination, to enhance immune responses in sheep to the F. hepatica pro cathepsin B antigen (FhCatB) [37], using a DNA prime/recombinant protein boost strategy.

2. Materials and methods

2.1. Plasmid construction

The plasmids pMASIA and pBISIA-40 (containing 40 ruminant specific CpG motifs) [17] were both kindly supplied by Dr. R. Hecker, Qiagen GmbH, Hilden, Germany. The cDNA for F. hepatica pro cathepsin B was amplified from pFLAG/CatB-WT [37] with Vent® polymerase (New England Biolabs Inc., Beverly, Massachusetts) using the primers 5′ GAGGAGGATCCATGAAAACACATAAACC and 5′ GAGGAGGGATCCCAAGACGGTGGGATTCC. The resulting product was digested with BamHI and ligated into the pMASIA and pBISIA-40 plasmids digested with the same enzyme to create the DNA vaccines pM:CatB and pM:CpG-CatB. The absence of a encoded extracellular secretion signal in both the pMASIA and pBISIA-40 plasmids means that the FhCatB antigen will be expressed in the cytoplasm and not secreted into the extracellular matrix. The plasmid pCI:oCTLA-4-hlg containing the secretion signal and extracellular domains of ovine CTLA-4 [40] was linked to the human Ig domain was obtained from M. Tachedjian (CSIRO, Geelong, Australia). The cDNA for F. hepatica pro-Cathepsin B was amplified as above using the primers 5′ GAGGAGACGCGTAAACCAAAC GAGGAGACGCGTTCAAAGACGTGGCA TTCC. and 5′ GAGGAGACGCGTCCCAAGACGGTGGGATTCC and subsequently digested with MluI and ligated into pCI or oCTLA-4-hlg cut with the same enzyme to create pCI:oCTLA-4-hlg:CatB. The oCTLA-4-hlg:CatB cDNA was excised from pCl:oCTLA-4-hlg:CatB following XhoI/Kpn digestion and ligated into pBluescript SK+ (Stratagene Corporation, La Jolla, CA) cut with the same enzymes to form pBS:oCTLA-4-hlg:CatB. The resulting product was digested with XhoI/Kpn digestion and ligated into pBluescript SK+ (Stratagene Corporation, La Jolla, CA) cut with the same enzymes to form the DNA vaccines pM:oCT-CatB and pM:CpG-oCT:CatB. The excision of CatB from pM:CpG-oCT:CatB with MluI generated the control pM:CpG-oCT. All constructs were subsequently sequence verified to ensure construct integrity.

2.2. In vitro expression of DNA vaccines

All plasmids were tested for the expression of encoded protein in COS 7 cells prior to use in animals. Freshly grown COS 7 cells were seeded at 2 × 10⁵ cells/ml and grown in
LipofectAMINE® 1000® (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. Cells were then cultured in 5% CO₂ at 37°C for 48 h before supernatants were collected and the cells washed with PBS. The media was replaced with serum free media to remove FCS that may mask protein detection. Cells were grown for a further 24 h before the cell pellets and supernatants were collected and analysed for the presence of expressed FhCatB protein by SDS-PAGE and Western blotting. Briefly, supernatants were fractionated by SDS-PAGE on 12% polyacrylamide gels under reducing conditions and transferred electrophoretically to nitrocellulose membranes that were blocked overnight in 5% skim milk. Western blots were probed with rabbit α-FhCatB (1:500 dilution) antisera followed by detection with HRP conjugated goat α-IgG (Southern Biotech, USA) at a 1:1000 dilution. For detection of human Ig, an identical western blot was probed with a HRP conjugated goat α-IgG (Southern Biotech, USA) monoclonal at 1:1000 dilution.

Plasmids were maintained and propagated in E. coli strain DH5α. The large-scale production of endotoxin-free DNA was achieved by using the Endofree® Plasmid Giga Kit (Qiagen, GmbH, Germany) according to the manufacturer’s instructions.

2.3. Animals and immunisations

Three-month-old Merino crossbred wethers were selected from a flock with no history of pre-exposure to F. hepatica and randomly assigned into seven groups of five sheep. On week 0 and 4, animals receiving DNA vaccines were vaccinated intramuscularly with 100 μg of DNA in 5 ml of PBS. As a positive control, another group of animals was vaccinated intramuscularly with 100 μg of yeast-expressed FhCatB (from clone pFCatB) [37] mixed with 100 μg of Quil A in 2 ml of PBS at weeks 0 and 4. At week 8, all animals were boosted with the same dose of FhCatB in Quil A adjuvant.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Sera were collected at bi-weekly intervals and assayed for the presence of antibodies to FhCatB by standard ELISA. Briefly, 96-well maxisorb micro titre plates (Nuclon, Denmark) were coated with FhCatB [37] at 2 μg/ml in 100 μL of carbonate-bicarbonate buffer and incubated overnight at 4°C. Plates were subsequently washed with PBS and 0.05% Tween® 20 (ICI Americas Inc., Bridgewater, NJ) (PBS-T) and then blocked for 1 h at 37°C in 0.5% skim milk powder in PBS-T, before the addition of serially diluted sera in the same buffer for 1 h at 37°C. Plates were again washed and bound antibody detected by using HRP conjugated rabbit anti-sheep IgG (Southern Biotech, USA) at a 1:1000 dilution for 1 h at 37°C followed by washing and the addition of substrate. Antibody isotypes were detected using mouse anti-sheep IgG and IgG2 monoclonals [41] followed by incubation with HRP conjugated sheep anti-mouse Ig (Silenus, Australia). The ELISA was developed by addition of substrate 3,3′,5,5′-tetramethylbenzidine (Sigma, USA), and the endpoint titres were defined as the highest dilution of sera yielding an OD₄₅₀ of 0.2.

2.5. Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation of heparinised blood at 800 × g for 20 min followed by centrifugation of the buffy coat at 1200 × g for 30 min on a Ficoll-Paque® density gradient (1.077 specific gravity; Pharmacia Biotech AB, Sweden). Cells were collected from the Ficoll/PBS interface and washed twice in PBS at 350 × g for 7 min before a final wash at 200 × g for 7 min to remove platelets. Cells were resuspended at 1 × 10⁶ cells/ml in 200 μL of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg 2-mercaptoethanol and 2 mM sodium pyruvate. Triplicate wells containing PBMCs were cultured for 5 days in the presence of FhCatB and Con A (positive control) each at 5 μg/ml. Proliferation was measured by the addition of 1 μCi/well of [³H]-thymidine for the final 18 h of culture. Cells were harvested onto multiscreen filtration system 96-well plates (Millipore) and the counts per minute (CPM) of incorporated [³H]-thymidine determined by gas phase scintillation counting. The stimulation index (SI) was calculated as the ratio between the CPM of cells in the presence of antigen and the CPM of cells in the absence of antigen.

2.6. IFN-γ assay

PBMCs were prepared as above and cultured at 5 × 10⁶ cells/ml in 1 ml of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg 2-mercaptoethanol and 2 mM sodium pyruvate. Duplicate wells containing medium alone, 5 μg/ml FhCatB or 5 μg/ml Con A were incubated for 24 h before culture supernatants were collected and frozen. The concentration of IFN-γ in each sample was determined by capture ELISA using the Bovigam® bovine γ interferon test kit (Pitzer Inc., New York, NY) according to the manufacturer’s instructions.

2.7. Statistical analysis

Prior to analysis, all data was log transformed to obtain a normal distribution. Differences between groups were analysed using one-way ANOVA followed by Tukey’s multiple comparison test. p-values below 0.05 were considered significant.
3. Results

3.1. In vitro expression in COS 7 cells of FhCatB from vaccine plasmids

Expression of FhCatB protein (∼41 kDa) from DNA vaccine vectors was confirmed by transient transfection of COS 7 cells. Analysis of Western blots probed with rabbit polyclonal FhCatB antisera revealed the expression of FhCatB in COS 7 cell lysates from the non-targeted (pM:CatB) and CpG-augmented non-targeted (pM:CpG:CatB) constructs (Fig. 1A). Similarly, expression of oCTLA-4:CatB fusion proteins (∼104 kDa) from the CTLA-4 targeted (pM:oCT:CatB) and CpG-augmented CTLA-4 targeted (pM:CpG:oCT:CatB) constructs was confirmed by probing western blots with a mouse anti-human Ig monoclonal antibody (Fig. 1B). Expression of the control CTLA4-Ig fusion protein was also observed (pM:CpG:oCT, ∼63 kDa) (Fig. 1B). This antibody detects the Ig portion of the CTLA-4-Ig targeting protein, thus confirming the integrity of the targeting moiety. The presence of FhCatB in the fusion proteins (∼104 kDa) was confirmed by probing an identical western blot with rabbit polyclonal FhCatB antisera (Fig. 1C). The extra band with a relative mobility of ∼83 kDa appears to be a component of the fetal calf serum that is recognised by the rabbit anti-FhCatB polyclonal antibody.

3.2. CTLA-4 DNA vaccine targeting enhances the primary IgG response and primes for a potent memory response to rFhCatB

CTLA-4 targeted DNA vaccination (pM:oCT:CatB) induced a significant increase in the primary IgG antibody response at week 6 compared to non-targeted DNA vaccination irrespective of CpG augmentation (Fig. 2). Specifically, the mean IgG titre (1/6500) in animals vaccinated with the targeted construct (pM:oCT:CatB) were significantly higher than the IgG titres generated in animals vaccinated with the non-targeted pM:CatB (1/2100, p < 0.05), pM:CpG:CatB (1/1600, p < 0.01) or pM:CpG:oCT (1/1800, p < 0.01) constructs. Similarly, CpG-enhanced targeted DNA vaccination (pM:CpG:oCT:CatB) induced significant increases in the primary IgG antibody response (titre 1/5200) compared to the CpG-augmented non-targeted (pM:CpG:CatB, p < 0.01) and control (pM:CpG:oCT, p < 0.01) groups at week 6. Although higher, IgG titres in the animals vaccinated with the CpG-enhanced targeted construct (pM:CpG:oCT:CatB) were not significantly different to the non-targeted construct without CpG augmentation (pM:CatB) at week 6 (Fig. 2). The CpG-enhanced targeted group (pM:CpG:oCT:CatB) also displayed a significantly higher IgG titre at weeks 2 (1/3900, p < 0.05) and 4 (1/12000, p < 0.01) compared to the CpG-augmented non-targeted group (pM:CpG:CatB) at weeks 2 (1/1100) and 4 (1/1500).

Within 1 week following protein boosting with recombinant FhCatB (week 9) it was evident that targeting ±CpG...
Fig. 2. Kinetics of the IgG response of sheep to FhCatB following DNA vaccination and protein boosting. All animals were vaccinated intramuscularly with 500 μg of DNA at weeks 0 and 4 followed by an intramuscular booster vaccination at week 8 with 100 μg of yeast-expressed FhCatB protein emulsified in 100 μg of Quil A. FhCatB-specific antibody titres were measured by ELISA from individual serum samples (n = 5/group). Values represent mean IgG titres plus one S.D. (⁎) pM:CpG:oCT:CatB significantly different to pM:CpG:CatB (p < 0.05). (**) pM:CpG:oCT:CatB significantly different to pM:CpG:CatB (p < 0.01) and pM:CpG:oCT (p < 0.05). (***) pM:CpG:oCT:CatB significantly different to pM:CpG:CatB (p < 0.01) and pM:CpG:oCT (p < 0.01). (a) pM:oCT:CatB and pM:CpG:oCT:CatB significantly different to all other groups (p < 0.001). (b) pM:oCT:CatB and pM:CpG:oCT:CatB significantly different to pM:CpG:CatB (p < 0.05) and to all other groups (p < 0.01).

Augmentation primed for a significant increase in memory IgG titres (1/175,800 and 1/102,200, respectively, p < 0.01) compared to groups vaccinated with the non-targeted DNA constructs ± CpG augmentation (1/8300 and 1/4200, respectively, Fig. 2). Although CpG-augmented CTLA-4 mediated targeting (pM:CpG:oCT:CatB) appears to enhance the IgG titres compared to CTLA-4 mediated targeting alone (pM:oCT:CatB), at weeks 9 and 10, these differences were not significant.

Conventional non-targeted DNA vaccination ± CpG augmentation (groups pM:CatB and pM:CpG:CatB) failed to induce a primary or memory (post week 8) IgG response (Fig. 2). The apparent induction of a memory IgG response (post week 8) in animals vaccinated with the non-targeted CpG-augmented plasmid (pM:CpG:CatB) is due to one animal responding well to protein boosting and is not significantly different to the non-targeted group (pM:CatB).

A comparison of animals primed with DNA or recombinant protein in Quil A followed by recombinant protein boost revealed that priming twice with either of the DNA targeting constructs (DDP) generated IgG titres that were comparable to those seen in animals primed once (PP) with protein in Quil A (PPP). The IgG titres were still not significantly higher than those seen in animals primed twice with either of the targeted DNA constructs. However, one animal in the protein prime group PPP did fail to respond to FhCatB protein boosting. From these data, it appears that priming twice with targeted constructs without adjuvant was just as effective as priming once with FhCatB protein in Quil A adjuvant.

3.3. CpG augmentation has no effect on the isotype profile following CTLA-4 targeted DNA vaccination

Analysis of the IgG1 antibody titres revealed that CTLA-4 mediated targeting, irrespective of CpG augmentation, induced significantly higher IgG1 titres compared to all other groups at week 9 (Fig. 4A). Although there was a trend for the mean IgG1 titres in the targeting groups to be higher than IgG1 titres in the non-targeted groups at week 6, these differences were not significant. An analysis of the mean IgG2 titres (Fig. 4A) at weeks 6 and 9 revealed no significant differences, although groups receiving the targeted or non-targeted constructs ± CpG augmentation showed a trend for higher mean IgG2 titre than their respective controls (pM and pM:CpG:oCT:CatB). Collectively, there appeared to be no differences in the mean IgG1 or IgG2 titres afforded by CpG augmentation of either the targeted or non-targeted constructs (Fig. 4A).

In order to assess whether individual animals have responded to CpG-augmented targeting, IgG1/IgG2 ratios were calculated for individual animals in each group (Fig. 4B). An analysis of these data revealed an even spread of individual IgG1/IgG2 ratios between the targeting ± CpG groups.
Fig. 4. Isotype of the IgG response to FhCatB following DNA vaccination. (A) Mean IgGl and IgG2 titres for weeks 6 and 9. (*) pM:oCT:CatB significantly different to pM:CatB and pM:CpG;CatB (p < 0.01) and to pM:CpG:oCT:CatB significantly different to all other groups (p < 0.001). (**) pM:CpG:oCT:CatB significantly different to all other groups (p < 0.001). (B) IgGl/IgG2 ratios for sera from sheep vaccinated with targeted DNA vaccines. Mean IgGl/IgG2 ratios and individual IgGl/IgG2 ratio analysis for weeks 0, 2, 6 and 9 from animals vaccinated with pM:oCT:CatB or pM:CpG:oCT:CatB are shown. Black dots represent individual animals (n = 5/group). Values represent mean IgG titres plus one S.D.

DNA groups, indicating that CpG augmentation has not biased the individual isotype response that was induced by CTLA-4 mediated targeting alone.

3.4. CpG augmentation enhances the FhCatB-specific lymphocyte proliferative responses in sheep vaccinated with CTLA-4 targeted plasmids

The CpG-augmented CTLA-4 targeted construct (pM:CpG:oCT:CatB) induced a significantly higher proliferative response (p < 0.01) than the non-targeted construct (pM:CatB) and the control (pM:CpG:oCT) at week 6 (Fig. 5). One week after FhCatB protein boosting at week 9, only the response of lymphocytes in the CpG augmented targeted group (pM:CpG:oCT:CatB) was significantly different to the response observed in the control group (pM:CpG:oCT, p < 0.01). Individual SI data for the CpG augmented CTLA-4 targeted group suggests that, while one animal appeared not to respond, compared to vector control (pM:CpG:oCT), the remaining four animals responded to varying degrees at weeks 6 and 9. Although it appeared that CpG augmentation of the non-targeted construct (pM:CpG:CatB) induced an enhanced proliferative response at week 9, individual data points indicate that this was solely due to one animal responding well with an SI of 9.19. This is the same animal responsible for the apparent increase in IgG titre for the CpG-augmented non-targeted group (pM:CpG:CatB) over weeks 9–12 (Fig. 1). While one animal may have responded at week 6 in the CTLA-4 mediated targeting group (pM:oCT:CatB), it is clear that only those animals that were in groups receiving CpG-augmented constructs were capable of responding upon antigen re-stimulation. Although some animals appeared to display lymphocyte proliferative responses following protein vaccination, these responses were not significantly different to the empty vector controls (pM and pM:CpG:oCT) at any time point.

Although IFN-γ production was evident in culture supernatants stimulated with Con A, there was no antigen specific IFN-γ production observed for any treatment group at any time point (data not shown).

4. Discussion

In this study, we have demonstrated for the first time, that both humoral and cellular immune responses can be generated in sheep to a DNA vaccine encoded antigen by combining the use of CpG motifs with CTLA-4 mediated targeting. While CTLA-4 mediated targeting alone induced a primary antibody response to FhCatB and a potent memory response following protein boosting (compared to non-targeted DNA
While significant enhancements in DNA vaccine efficacy have been observed in mice using CTLA-4 mediated targeting [6,42], there are few reports detailing its effectiveness in larger animal species [7,43]. In this study, we have demonstrated that the intramuscular vaccination of sheep with a DNA vaccine expressing the F. hepatica pro cathepsin B antigen fused to CTLA-4, enhanced the speed and magnitude of the humoral response, both before and after protein boosting, compared to non-targeted DNA vaccination. Although the CTLA-4 induced IgG responses did not appear until 2 weeks after the second DNA vaccination, the non-targeted constructs failed to induce detectable antibody responses even with CpG augmentation. Our results are in agreement with Chaplin et al. [8], who demonstrated that the intramuscular vaccination of sheep with the phospholipase D (PLD) antigen from Corynebacterium pseudotuberculosis fused to CTLA-4 enhanced the speed and magnitude of the humoral response compared to animals vaccinated with the non-targeted constructs. Similarly, the gene gun mediated vaccination of pigs with a CTLA-4 plasmid significantly enhanced the speed and magnitude of the humoral response to the model antigen ovalbumin [7]. In contrast, the vaccination of sheep with a CTLA-4 construct encoding the 45W antigen from T. ovis induced antibody responses that were not significantly different from those generated by the non-targeted constructs [43]. In this study, the authors noted that the binding efficiency of the CTLA-4:45W fusion protein to its B7 ligand was lower than for the CTLA-4 control. This indicates that the effectiveness of CTLA-4 mediated targeting may be, at least partially, dependent on particular characteristics of the chosen antigen.

While studies in mice have shown that enhanced humoral immune responses can be obtained by adding CpG motifs to the plasmid backbone [15,44], this is the first study to explore the potential adjuvant effects of ruminant specific CpG motifs on DNA vaccine induced immune responses in sheep. In contrast to the studies in mice, the addition of CpG motifs to the non-targeted construct in this study still failed to induce a detectable humoral response. Similarly, the addition of CpG motifs to the backbone of the CTLA-4 targeted construct did not further enhance the magnitude or quality (IgG/IgG2 ratio) of the FhCatB specific IgG titres, either before or after protein boosting, when compared to the IgG responses induced by the targeting construct alone. However, it did appear that the speed of the IgG response to FhCatB in the CpG-augmented CTLA-4 mediated targeting group (pM:CpG:oct:CatB) was slightly enhanced, with significant IgG titres (p < 0.05) appearing at week 2 that were absent in the CTLA-4 mediated targeting alone group (pM:oct:CatB). Although there are no other reports concerning the effects of CpG motifs on immune responses induced by DNA vaccines in sheep, a recent study in cattle showed no differences in the resultant IgG titres induced following vaccination with plasmids containing 40 or 88 ruminant specific CpG motifs. While there was a dramatic decrease in the IgG/IgG2 ratio in animals receiving the plasmid containing 88 CpG motifs, this decrease was not observed in cattle vaccinated with the 40 CpG motif containing plasmid. While our study showed no changes in the IgG/IgG2 ratios using the same plasmid containing 40 ruminant specific CpG motifs (Fig. 4B), it remains to be seen whether the vector containing 88 CpG motifs could alter the isotype response in sheep. The authors suggested that a critical threshold of CpG motifs may be required to influence the isotype ratios in large animals.

Analysis of the antigen-specific T-cell responses at week 6 revealed that the combined use of CTLA-4 mediated targeting and CpG motifs induced proliferative responses in 45 animals that were significantly different (p < 0.01) to the responses observed using either strategy alone, suggesting that the induction of efficient T cell responses appears to be a result of this unique combination of strategies. The lack of T cell responses in most groups may not be surprising, as other papers have failed to demonstrate an ability of DNA vaccines to induce T-cell proliferative responses in sheep [45,46]. This is the first report showing the induction of significant cellular responses in sheep following DNA vaccination. Furthermore, the efficiency of the CTLA-4/CpG combination in inducing cellular responses in sheep is highlighted by the observation that the delivery of CatB protein in Quil A adjuvant failed to induce significant proliferative responses at any time point. One of the hallmarks of DNA vaccination has been its ability to prime for a strong memory response even in the absence of a detectable primary antibody response [8,46,47]. Similarly, we observed that boosting with recombinant FhCatB protein in Quil A adjuvant induced potent memory IgG responses in those animals that were primed with the CTLA-4 constructs, which remained significantly different until 5 weeks post protein boost when the experiments were terminated. The efficiency of CTLA-4 priming was evident as animals receiving CTLA-4, irrespective of CpG augmentation, displayed memory IgG titres (post week 8) that were not significantly lower than those observed in control animals primed with one or even two doses of FhCatB protein in Quil A (Fig. 5). The failure of the non-targeted DNA vaccines to prime for an anamnestic response suggests that, even in the presence of CpG, the levels of antigen production in transfected cells was insufficient for priming an immune response. This observation may be partly explained by the intracellular expression of encoded antigen, which would presumably act to limit the amount of antigen available for sampling by APCs. In fact, several papers have reported that the cytoplasmic expression of a DNA vaccine encoded antigen results in humoral responses up to 100-fold lower than those induced by the secreted form of the antigen [48,49]. An analysis of the antigen specific lymphocyte proliferation responses 1 week
following protein boost (week 9) revealed that only those animals primed with the CpG-augmented/CTLA-4 mediated targeting construct were capable of mounting a significant lymphocyte proliferative response.

Considering that as much as 90% of injected DNA never enters the cell [50], but instead circulates through the local lymphatic system, it is conceivable that the exposure and thus activation of APCs by circulating CpG motifs would occur prior to CTLA-4/FhCatB fusion protein expression from transfected cells. Thus, if the circulating APCs were activated and displayed more costimulatory molecules (especially CD80/86) and had improved antigen processing and presentation capabilities [10], a more efficient immune induction would have resulted. This may at least partially explain the observed enhancement of cellular responses by the CTLA-4/CpG motif combination. The lack of cellular responses in animals vaccinated with the non-targeted CpG-augmented DNA vaccines, even in the presence of CpG, again suggests that there was not enough antigen sampled by APCs for efficient immune induction.

Recently, it has been observed that the enhancement of antigen processing and presentation favors antigens sampled at the time of CpG exposure [51], suggesting that timing of CpG delivery could be important in obtaining optimal immune responses to a DNA vaccine. In agreement with this observation, the delivery of a CpG containing plasmid into mice at day 2 after DNA vaccination induced a five-fold increase in antibody titre and a 20% increase in specific immune responses to a DNA vaccine. In summary, we have confirmed that CTLA-4 mediated targeting can enhance antibody responses in sheep following DNA vaccination, and for the first time, we have shown the induction of significant cellular responses in sheep to a DNA vaccine encoded antigen by the incorporation of CpG motifs into the backbone of the targeting plasmid. Collectively, our results suggest that exploring various combinations of strategies may be an effective approach to further enhance the potency of DNA vaccines in large out-bred animal models.

Acknowledgments

This work was carried out with the support of the Australian Government’s Cooperative Research Centres Program, the Australian Research Council Strategic Partnerships with Industry—Research and Training Scheme (ARC-SPIRT); CSL; Monash University and the Centre for Animal Biotechnology, University of Melbourne.

References


N. J. Kennedy et al. / Vaccine 24 (2006) 970–979 977


