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The serological response of sheep to DNA immunisation against *Toxoplasma gondii*

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Abstract of a dissertation submitted in partial fulfilment of the requirements for the
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ABSTRACT

The administration of DNA vaccine has been suggested as a promising method to increase specific humoral and cellular immune response against *Toxoplasma gondii* (*T. gondii*) infection. In the current study, the serological (immunoglobulins IgG1 and IgG2) responses of sheep to DNA vaccination against *T. gondii* were evaluated.

The cloned plasmid DNA containing protective *T. gondii* antigens, ROP1, GRA7, MIC3, GRA1, GRA6 and MAG1 were transformed and grown in *Escherichia coli* cells and purified. Three groups of coopworth twelve month old ewes (n=40) were immunised by intramuscular injection with the combination of purified plasmids pVAXROP1/CD154+pVAXIgGRA7+pVAXMIC3 and/or pVAXIgGRA1+pVAXIgGRA6+pVAXMAG1 separately, as well as plasmid encoding CpG oligodeoxynucleotide and liposomes as adjuvants. Another group of ewes (n=40) were injected with phosphate buffered saline solution as controls. The injections were applied twice intramuscularly with a four weeks interval between injections.

Production of IgG1 and IgG2 antibodies were studied by the use of enzyme linked immunosorbent assays, with the optical density ratio representing antibody titre response. The first and second immunisations with the combination of plasmids pVAXROP1/CD154+pVAXIgGRA7+pVAXMIC3 produced high ($P<0.001$) level of specific anti-ROP1 IgG1 antibody in the serum. This indicates that this technique could stimulate strong humoral immune response, and therefore has promise for development of a commercial *Toxoplasma* vaccine.

Keywords: sheep, DNA vaccine, *Toxoplasma gondii*, antibody.

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Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite that causes toxoplasmosis in a variety of mammals and in more than 30 species of birds worldwide (Bhopale 2003; Gatkowska et al., 2006). Although an infection is usually asymptomatic, toxoplasmosis is still a significant medical and veterinary issue. Infection during human pregnancy, especially during the third trimester, causes severe health problems to the foetus, such as neurological diseases (Roque-Resendiz et al., 2004; Mévélec et al., 2005; Fatoohi et al., 2002). The infection also causes loss of offspring in pregnant animals, especially in sheep and goats, causing it to become a potential economic problem worldwide. The major causes of toxoplasmosis infections in humans are consuming either undercooked meat containing tissue cysts or vegetables contaminated with oocysts released in cat faeces, and the infection in sheep are due to eating oocysts contaminated pasture.

In New Zealand, *T. gondii* has been recognized as an economically important parasite in the sheep industry. It is one of the major reasons leading to reduction in the number of lambs born, and therefore, affects meat production on farms (Desolme et al., 2000). Human infection by *T. gondii* is also common in New Zealand. A study carried out by Cursons et al. (1982) showed that more than 55% of pregnant women had antibodies against *T. gondii*.

At present, there is a live attenuated vaccine (Toxovax) available on the market, and was first discovered by Wilkins et al. (1987) and his colleagues. This uses live tachyzoites of strain S48 and is a suitable as a veterinary medicine for ovine toxoplasmosis (Dautu et al., 2007). However, there are limitations with this vaccine such as its short shelf life, high storage costs and even vertical transmission of *T. gondii*

from the ewe to foetus upon application. Moreover, this vaccine is not suitable for humans.

To overcome those limitations it was decided to use DNA vaccines technology that was first introduced by Wolff et al. (1990). This new type of vaccine allows protein expression in mammalian cells after the introduction of plasmids encoding selected protective antigens. The protective immunity of using DNA vaccines has shown encouraging results against various diseases such as human immunodeficiency virus (HIV) (Calarota and Weiner, 2004), malaria (Moore and Hill, 2004), tuberculosis (Lowrie et al., 1994) and cancer (Stevenson et al., 2004) by inducing both humoral and cellular immunity. In the current study, ewes were immunized with individual constructs of plasmid DNA encoding *T. gondii* antigens (GRA1, GRA6, GRA7, MIC3, ROP1/CD154 and MAG1). CpG ODN was used as immunostimulatory agents and liposomes as adjuvants to determine the ability of stimulating humoral immune response.

2 Literature Review

2.1 Overview of *T. gondii*

The natural life cycle of *T. gondii* exhibits three distinct infectious stages: tachyzoites, bradyzoites (in tissue cysts) and sporozoites (in oocysts) (Cristina et al., 2004). The tachyzoite is normally crescent-shaped and $2\ \mu\text{m} \times 6\ \mu\text{m}$ in size. It enters the host cell by active penetration of the cell membrane and is then wrapped by a parasitophorous vacuole that protects it from host defence mechanism (Dubey, 2004). After a number of active divisions, tachyzoites become bradyzoites, which are contained in tissue cysts. The tissue cysts remain intracellular, and grow to various sizes from 5 to 70 μm (Dubey et al., 1998). Unlike tachyzoites, bradyzoites are more slender and less likely to be destroyed by proteolytic enzymes (Dubey, 2004). The third stage of *T. gondii* cycle is forming oocysts in cats only. Cats shed oocysts after ingesting tachyzoites, bradyzoites or sporozoites. Oocysts in freshly passed faeces are unsporulated, while sporulation occurs within 1 to 5 days depending on temperature. A sporulated oocyst contains eight sporozoites, which is $2\ \mu\text{m} \times 6\text{-}8\ \mu\text{m}$ in size (Dubey et al., 1998).

Initially infection by *T. gondii* leads to an acute systemic phase with rapid multiplication of tachyzoites in the cells of a non-immune host, during which they spread throughout the body via the bloodstream. After immunity develops, the host immune response controls the acute infection, and a chronic phase takes over the infectious process, in which bradyzoites multiply slowly. Bradyzoites are enclosed in cysts in a dense matrix and surrounded by a thick cyst wall; persist in the brain, the heart and other tissues (Bhopale, 2003; Nielsen et al., 2006). The tissue cysts contain hundreds of bradyzoites, which are also long-lived and do not cause disease. If non-feline hosts (humans and all the other warm-blooded vertebrates) consume the tissue

cysts, an asexual multiplication occurs (Fig 1). If cat family members eat the tissue cysts, a sexual cycle of the parasite occurs in the intestine to produce oocysts, which are released in their faeces. When humans or sheep consume sporulated oocysts or infective tissue cysts from meat of infected animals, the sporozoites are released and infect the intestinal epithelial cells of humans or sheep. Then tachyzoites are multiplying rapidly and first produce acute, and then chronic infection.

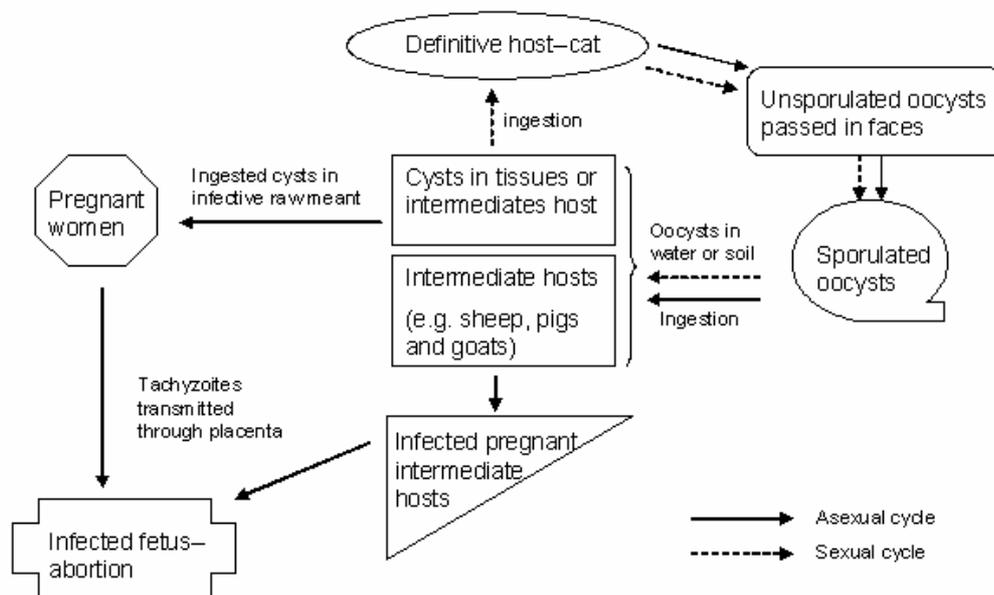


Figure 1. The life cycle of *Toxoplasma gondii*.

2.2 Mechanisms of immunity

Natural infection with *T. gondii* generally leads to a state of long-lasting protective immunity (Leyva et al., 2001). It is well established that both humoral and cell-mediated immune responses, include T-cell mediated immune response that involves both CD4+ and CD8+ T lymphocytes (CTL), and antibodies, are important in conferring immunity to *T. gondii* infection (Buxton, 1993; Vercammen et al., 2000). In general, humoral antibodies are effective against blood-borne stages of the *T. gondii* life

cycle, but once they have infected host cells, cell-mediated immunity is necessary (Goldsby et al., 2003).

Humoral immunity refers to antibody (immunoglobulin, Ig) production. In the humoral response, an antigen-binding cell, B cell, interacts with invading microbes (such as *T. gondii*) and then differentiate into antibody-secreting plasma cells (Goldsby et al., 2003). The infection is generally diagnosed by the presence of these specific antibodies to *T. gondii* antigens in the sera of infected mammals. During acute toxoplasmosis, the presence of *Toxoplasma* specific IgM antibodies is the most valuable serological marker and may be detected for a long time after this phase of infection (Marcolino et al., 2000; Beghetto et al., 2003). Another marker showing a significant increase is the specific IgG antibody titers against *Toxoplasma* infected serum samples (Marcolino et al., 2000; Pfrepper et al., 2005). This suggests that IgG antibody production will be stimulated if vaccination is effective against *T. gondii* infection.

The cell-mediated response in a host protects against acute *T. gondii* infection and also control of the chronic steps (Prigione et al., 2006). CD4⁺ type 1 helper T (Th1) cells and major histocompatibility complex-I (MHC-I) restricted CD8⁺ T lymphocytes (CTL) are considered the major effector cells that are responsible for protection against *T. gondii* (Fux et al., 2003). When tachyzoites are released, CD4⁺ Th1 cells and then CD8⁺ T cells secrete various cytokines, particularly interferon-gamma (IFN γ), which is the major mediator of resistance against toxoplasmosis (Leyva et al., 2001; Martin et al., 2004; Bradley and Boothroyd, 2001). Tachyzoites also stimulate macrophages, which in combination with CD4⁺ Th1 cells protect the host from infection by producing IFN γ , interleukin-12 (IL-12), tumour necrosis factor- α (TNF α) and natural killer (NK) cells

(Bhopale, 2003). Those cytokines are crucial for resistance and contribute to killing tachyzoites by antibodies and/or macrophages. This indicates that effective vaccines against *T. gondii* should be based on parasite antigens, which induce a T cell-mediated response, particularly Th1 protective immunity.

Currently, there is only one live attenuated vaccine, Toxovax® (Intervet Ltd, New Zealand), which is available on the market and consists of live tachyzoites of strain S48, is used as a veterinary medicine for ovine toxoplasmosis (Wilkins et al., 1987; Buxton et al., 1991; Roque-Resendiz et al., 2004; Dautu et al., 2007). However, there are limitations with this live vaccine, such as its short shelf life, high storage costs and vertical transmission of *T. gondii* from the ewe to foetus upon application. The live vaccine cannot be used in humans, as it may revert to a pathogenic strain and give unexpected harmful mutations. Therefore, DNA vaccination is suggested as an ideal alternative for the stimulation of immunity against toxoplasmosis for both animals and humans.

2.3 DNA vaccine

2.3.1 Overview of DNA vaccine

Over the last 17 years, DNA immunization, also known as genetic or polynucleotide immunization is a rapidly developing area of vaccines for the prevention and treatment of diseases, such as HIV (Calarota and Weiner, 2004), hepatitis C (Geissler et al., 1997), malaria (Moore and Hill, 2004), tuberculosis (Lowrie et al., 1994) and tumor (Stevenson et al., 2004) by inducing both humoral and cellular immunity.

DNA vaccine utilizes *in vivo* DNA expression vectors as plasmids encoding for a specific foreign antigen under the control of a eukaryotic promoter, to stimulate the synthesis of immunogenic proteins and immune response. DNA vaccines against *T. gondii* in most animal studies have used *Escherichia coli* (*E. coli*)-derived plasmid DNA (Koide et al., 2000). The plasmid DNA used for immunization contains the following basic elements: a prokaryotic origin of replication (*ori*) in *E. coli*, a strong mammalian promoter to drive expression in eukaryotic cells, an antibiotic resistance gene for growth selection, a polyadenylation termination sequence, and an antigen-encoding gene whose expression is regulated by the promoter (Donnelly et al., 1997; Cui, 2005).

The generation of a recombinant plasmid DNA is via a simple process. A selected fragment of antigen-encoding gene is amplified by PCR from genomic DNA of *T. gondii*. Specific primers (forward and reverse) are used to amplify the selected gene by introducing two restriction enzyme sites for ligation. The amplified DNA fragments are cloned in the two enzyme sites of the vector, under the transcriptional control of the promoter, to give a recombinant plasmid DNA. The recombinant plasmid DNA is then isolated from transformed cells by a purification process, and dissolved in sterilized PBS.

Immunization of DNA can be administrated in two different ways: a simple intramuscular (i.m.) injection of the plasmid DNA alone (delivery vaccine via needles and syringes) and gene gun (use gold beads coated with plasmid DNA, and transfect cells in gun-bombarded skin) (Kalinna, 1997). DNA vaccine delivered by i.m. injection has gene expression predominantly in the myoblasts. The majority of gene expressions

are found in fibroblasts after gene gun mediated administration. Studies found that i.m. injections are very effective in small animal models such as mice (Cui, 2005). The gene gun delivery system is highly reliable in larger animals such as sheep, and has resulted in a better immune response than i.m. injection in clinical trials. For both types of immunization, activated antigen presenting cells (APCs) express antigen with MHC molecule to induce cytotoxic T-lymphocyte (CTL) response. Transfection of APCs was found to be particularly efficient after gene gun immunization, as epidermal cells and macrophages act as APCs. Thus, gene gun immunization allows rapid delivery of a vaccine to large populations, results in broad expression and enhances vaccine potency.

2.3.2 Immunity of DNA vaccine

DNA immunization has been used to elicit humoral (protective antibodies) immunity and cell-mediated immune responses in a wide variety of animal models for viral, bacterial, and parasitic diseases and clinical human diseases, such as HIV (Donnelly et al., 1997). In general, when a plasmid DNA encoding the antigenic peptide is injected into a body tissue, mostly in muscle cells, the encoded information is transcribed and translated into proteins, which are then processed into small peptides and bound to MHC (Fig 2). Then they migrate together to the cytoplasmic membrane, present themselves to CD8 T-cells, and stimulate a of cytotoxic T cell response. Meanwhile, the expressed antigen protein is also released from the muscle cell in order to bind and stimulate B cells (a kind of antigen presenting cell, APC) for antibodies production. Furthermore, the muscle cell itself makes contact with an APC, which presents the expressed protein by MHC-I and MHC-II pathways to stimulate T helper cells and thus regulate the B cell response.

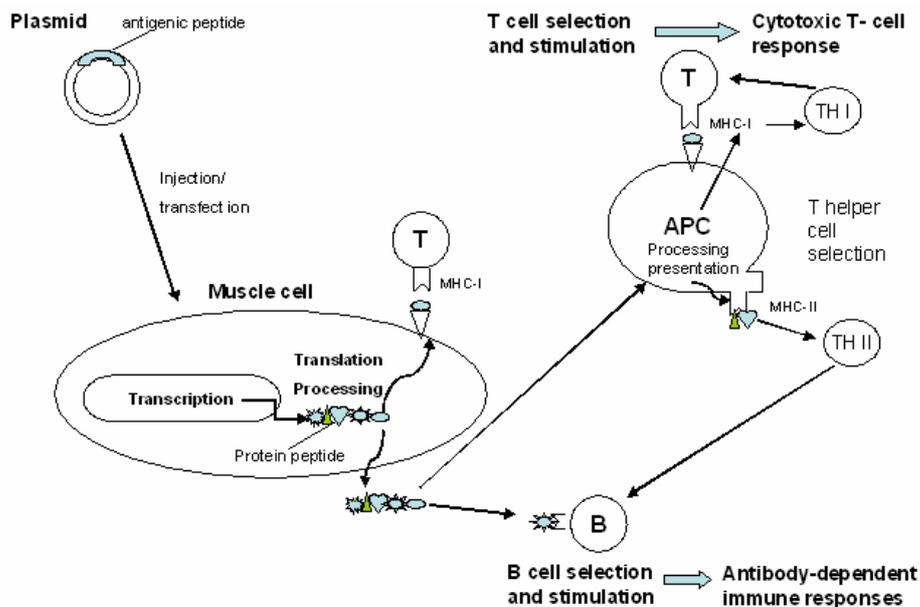


Figure 2. Immune mechanism induced by inoculation with naked DNA. The diagram was drawn based on a figure by Gregersen (2001).

DNA vaccine administration has been shown to induce a strong specific antibody response to a variety of proteins, such as virus, bacteria, parasite, tumour and eukaryote (Donnelly et al., 1997). The technique was first discovered in mice in 1992 by Tang et al. who found that the antibodies were produced against human growth hormone after a gene gun delivered DNA coding for growth hormone. They detected the antibodies in the sera for years, inferring there is continued antigen stimulation. Antibodies against viral proteins were detected with i.m. injection of a DNA vaccine encoding for influenza HA (Ulmer et al., 1993), and HIV (Wang et al., 1993). Cohen et al. suggested that the production of antibody type is dependent on the mode of DNA administration and the injection dose (Cohen et al., 1998). The antibody production is increased in a dose-responsive manner with a single injection or multiple injection of DNA. However, irrespective of the time of injecting an optimal dose of DNA, the amount of antibody produced could not be dramatically influenced once a plateau had been reached.

DNA vaccine is different from protein and live vaccines. The duration of antibody responses induced by DNA vaccination is long-lived in mice with influenza HA

(Justewicz et al., 1995). The antibody isotypes induced are generally IgG, but serum IgM and IgA also have been detected. Among the detected antibodies, IgG2a in mice is predominantly induced by DNA vaccination, indicating that a Th1 helper T cell response is induced. In some cases, antibodies were also able to protect against challenges from relevant infectious pathogens, suggesting that after application of the DNA vaccine that the in vivo expressed antigens could provide a native configuration with whole epitopes capable of inducing neutralizing antibodies (Donnelly et al., 1997). Thus, DNA vaccines may generate antibody responses that resemble more closely antibodies produced after natural infection and therefore provide a potential advantage over conventional vaccines.

The cellular immunity comprises primarily CD4 T helper cell responses and CD8 CTL responses. Both CD4 and CD8 T cells recognize foreign antigens, which are processed and presented by APCs with MHC-II (associated with CD4 cells) and MHC-I (associated with CD8 cells) molecules, respectively. CD4 T cells play a central role in specific immune responses. Without CD4 T cells antibodies cannot be generated against most antigens. The CD4 T cells provide “help” to other immune cells, including B cells, T lymphocytes and macrophages by interacting with them. They are required for B cells to differentiate into plasma cells, for naïve CD8 T cells to become effector T cells and for the activation of macrophages in a delayed type hypersensitivity response. CD4 T cells also secrete a number of cytokines, which contribute to antibody production. For mice, specific cytokines like IL-2 and IFN γ are produced by Th1; all these biological components support cellular immunity as illustrated by CTL and IgG2a immunoglobulin responses. Other cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 produce type-2 like helper T cells (Th2), which induce B cell activation and the

production of IgG1 immunoglobulin. The CTL immune response contributes to the generation of endogenous antigens after the applied DNA vaccines interact with the MHC-I pathway.

2.3.3 Adjuvant and DNA vaccines

Vaccine adjuvants are substances that are used to combine with vaccine specific antigen to produce a higher immune response than the antigen alone (Sesardic and Dobbelaer, 2004). An efficient vaccine consists of two parts, a specific protective antigenic part and a non-specific adjuvant part, which is able to bind adjuvant. Traditional live vaccines often contain the feature of a pathogen that is effective in inducing protective immunity. Even though the naked recombinant DNA vaccines (plasmid DNA in PBS) have been shown to be effective in preclinical animal models, they are highly purified and lack some features of the original pathogens, and may require immunological adjuvants to assist the vaccines to mimic infections more closely and so initiate a potent immune response (Donnelly, 1997).

Adjuvants are divided into two types, immuno-stimulatory adjuvants and vaccine delivery systems. The immuno-stimulatory adjuvants (such as unmethylated cytosine-phosphate-guanosine [CpG]) are derived from the pathogens and usually mimic some of their structures.

Unmethylated CpG oligodeoxynucleotide (ODN) in the context of selective flanking sequences within the DNA backbone of DNA vaccines are known to stimulate innate immunity in a number of different mammals, such as cattle (Nichani et al., 2004), sheep (Nichani et al., 2004) and piglets (Zhang et al., 2006). Subsequent studies have

confirmed that CpG motifs are able to stimulate B-cell proliferation (Krieg et al., 1996), expression and synthesis of cytokines and NK cell cytotoxicity (Ballas et al., 1996; Boggs et al., 1997). The responses to CpG are mediated by binding to a specific receptor present on dendritic cells (DCs), and Toll-like receptor 9 (TLR9), which induces cell signalling and subsequently activates a pro-inflammatory cytokine response. As CpG motifs contain the above immunostimulatory properties, they have been used as an adjuvant in DNA vaccines and enhance its immunity by inducing Th1 and mixed Th1/Th2 immune responses and producing detecting a higher IgG antibody response and greater IFN γ production than the original vector (Mutwiri et al., 2003).

Liposomes are very efficient transfection reagent and are suitable for use in transfecting nucleic acids into eukaryotic cells. Liposomes are phospholipids vesicles and have been successfully employed as adjuvants that package naked DNA vaccines and increase the efficiency of delivering DNA to certain mucosal surfaces. For example, in vaccination against *T. gondii*, immunized p30 (SAG1) protein with liposomes resulted in a higher level of protection than the injection of p30 alone (Bulow and Boothroyd, 1991). Liposome-entrapped plasmid DNA could be taken up directly by APCs (such as DCs). This would result in transfection, both MHC-I and II classes expression, and subsequent stimulation of the CD4⁺ and CD8⁺ T cells by antigenic peptide (Gregoriadis et al., 2002). It also induces CTL responses and B cells to produce antibodies. DNA vaccines delivered with liposomes induced significant increases in the serum levels of IgG antibody and CD8⁺ CTL activity (Lewis and Babiuk, 1999). The injection of liposome with DNA vaccines has the advantage of being chemically stable, relatively mild in their immune stimulation, and ideally suited for use with a membrane antigen that possess a hydrophobic lipid anchor.

2.3.4 Advantages of DNA vaccine

DNA vaccine is one of the most promising applications of gene therapy. In this technique, plasmid DNA encoding a foreign protein is injected into an animal. The *in vivo* expressed protein from the DNA code elicits humoral and cell-mediated immune responses against the pathogen from which the DNA coded protein was derived. DNA vaccines have the following advantages over traditional vaccine technology:

- First, the encoded protein is expressed in the host, and there is no denaturation and modification. However, this does not occur when antigens for vaccination are produced in bacteria (Kofta and Wedrychowicz, 2001).
- Second, multiple antigens may be combined into one plasmid to target multiple pathogens or multiple components of a single pathogen, and thus the same manufacturing techniques can be used for different DNA vaccines, each encoding an antigen from a different pathogen.
- Third, it is relatively easy to clone a DNA fragment into an appropriate vector and to obtain a considerable amount of DNA. Because DNA is also quite resistant to heat, DNA vaccine is relatively more stable, easier to handle, and thus potentially more cost-effective to manufacture and store in a nearly pure form than live vaccines (Cui, 2005). These benefits are important for distribution of vaccines to developing countries.
- Fourth, DNA itself can act as an adjuvant. Unmethylated CpG motifs with the right flanking sequences are usually built into the backbone of the plasmid. The CpG motif is immuno-stimulatory and induces the production of Th1 cytokines and up-regulation of co-stimulating molecules (such as CD80/CD86) on APCs.

- Fifth, DNA vaccine could extend antigen expression in the host, which generates significant long-lived immune response (Goldsby et al., 2003).

2.3.5 Safety issues

A number of safety issues have been considered on the application of DNA vaccines to both humans and veterinary animals (Donnelly et al., 1997). The first concern is the random integration of injected plasmid DNA into the genome of host cells which potentially increases the risk of malignancy (Donnelly et al., 1997). Another issue to be considered is that if the vaccine induces responses against transfected cells, it might induce immune tolerance or have autoimmunity (Gregersen, 2001). The third concern is the potential induction of antibodies and the stimulation of the production of cytokines by using of CpG motifs that may disrupt the immune homeostasis and thus alter the host's ability to respond to other vaccines and resist infection (Donnelly et al., 1997).

2.4 Previous studies employing DNA immunisation against *T. gondii*

During the past two decades, a number of experiments have identified vaccine candidates that can induce protective immunity against *T. gondii* infection. Most studies have focused on the parasite molecules belonging to four major protein families: the cell surface antigens, the dense granule excreted-secreted antigens, the rhoptry antigens and the micronemal antigens (Nielsen et al., 2006).

2.4.1 Immunisation with cell surface antigens

Among the defined antigen candidate vaccines, SAG1 (30kDa), a major surface antigen of tachyzoites, is the most studied antigen (Bhopale, 2003). SAG1 antigen not only displays a strong reactivity with chronic toxoplasmosis in medical diagnosis

(Gatkowska et al., 2006), but also plays a key role in eliciting strong humoral and cell-mediated immunity of host cells (Chen et al., 2003; Chen et al., 2003; Kato et al., 2005). In *in vivo*, SAG1 stimulates NK cells proliferation and activity, induces IFN γ and IL-12 expression, and induces cytotoxic T cell protection. Apart from the induction of IgG1 and IgG2 antibodies, significantly higher IFN γ , IL-12 and IL-10 expressions were also observed. During infection, IL-10 inhibits IFN γ synthesis, while IL-12 induces Th1 cell activation. The induction of IFN γ and IL-12 also provide largely cytotoxic T cell protection. SAG1 has also been suggested as the best vaccine candidate for protection against congenital toxoplasmosis in mice (Letscher-Bru et al., 2003) and guinea pigs (Haumont et al., 2000). Therefore, stimulating high levels of IFN γ , IL-12 and IL-10 expression, particularly by SAG1, is clearly suggestive of the activation of protective immunity against *T. gondii* and acting as an appropriate candidate vaccine for immunization of humans and/or domestic livestock.

2.4.2 Immunisation with dense granule antigens

Excretory secretory dense granule antigens of *T. gondii* also play an important role in the stimulation of the protection from toxoplasmosis. These antigens are expressed by both tachyzoites and bradyzoites. The major components of these antigens are GRA family: GRA1 (24kDa) (Kato et al., 2005), GRA4 (40kDa) (Mévélec et al., 2005), and GRA7 (29kDa) (Vercammen et al., 2000), which have been proved to be promising candidates to induce strong immune response against toxoplasmosis.

GRA1 is a calcium-binding protein located in the vacuolar matrix and membranes (Kato et al., 2005) and is capable of stimulating humoral immune responses in mice and human in the chronic phase of the infection (Cesbron-Delauw et al., 1989; Fatoohi et

al., 2002; Scorza, et al., 2003). GRA1 based protein and DNA vaccine lead to a high level of anti-GRA1 antibodies. Protective immune responses against *T. gondii* are clearly associated with Th1 type responses, characterized by the production of IFN γ , IgG2a and CTL (Bivas-Benita et al., 2003). Later studies on GRA1 DNA vaccination on BALB/c mice assayed positive response for both IgG1 and IgG2 antibodies (Kato et al., 2005).

GRA6 antigen has been shown to trigger a high immune response during acute *T. gondii* infection (Gatkowska et al., 2006). Lecordier et al. (2000) also found that the N-terminal hydrophilic part of GRA6 was recognized by positive IgG human sera in an immunoblot, indicating that GRA6 is an indicator of an immunological response to toxoplasmosis. In another study, a plasmid consisting of GRA6 and SAG1 was injected into *T. gondii* infected women (Li et al., 2003). The expression of GRA6 in this experiment reached a higher sensitivity for detection of antibodies to *T. gondii* compared to the experiment in which a plasmid containing GRA6 only was injected.

GRA7 could react with both acute and chronically infected human sera (Jacobs et al., 1999; Pietkiewicz et al., 2004). The vaccination with plasmid DNA encoding with GRA7 also induces a strong antibody response, especially in the vaccinated C3H and BALB/c mice strains (Vercammen et al., 2000).

GRA4 vaccine also induced the secretion of IFN γ . Immunization with a mixture of plasmids expressing GRA4 and SAG1 resulted in an improvement of the protective immunity against the acute phase of parasite infection with about 75% survival rate

(Mévélec et al., 2005). The results suggest GRA families give significant protection against acute and chronic infection and a partial protection against toxoplasmosis.

2.4.3 Immunisation with rhoptry antigens

Both ROP1 (66kDa) and ROP2 (56kDa) antigens are two subclasses of rhoptry proteins. ROP1 is expressed by tachyzoite, and ROP2 is expressed in the tachyzoite and bradyzoite life stages of the parasite (Bhopale et al., 2003; Vercammen et al., 2000). They have also been used as vaccine candidates against toxoplasmosis. These two classes of rhoptry proteins are secreted into the host cells during the early stages of invasion (Bradley and Boothroyd, 2001).

The ROP1 is a soluble protein, which is secreted into the parasitophorous vacuole (PV) during entry and then quickly disappears, suggesting a role in the process of invasion. ROP2 is a tyrosine-based motif of trans-membrane protein that is produced into the PV membrane. ROP1 plays a key role in the penetration of host cells, and it is one of the important antigens of *T. gondii*. Both cellular and humoral immune responses in immunized BALB/c mice were increased (Chen et al., 2001). CD8⁺ CTL was also stimulated, but the mechanism is not clear. The immunization resulted in higher activity of NK cells and T cells proliferation. Meanwhile, IgG titer in sera from the immunized mice was significant. Another experiment carried out to evaluate the immune responses induced by experimental DNA construct encoding *T. gondii* ROP1 and SAG1 in mice as a hybrid gene (Chen et al., 2003). The vaccines based on encoding SAG1 and ROP1 could elicit strong protective immunity. Several weeks after i.m. injection with pSAG1-ROP1, the analyzed blood samples showed a strong IgG response. The immunization also generated large amount of IFN γ . Vaccination of mice with plasmid DNA encoding

ROP2 also induced a strong antibody response, generated a mixed Th1/Th2 response (Leyva et al., 2001) and showed partial protection particularly in immunized BALB/c mice (Martin et al., 2004). ROP2 was identified in human T cell clone that produced high levels of IFN γ (Vercammen et al., 2000). However, Leyva et al. (2001) suggested that ROP2 expression is not satisfied, showing low levels of expression or degradation and solubility problems.

A study in sheep has shown that co-administration of plasmid-encoded bovine CD154, increased antibody responses to bovine herpesvirus 1 glycoprotein D injected as DNA vaccine (Manoj et al., 2003). CD154 belongs to the activated TNF superfamily and is expressed as a type II integral membrane protein on the surface of activated T cells and mast cells. Its receptor, CD40, is a surface protein and expressed on B cells, DCs, macrophages and epithelial cells. CD154-CD40 interaction between DCs and T cells provide signals for the activation and maturation of DCs.

2.4.4 Immunisation with micronemal antigens

The micronemal protein MIC3 (90kDa) has been as a promising vaccine candidate, as it is a potent adhesion of *T. gondii* (Bhopale, 2003; Ismael et al., 2003). Intramuscular vaccination of mice with a plasmid encoding an immature form of MIC3 protein (pMIC3i) produces specific anti-MIC3 IgG antibodies. A co-administration of a plasmid encoding GM-CSF (granulocyte-macrophage colony-stimulating factor) was also evaluated. A very high level of IgG2a antibody titers was observed in the sera of immunized mice with pMIC3i, and this response was increased by co-injection of pGM-CSF DNA. Thus, this indicates a shift to Th1 response. Moreover, the vaccinations induced a cellular proliferation response. Large amounts of IFN γ were produced by

mice immunized with pMIC3i combined with pGM-CSF. In later studies, MIC3 knockout mice induced a strong humoral and cellular Th1 response and conferred highly significant protection against chronic infection (Ismael et al., 2006). Therefore, MIC3 protein elicits a strong specific immune response as well as providing effective protection against *T. gondii* infection, and subsequently is a good candidate vaccine against the parasite.

2.4.5 Immunisation with bradyzoite and matrix antigen

Apart from the four major families of parasite proteins above, the induction of humoral immune responses against BAG1 and MAG1 antigens has also been demonstrated in mice and are particularly promising during the chronic phase of parasite infection (Chen et al., 2001). The bradyzoite antigen BAG1 (30kDa) is a cytoplasmic protein, and the matrix antigen MAG1 (65kDa) is expressed within the cyst and cyst wall surrounding the bradyzoites. Analysis of the antibodies and lymph-proliferation tests to recombinant antigens demonstrated that both BAG1 and MAG1 fragments are recognized by antibodies and T cells from infected individuals (Cristina et al., 2004). BAG1 fragments induced T cell growth in about 34% of individual exposed to *T. gondii*, and MAG1 fragments induced about 17%. The T cells responding to BAG1 also secreted IFN γ . About 50% parasite-infected tested candidates produced specific IgG antibodies after the injection of a DNA vaccine encoding BAG1, and even 73% of those individuals gave IgG response with MAG1 inserted DNA vaccines. They also found antibodies against bradyzoites of BAG1 and MAG1 occur early after infection in humans. In another study Nielsen et al. (2005) designed DNA vaccines with a mixture of MAG1 and BAG1 that express plasmids to give protective immunity in mice after an oral challenge with *T. gondii*. The MAG1/BAG1 DNA vaccination induced the synthesis of

specific anti-MAG1 and anti-BAG1 IgG2a antibody in about 80% of immunized mice. The result also indicated that the immunization induced a Th1-like response. Recently, the matrix antigen MAG1 detected antibodies more frequently from the acute stage than from the chronic stage of toxoplasmosis (Holec et al., 2007). Thus, researchers suggested that BAG1 and MAG1 antigens should be considered in the design of vaccines against *T. gondii* both in animals and human.

2.5 Conclusions

In conclusion, infection by *T. gondii* causes a serious medical condition in humans and is also a significant problem in the farm industry, especially in New Zealand, due to reduced numbers of lambs being born because of abortions. The only current commercial vaccine against *T. gondii* is an expensive live attenuated vaccine, which is not suitable for humans because of the risk of conversion back to a pathogenic strain and provides functional not sterile immunity. It means that meat from lambs or ewes previous vaccinated may still contain live parasites and their infections could be continuously affecting any intermediate host consuming the meat. Development in the field of DNA vaccine technology suggests that DNA vaccine would be ideal to replace the traditional vaccine and provide strong protection against *T. gondii* infection by stimulating both humoral and cell-mediated immunity. Research on several toxoplasma antigens (such as SAG1, GRA1, GRA4, GRA6, GRA7, ROP1, ROP2, MIC3, MAG1 and BAG1) using mice models suggests that these are promising vaccine candidates and could elicit strong or partial protection against *T. gondii* infection. The current research is the first sheep study on the Toxoplasma antigens, ROP1, GRA1, GRA6, GRA7, MIC3 and MAG1.

3 Aim

The aim of this study is to test the immunogenic ability of DNA protective antigens (ROP1, GRA1, GRA6, GRA7, MIC3, MAG1 and native toxoplasma antigen) against *T. gondii* in sheep.

4 Hypothesis

The hypothesis of this experiment is that after immunisation of sheep with DNA constructs containing the *T. gondii* antigens, the specific IgG1 and IgG2 would develop if the protective antigens are expressed and recognised by the immune system of sheep. It is also hypothesised that antibody levels in immunised sheep will change over time, increasing after the 1st and 2nd administration of the DNA constructs, which are absent in sheep injected only with phosphate buffer solution (control sheep).

5 Objectives

To achieve the aim, ewes were immunised with DNA vaccines encoding antigens ROP1/CD154, GRA1, GRA6, GRA7, MIC3 and MAG1 (Table 1). The humoral immune responses were evaluated. The first objective was to determine whether the DNA construct containing the above *T. gondii* antigens stimulate the production of IgG1 and IgG2 antibodies against *T. gondii* in sheep. The second objective was to determine the antibody level responses against individual recombinant antigens in sheep immunised with the same DNA vaccines. The third objective was to determine the change in antibody levels in sheep sera over time after two DNA vaccinations in comparison with antibody levels in the control sheep.

6 Materials and Methods

6.1 Location

The experiment was conducted in the Immunology Laboratory of Lincoln University, the Johnstone Memorial Laboratory (JML), and the Lincoln University sheep farm. All experimental procedures were carried out under the authority of Lincoln University Animal Ethics Committee (ACE # 123) and the Institutional Bio-safety Committee.

6.2 Animal allocation and experimental design

A total of 200 twelve month old (two-tooth) non-pregnant Coopworth ewes were tested for *Toxoplasma* antibodies using the Latex Agglutination test kit (TPXOREAGENT--Eiken, Japan) and an ELISA *T. gondii* serum screening commercial kit (Institut Pourquier, France). One hundred and sixty *Toxoplasma* antibody negative sheep were selected and randomly allocated to four groups of 40 animals of similar weight (51 ± 6 kg). All selected ewes were drenched orally with a Q-drench multi-combination drench (Jurox, New Zealand) to ensure they were nematode free.

	Vaccine 1st injection	Vaccine 2nd injection (Booster)
Group 1 (40 sheep)	DNA vaccine: pVAXROPI/CD154 + pVAXIgGRA7 + pVAXIgMIC3 + CpG + Liposome	DNA vaccine: pVAXROPI/CD154 + pVAXIgGRA7 + pVAXIgMIC3 + CpG + Liposome
Group 2 (40 sheep)	DNA vaccine: pVAXIgGRA1 + pVAXIgGRA6 + pVAXIgMAG1 + CpG + Liposome	DNA vaccine: pVAXIgGRA1 + pVAXIgGRA6 + pVAXIgMAG1 + CpG + Liposome
Group 3 (40 sheep)	DNA vaccine: pVAXROPI/CD154 + pVAXIgGRA7 + pVAXIgMIC3 + CpG + Liposome	DNA vaccine: pVAXIgGRA1 + pVAXIgGRA6 + pVAXIgMAG1 + CpG + Liposome
Group 4 (40 sheep)	No immunization, injection of PBS	No immunization, injection of PBS

MAG = the tissue cyst antigen that protects against *T. gondii*
GRA = the dense granule antigen that protects against *T. gondii*
SAG = the surface antigen that protects against *T. gondii*
ROP = the roptry antigen that protects against *T. gondii*
MIC = the microneme antigen that protects against *T. gondii*

Table 1. Treatments to be allocated to each group

Immunisation was carried out as described in Table 1. The first vaccination was immunised intramuscularly in the dorsal neck region of all sheep in the PC2 laboratory

of JML on week 0, and a second vaccination on week 4 (Table 2). Each of the forty sheep in group 1 was vaccinated intramuscularly with plasmids pVAXROP1/CD154 (pROP1/CD154), pVAXIgGRA7 (pIgGRA7) and pVAXIgMIC3 (pIgMIC3) separately in different places for both injections. The ewes in group 2 were vaccinated with plasmids pVAXIgGRA1 (pIgGRA1), pVAXIgGRA6 (pIgGRA6) and pVAXIgMAG1 (pIgMAG1) in the same way as in group 1. The sheep in group 3 were primed (1st injection) with plasmids pVAXROP1/CD154, pVAXIgGRA7 and pVAXIgMIC3 and boosted (2nd injection) with plasmids pVAXIgGRA1, pVAXIgGRA6 and pVAXIgMAG1 as the same way in group 1. The group 4 sheep, as negative controls, were only injected with phosphate buffered saline (PBS).

Week	Blood sampling	Immunisation
0	Yes	Yes
1	Yes	
2	Yes	
3	Yes	
4	Yes	Yes
5	Yes	
6	Yes	
7	Yes	
8	Yes	
12	Yes	
16	Yes	

Table 2. Schedule of trial: sheep bleeding and DNA immunisation.

A 10 ml blood sample was collected weekly using a red top tube (BD vacutainer serum, NJ USA) weekly from each experimental sheep after the 1st and 2nd injections. After eight weeks from the start of the experiment, the last two samples were taken at four-week intervals (Table 2). The blood samples collected were centrifuged at 800 × g for 20 min at 4°C and then the sera were decanted off into appropriately labelled Eppendorf tubes and frozen at -20°C until the ELISA IgG (IgG1 and IgG2) tests were performed.

6.3 DNA vaccine preparation

6.3.1 Plasmid construction

All DNA constructs used for vaccination have been prepared by Drs. Gabriela Oledzka and Ela Hiszczyńska-Sawicka based on the plasmid expression vector pVAX1 (Invitrogen, USA). Details of the vector pVAX1 are described by the manufacturer (www.invitrogen.com/content/sfs/manuals/pvax1_man.pdf). This vector is approved by the Food and Drug Administration in USA and is specifically designed for use in the development of DNA vaccines. The small size of pVAX1 simplifies transfection into the mammalian cells. The plasmids DNA that used in current study were pVAXROP1/CD154, pVAXIgGRA7, pVAXIgMIC3, pVAXIgGRA1, pVAXIgGRA6 and pVAXIgMAG1 (Table 1).

6.3.2 Plasmid generation and isolation

All steps were carried under sterile conditions.

For competent cell preparation, *E. coli* DH5 α (Invitrogen, USA) was transformed with plasmids, as listed above (6.3.1). A single white colony from fresh *E. coli* DH5 α growing in the petri dish was inoculated into 10 ml of Luria Broth (LB) medium (Difco, Miller). After vigorous shaking (300 rpm) overnight at 37°C, the culture was diluted 1:50 in fresh LB medium and incubated at 37°C with vigorous shaking until an $OD_{575} = 0.2$ (approximately 3 h). About 50 ml of the culture was then transferred to a sterile 50 ml of tube and centrifuged at $800 \times g$ for 10 min at 4°C. The cell pellet was resuspended gently in 25 ml of chilled 100 mM CaCl₂. After 30 min incubation on ice, the suspension was centrifuged at $800 \times g$ for 10 min at 4°C. The cell pellet was resuspended in 2 ml of ice-cold 100 mM CaCl₂. After further 1 h incubation on ice, the preparation was then ready for *E. coli* transformation.

For *E. coli* transformation, 3 μ l (5 μ g) of plasmid DNA was transferred into 150 μ l (1×10^6 transformants) competent *E. coli* cells in chilled sterile micro-centrifuge tubes and the mixture was incubated on ice for 1 h. The mixture was heat shocked at 42°C for 1 min, and then immediately transferred back onto ice for 1 min. LB medium (1 ml) was added to the tube which was then incubated for 1 h at 37°C with vigorous shaking (750 rpm). Aliquots (150 μ l) were spread on Luria Broth Agar (LA) plates (Sigma, New Zealand) containing 50 μ g/ml kanamycin and the plates were incubated at 37°C overnight to produce colonies. A reference plate spread only with 100 μ l component cells was also included as a negative control to check antibiotic activity.

For each plasmid purification, a single colony of *E. coli* DH5 α cell transformed with different plasmids DNA was inoculated into 10 ml of LB medium containing 50 μ g/ml of kanamycin, followed by vigorous shaking for 8 h at 37°C. This culture was diluted 1000-fold in fresh LB medium and incubated at 37°C with vigorous shaking overnight. The cells were harvested by centrifugation at 6000 \times g for 10 min at 4°C. The plasmid DNA was purified from the cell pellets using an EndoFree Plasmid Giga kit (Qiagen, Germany) as follows. The bacterial pellets were resuspended with 125 ml of buffer P1 (50 mM Tris-HCl pH=8.0, 10 mM EDTA, 100 μ g/ml RNase A) and completely lysed with 280 ml of buffer P2 (200 mM NaOH, 1% w/v SDS) for 5 min. After the lysates were neutralised with 280 ml of chilled buffer P3 (3.0 M potassium acetate pH 5.5), they were poured immediately into a QIAfilter Mega-Giga Cartridge screwed on a glass bottle connected to a vacuum pump and incubated at room temperature for 10 min. After all the clarified liquid was pulled through by vacuum, the remaining white fluffy material was washed with 50 ml of FWB2 buffer (1 M potassium acetate pH 5.5) and

the liquid was cleared by vacuum filtration. Applying the filtered lysate into a pre-equilibrated QIAGEN-tip 10000 allowed it to enter the resin by gravity flow. The tip was washed with a total of 600 ml of washing buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% volume/volume [v/v] isopropanol) and the plasmid DNA eluted with 100 ml of buffer QF (1.25 M NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol). The eluted plasmid was then precipitated with 70 ml of isopropanol at room temperature and immediately centrifuged at $15,000 \times g$ for 30 min at 4°C. The DNA pellet was washed with 10 ml of endotoxin-free 70% ethanol and the purified DNA pellet was collected by centrifugation at $15,000 \times g$ for 30 min at 4°C and re-dissolved in sterilised PBS buffer.

The concentration of plasmid DNA was tested using a NaNo Drop spectrophotometer (ND-1000, 3.3.0, USA) and the size of the plasmid DNA was checked by electrophoresis on 1% (weight/volume [w/v]) agarose gel (Sigma, USA) containing 0.1% (v/v) ethidium bromide.

6.3.3 Vaccine preparation

The individual DNA vaccine preparations were prepared by mixing 1 mg of each plasmid DNA with 1 mg of liposomes (ESCORT transfection reagent, Sigma, Australia) and 150 µg of CpG oligodeoxynucleotide (ODN) (Table 3).

Name	µg of DNA vaccine	CPG µg/1mg DNA	Adjuvant 1mg/1mg DNA
pVAXROP1/CD154	1000	150	liposomes
pVAXIgGRA1	1000	150	liposomes
pVAXIgGRA7	1000	150	liposomes
pVAXIgGRA6	1000	150	liposomes
pVAXIgMIC3	1000	150	liposomes
pVAXIgMAG1	1000	150	liposomes

Table 3. The injection doses

6.4 Verification of Encoded Recombinant protein

6.4.1 Recombinant protein construction

All recombinant protein constructs used for the ELISA were prepared by Dr. E. Hiszczynska-Sawicka, based on the plasmid expression vector pUET1 (Hiszczynska-Sawicka et al. 2003 and Holec et al. 2007). The gene fragments of the recombinant protein were as follows: pUETMAG1 (rMAG1), pUETGRA1 (rGRA1), pUETGRA6 (rGRA6), pUETGRA7 (rGRA7), pUETMIC3 (rMIC3), and pUETROP1 (rROP1).

6.4.2 Recombinant protein expression and purification

The *E. coli* Rosseta (DE3) pLysS was transformed with each plasmid of the recombinant protein listed above (6.4.1). The transformed cells were inoculated into LB medium containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and incubated at 37°C overnight with vigorous shaking. The overnight cultures (40 ml) were then diluted in 1 L of fresh LB medium supplemented with the same antibiotics and the culture grown with vigorous shaking at 37°C until $OD_{600} = 0.4$. Protein expression was induced by incubating with isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 1 mM for 4 h. The bacterial cell pellets were harvested by centrifugation at $8000 \times g$ for 10 min at 4°C.

The recombinant protein was purified on 4 ml of 50% Ni-NTA slurry column under denaturation conditions. The cell pellet was resuspended in 20 ml of buffer B (100mM NaH₂PO₄, 10mM Tris-HCl pH 8.0, 0.5% Triton X-100, 5 M urea). The cells were disrupted by sonication and the insoluble debris was removed by centrifugation at $14000 \times g$ for 30 min at 4°C. Supernatant (20 ml) was mixed with 4 ml of 50% Ni-NTA slurry (QIAGEN, Germany) with shaking for 30 min at room temperature. The lysate-

resin mixture was applied carefully on to a column (pre-washed with water) and then washed with buffer C (100 mM NaH₂PO₄, 10mM Tris-HCl pH 6.3, 0.5% Triton X-100, 5 M urea). The His-tagged MAG1 protein was eluted with buffer D (100 mM NaH₂PO₄, 10 mM Tris-HCl pH5.9, 0.5% Triton X-100, 5 M urea) and then with eluted with buffer E (100 mM NaH₂PO₄, 10 mM Tris-HCl pH 4.5, 0.5% Triton X-100, 5 M urea).

The recombinant protein fractions were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel stained with Coomassie blue. The amount of protein in the sample was measured by the Bradford assay with Quick start™ bovine serum albumin (Bio-Rad laboratories, USA) as the standard.

6.5 Determination of serum IgG in vaccinated sheep using indirect enzyme linked immunosorbent assay (ELISA)

ELISA was extensively used for the detection and/or titration of specific antibodies from serum samples (Crowther, 2005).

Ninety-six well ELISA plates (Costar plate, medium binding, DKSH Australia) were coated with 100 µl per well of a solution containing 10 µg/ml of recombinant *Toxoplasma* antigens MAG1, GRA7 and ROP1, or 5 µg/ml GRA6 and MIC3, or 5 µg/ml of *Toxoplasma* soluble antigen (TA), respectively, diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6 at 4°C overnight. Plates were then washed three times with PBS containing 0.05% Tween 20, pH7.4 (PBST) using an ELISA washer (ultrawash plus, 1UWA-2217, Dynex technologies, Inc. USA) The sensitised wells of plates were

post-coated with 200 µl of a blocking solution containing 12% w/v Bovine Calf Serum (BCS) in PBST (pH=7.4) for two hours at 37°C to eliminate non-specific binding.

For testing serum samples, 100 µl per well of serum samples diluted in PBST from individual experimental sheep was dispensed to the different antigen-coated plates in duplicate, along with a *Toxoplasma* positive control serum and a negative control serum from non- *Toxoplasma* infected foetal sheep. The plates were then incubated for one hour at 37°C, followed by three washes in PBST for one hour at 37°C.

The monoclonal mouse anti-sheep IgG1 (AgResearch Ltd, Upper Hutt, New Zealand) antibody at a dilution of 1:500 and IgG2 (AgResearch Ltd, Upper Hutt, New Zealand) antibody at a dilution of 1:100 in PBST, was added to each well (100 µl per well) and incubated for one hour at 37°C, followed by three washes in PBST as before.

For applying the conjugated antibody, each well was loaded with 100 µl of polyclonal rabbit anti-mouse immunoglobulins: HRP conjugated (med.bio, DakoCytomation) diluted at 1:2000 with PBST for the IgG1 detection assay and goat anti mouse IgG (multi species adsorbed): HRP conjugated (Serotec, Australia) diluted at 1:1000 with PBST for the IgG2 detection assay, respectively. The plates were then incubated for one hour at 37°C, followed by three washes in PBST as before.

The substrate solution was prepared by dissolving an o-phenyldiamine (OPD) tablet set (Sigmafast OPD, Zealand) in 20 ml of distilled water; 100 µl of substrate buffer was added into each well and the colour developed at room temperature with low light

intensity for 6 min for the IgG1 test and 12 min for the IgG2 test, respectively. The reaction was then stopped by adding 100 µl per well of 1.5 M sulphuric acid.

The optical density (OD) was measured in a microplate reader FluoStar, BMG Lab Technologies, Germany) at a wavelength of 492 nm against the negative control well that received anti-*T. gondii* reference serum. To account for inter-assay variation, a *T. gondii* positive serum was included in all assays performed. To interpret the result, Dr. L. Hong (personal communicate) suggested that the OD ratio was calculated using the formula: OD ratio = OD (test serum) / OD (negative serum).

6.6 Statistical analysis

Statistical analysis was carried out using GenStat (10th edition, VSN, UK) and SigmaPlot 8.0. Differences between the groups and tested antigens were tested by repeated measures of analysis of variance (ANOVA). A significant difference was indicated by a P value ($p < 0.001$).

7. Results

7.1 Purified plasmids DNA

The bright thick bands (lanes 2 lane 7) indicated that to each purified plasmids DNA was at a high concentration. Compared with the marker (lane 1) the sizes of the plasmids DNA were 3612bp in pIgGRA1, 3676bp in pIgGRA6, 3768bp in pIgGRA7, 4334bp in pIgMAG1, 3979bp in pIgMIC3 and 4768bp in pROP1/CD154 (Fig 3).

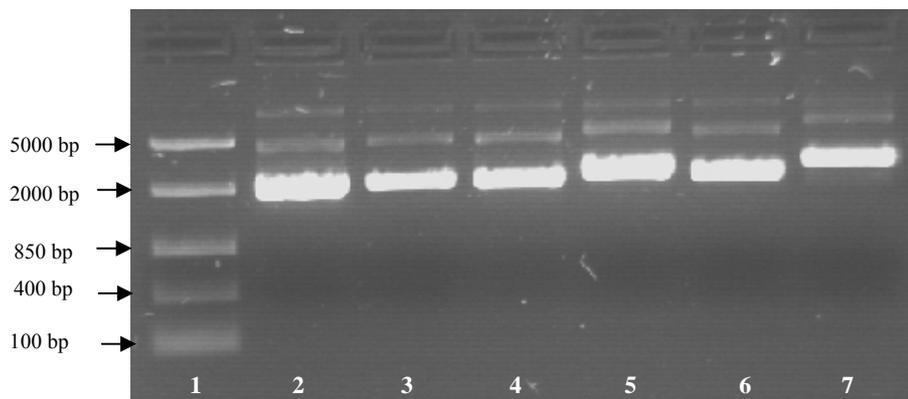


Figure 3. Electrophoresis of purified plasmid DNA contrasts. Lane 1, marker; Lane 2, pVAXIgGRA1, Lane 3, pVAXIgGRA6; Lane 4, pVAXIgGRA7; Lane 5, pVAXIgMAG1, Lane 6, pVAXIgMIC3; Lane 7, pVAXROP1/CD154.

7.2 Purified recombinant proteins

The result of purified MAG1, GRA7, GRA6 and MIC3 soluble fractions were clearly observed in a single dark lane with a protein marker (Fig 4). In this experiment, some protein constructs had only part of gene cloned into the vector, and thus the purified products were not the full size, such as MAG1 (24kDa), GRA6 (30kDa), MIC3 (36.5kDa), ROP1 (39.1kDa) and GRA1 (21.9kDa); while recombinant GRA7 were cloned with its full gene plus a fusion domain, so that the purified GRA7 is heavier in molecular weight (32.6kDa).

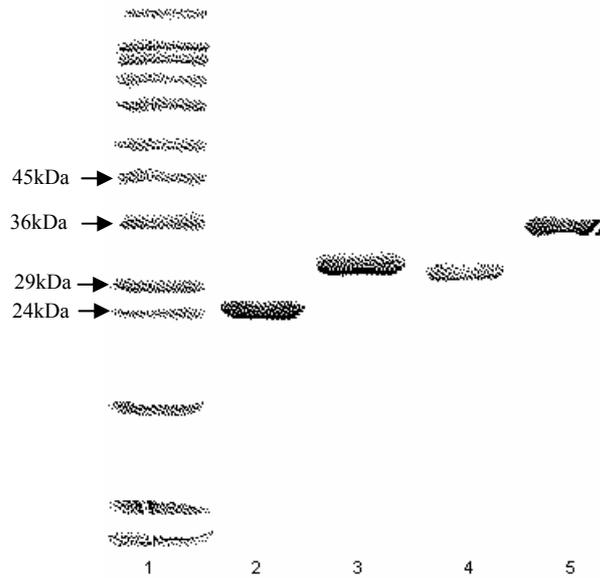


Figure 4. SDS-PAGE analyses of purified recombinant proteins. Lane 1, marker; Lane 2, MAG1; Lane 3, GRA7; Lane 4, GRA6; Lane 5, MIC3

7.3 Antibody responses

7.3.1 Antibody responses detected in each group of sheep immunized with different combinations of plasmid DNA

7.3.1.1 Group 1

In group 1, the vaccination induced significant ($P < 0.001$) increases in specific IgG1 anti-ROP1 antibody levels after two injections, with two peaks, one at week 2 and one at week 6 (Fig 5, A). The IgG1 levels against rGRA7 were induced two weeks after the 1st injection, and were increased three weeks after the 2nd injection. Low levels of IgG1 production against rMIC3 were only observed at week 4 and 5. The IgG1 response produced against natural *T. gondii* (TA) was only detectable at two and three weeks after the 2nd injection. The rROP1 responses were significant ($P < 0.001$) greater than the other antigens, tested from two weeks after 1st injection till three weeks after the 2nd

injection. The rGRA7 responses were also greater ($P < 0.001$) than those of the rMIC3 and *T. gondii* native antigen during two and three weeks after the 2nd injection.

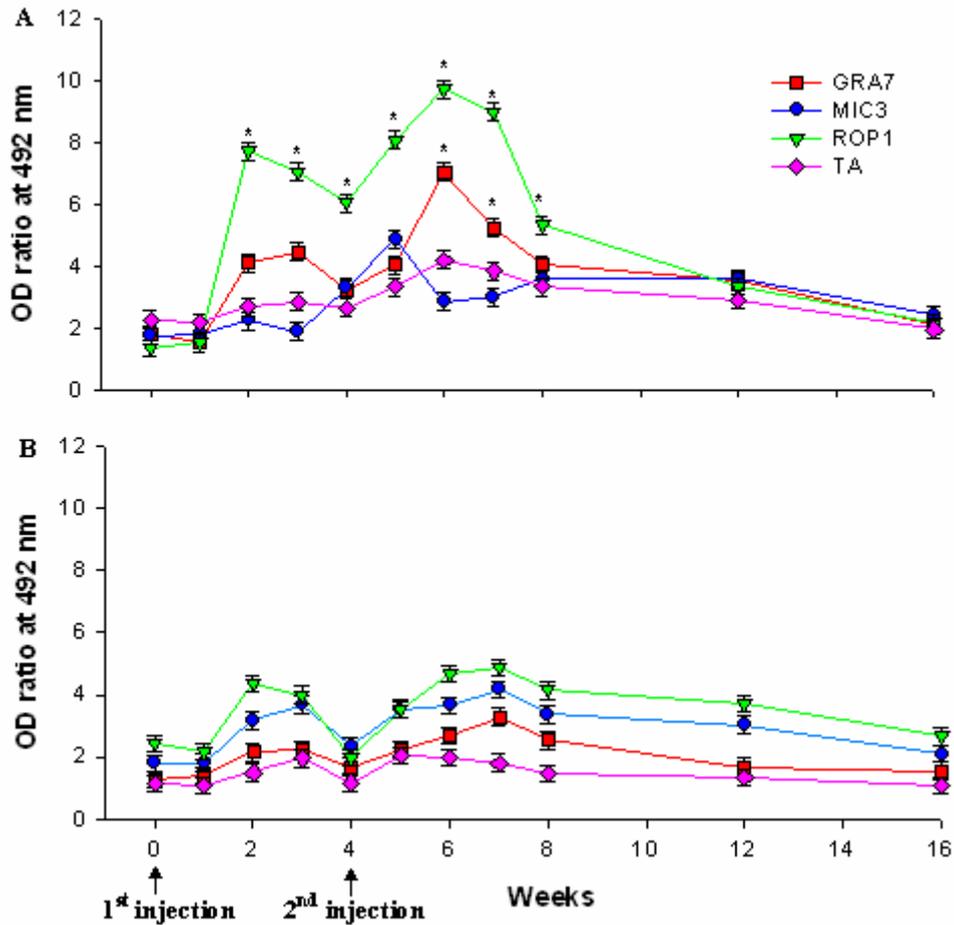


Figure 5. Changes in serum IgG1 (A) and IgG2 (B) response determined by specific toxoplasma antigens and anti-sheep IgG1 and IgG2 antibodies after immunizing group 1 ewes with pROP1/CD154+pIgGRA7+pIgMIC3. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some antigens. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p32-34).

All the IgG2 antibodies produced in group 1 ewes were detected from three weeks after the 1st injection with the highest levels at three weeks after the 2nd injection (Fig 5, B). Highest IgG2 levels were observed from anti-MIC3 antigens, followed by anti-GRA7, anti-ROP1 and then anti-native *T. gondii* proteins, but there was no significant

difference between them. The overall IgG2 levels were lower than IgG1 levels except the specific anti-MIC3 IgG2 levels were higher than the anti-MIC3 IgG1 levels.

7.3.1.2 Group 2

In group 2, the sheep induced a greater ($P < 0.001$) response with specific IgG1 anti- GRA6 antibody than with the other IgG1 antibodies after the 1st injection (week 2 to 4) (Fig 6, A). Two or three weeks after the 2nd injection, the injection produced a high ($P < 0.001$) level of IgG1 antibodies against *T. gondii* antigens: the highest peak value was found with rMAG1, followed by rGRA1 and rGRA6. The IgG1 response against native *T. gondii* antigen also increased after the 2nd injection compared with base-line, but the IgG1 levels were lower than those against the other *T. gondii* recombinant antigens.

The IgG2 levels of group 2 against all the tested antigens increased two weeks after the 1st injection, and then decreased at week 4 followed by an increase one week after the booster, reaching peak values at week 7 before decreasing in the following weeks (Fig 6, B). Even though there was no significant difference of IgG2 levels between each tested antigens, the strongest trend in IgG2 responses were found against rMAG1, followed by rGRA6, rGRA1 and then *T. gondii* native antigen. The overall IgG2 levels produced were lower than overall IgG1 levels in group 2 sheep.

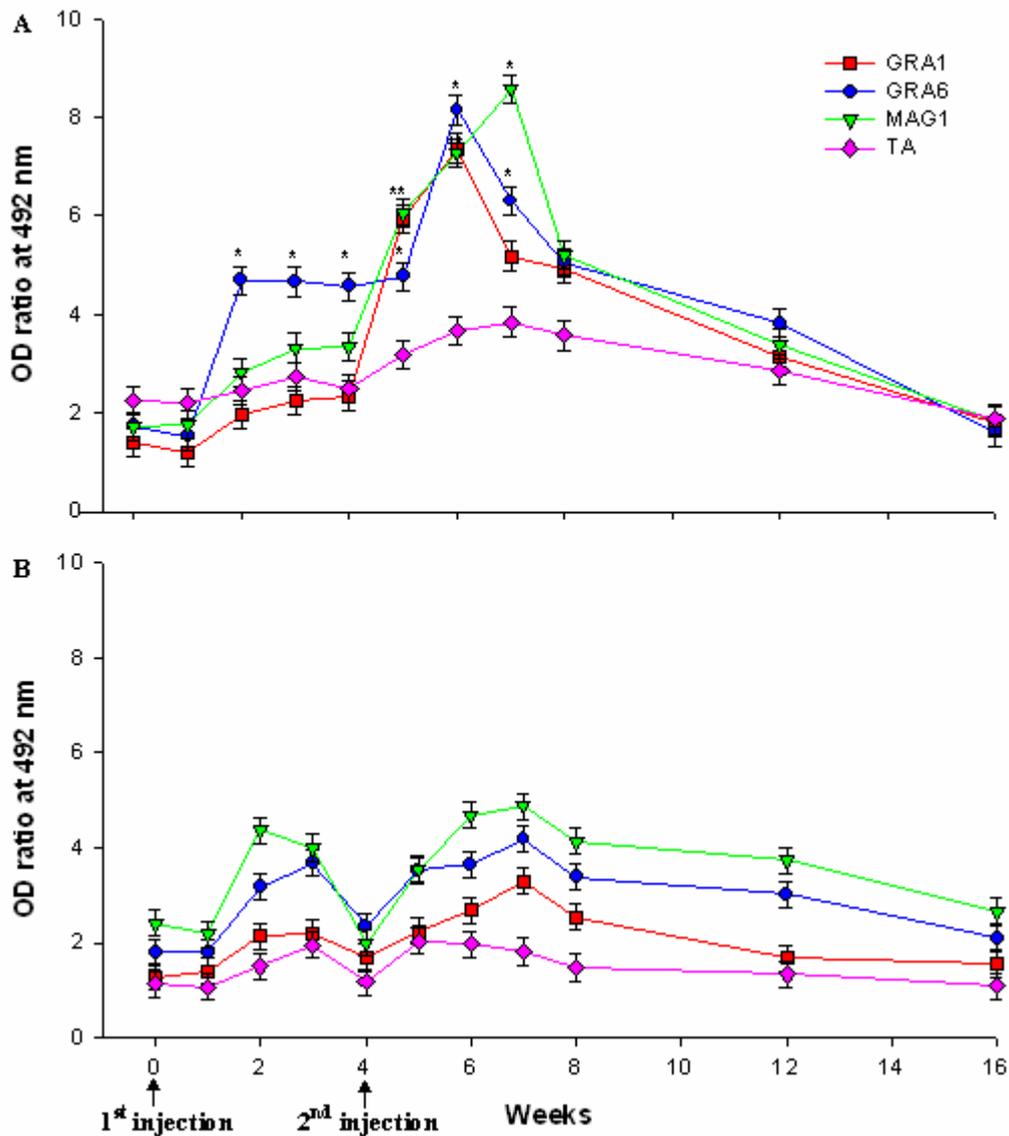


Figure 6. Changes in serum IgG1 (A) and IgG2 (B) response determined by specific toxoplasma antigens and anti-sheep IgG1 and IgG2 antibodies after immunizing group 2 ewes with pIgGRA1+ pIgGRA6+pIgMAG1. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some antigens. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p34).

7.3.1.3 Group 3

In group 3, sera from treated sheep injected with pROP1/CD154 + pIgGRA7 + pIgMIC3 at week 0 and with pIgGRA1 + pIgGRA6 + pIgMAG1 at week 4 were tested by ELISA against specific rROP1, rGRA7, rMIC3, rGRA1, rGRA6, rMAG1 and native *T. gondii* antigens responses (Fig 7, A and B). The increase of IgG1 levels against rROP1, rGRA7 and rMIC3 started two weeks after the 1st injection (Fig 7, A), reaching

peak values at week 3 and 4; while the increase of IgG1 levels against the other antigens started one week after the 2nd injection with peak values at week 7. The specific anti-rROP1 IgG1 level was significantly greater ($P < 0.001$) than the other tested antigens from two to three weeks after the 1st injection but the other *T. gondii* antigens were not significantly different from each other.

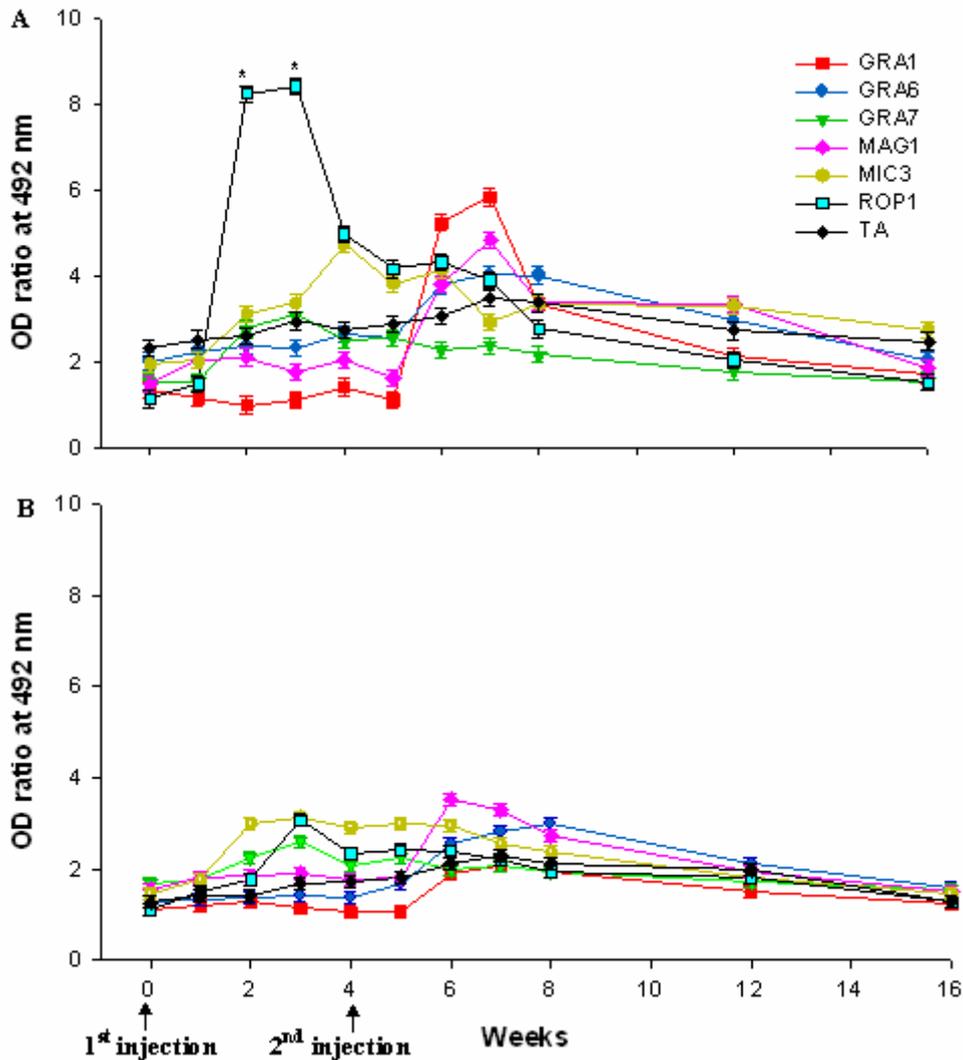


Figure 7. Changes in serum IgG1 (A) and IgG2 (B) response determined by specific toxoplasma antigens and anti-sheep IgG1 and IgG2 antibodies after immunizing group 3 ewes with pROP1/CD154+ pIgGRA7+pIgMIC3 and pIgGRA1+ pIgGRA6+pIgMAG1. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some antigens. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p35-37).

Although the induced IgG2 responses were much lower than IgG1 response (Fig 7, B), there were small increase of specific IgG2 levels against rROP1, rGRA7 and rMIC3

two weeks after the 1st injection and those against the rGRA1, rGRA6, rMAG1 and native *T. gondii* antigens two weeks after the 2nd injection. There were no significant differences between IgG2 levels of each tested antigen in group 3.

7.3.2 Antibody responses detected by *T. gondii* recombinant antigens

The sheep of immunized groups 1, 3 and control group 4 were used in testing both IgG1 and IgG2 responses against recombinant antigens ROP1, GRA7 and MIC3. The treated groups 2, 3 and the control group 4 were used for indicating both antibodies responses against recombinant antigens GRA1, GRA6 and MAG1. All three treated groups 1, 2, 3 and the control group 4 were also used to test both antibodies responses against native *T. gondii* antigen.

7.3.2.1 Antibody responses detected by anti-rROP1

The ewes from treated group 1 produced significantly ($p < 0.001$) increased *T. gondii*-specific IgG1 response against rROP1 compared with the control group 4 at two weeks after the 1st injection and one week after the 2nd injection (Fig 8, A), and after reaching a peak (OD ratio of 10) at week 6 the IgG1 level dropped down in the following weeks. The sheep of vaccinated group 3 also induced significantly ($p < 0.001$) increased *T. gondii*-specific IgG1 anti-ROP1 response compared with the control group two weeks after the 1st injection with a peak (OD ratio of 8.6) at week 3, and then this IgG1 response started to decrease in the following weeks. The plasmids DNA treated groups (group 1 and 3) produced detectable IgG2 response against rROP1 from two weeks after the 1st injection till four weeks after the 2nd injection (Fig 8, B). There was a significant ($p < 0.001$) difference of IgG1 production between both vaccinated groups and the control group from week 2 to 4, and between all these three groups after the 2nd

injection (week 5 to 8). No further significant difference was found between tested groups.

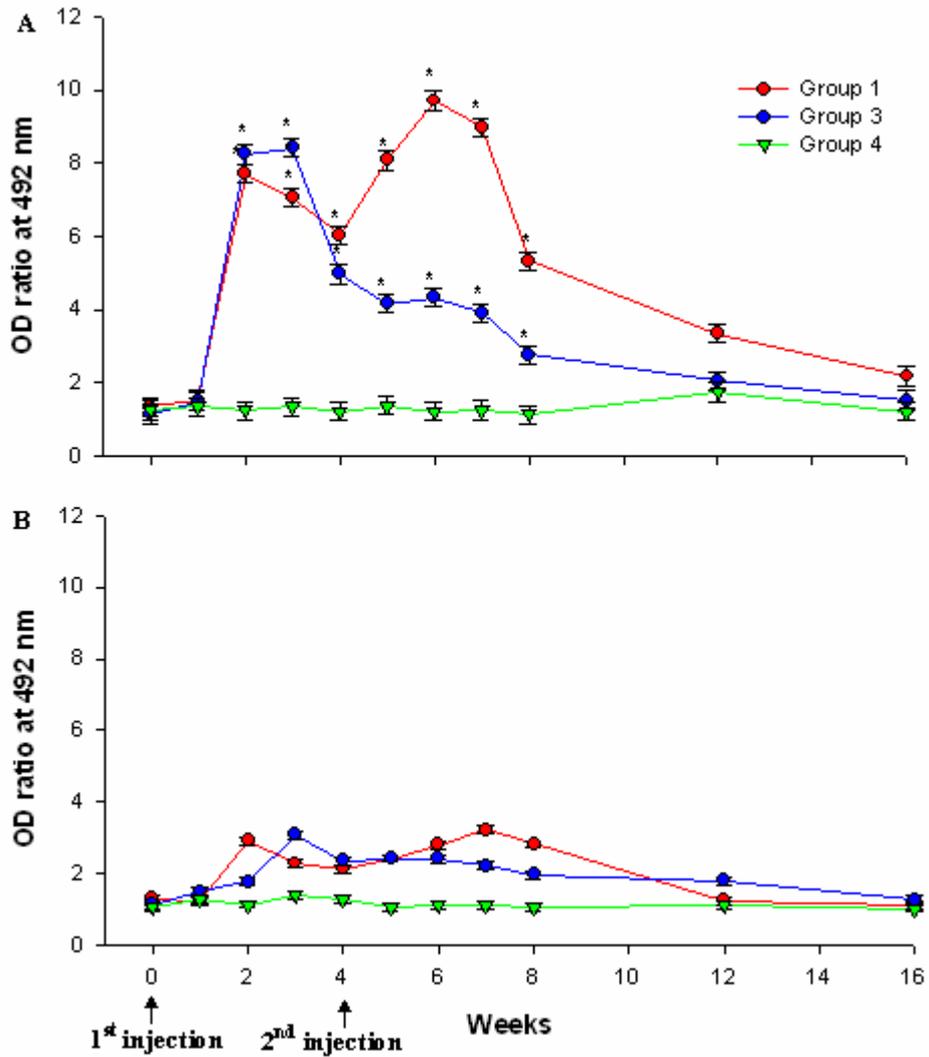


Figure 8. Specific IgG1 (A) and IgG2 (B) response to the injection of constructs against recombinant ROP1. Group 1, pROP1/CD154+pIgGRA7+pIgMIC3; Group 3, pROP1/CD154+pIgGRA7 +pIgMIC3 and pIgGRA1+pIgGRA6+pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p37-38).

7.3.2.2 Antibody responses detected by anti-rGRA7

The group 1 immunized with pROP1/CD154+ pIgGRA7+pMIC3 had greater ($p < 0.001$) increased *T. gondii*-specific IgG1 production against rGRA7 than the non-immunized group 4 two weeks after the 1st injection (Fig 9, A), and after reaching a peak value (OD ratio of 7.5) at week 7 the IgG1 levels declined in the following weeks. The treated

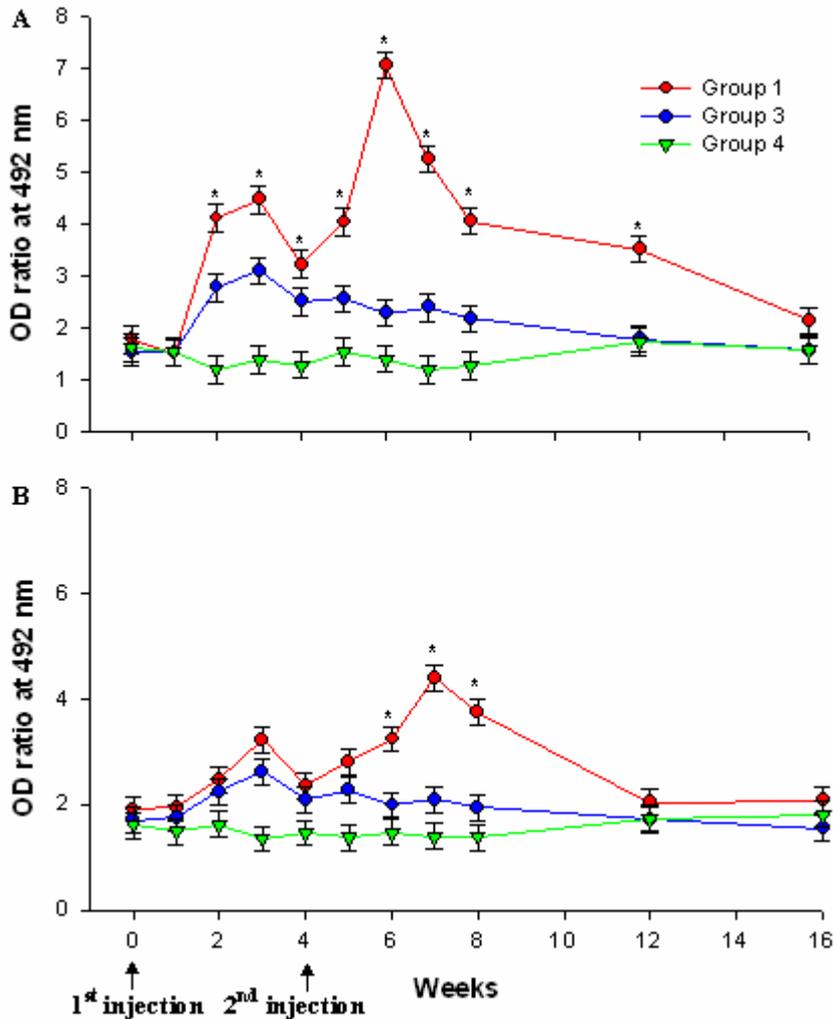


Figure 9. Specific IgG1 (A) and IgG2 (B) response observed by injection of constructs against recombinant GRA7. Group 1, pROP1/CD154+ pIgGRA7+pIgMIC3; Group 3, pROP1/CD154+ pIgGRA7+pIgMIC3 and pIgGRA1+pIgGRA6+pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p39-40).

group 1 also induced dramatically increased specific anti-GRA7 IgG2 response at week 3 and week 6 to 8 compared with the control group (Fig 9, B). The DNA immunized

group 3 produced low levels of IgG1 and detectable IgG2 anti-GRA7 responses from two weeks after the 1st injection till four weeks after booster. Generally, both treated group 1 and group 3 induced higher IgG1 responses against rGRA7 than the IgG2 response. Between group 1 and both group 3 and the control group, significant differences ($p < 0.001$) were observed in IgG1 production from week 5 to 12, and IgG2 production at week 6 to 8. No further significant difference was found between each tested groups.

7.3.2.3 Antibody responses detected by anti-rMIC3

Compared with the control group 4, significantly ($p < 0.001$) increased level of IgG1 response against rMIC3 antigen was produced by the vaccinated group 1 at three weeks after the 1st injection and one to four weeks post booster with reaching a peak (OD ratio of 4.7) at week 5 (Fig 10, A). The injection in the group 3 also induced a higher level of anti-rMIC3 of IgG1 from three weeks after the 1st injection to three weeks after the 2nd injection followed by reaching a peak (OD ratio of 4.6) at week 4. Compared with group 3 and group 4, a higher ($p < 0.001$) level of anti-MIC3 IgG2 response was stimulated by sheep of immunized group 1 only after the 2nd injection (week 5 to 8), reaching a peak (OD ratio of 5.4) at week 7 (Fig 10, B), which was higher than level of IgG1. The mean of anti-MIC3 IgG2 level in group 3 was lower than those in group 1, but is still produced from week 2 to 8.

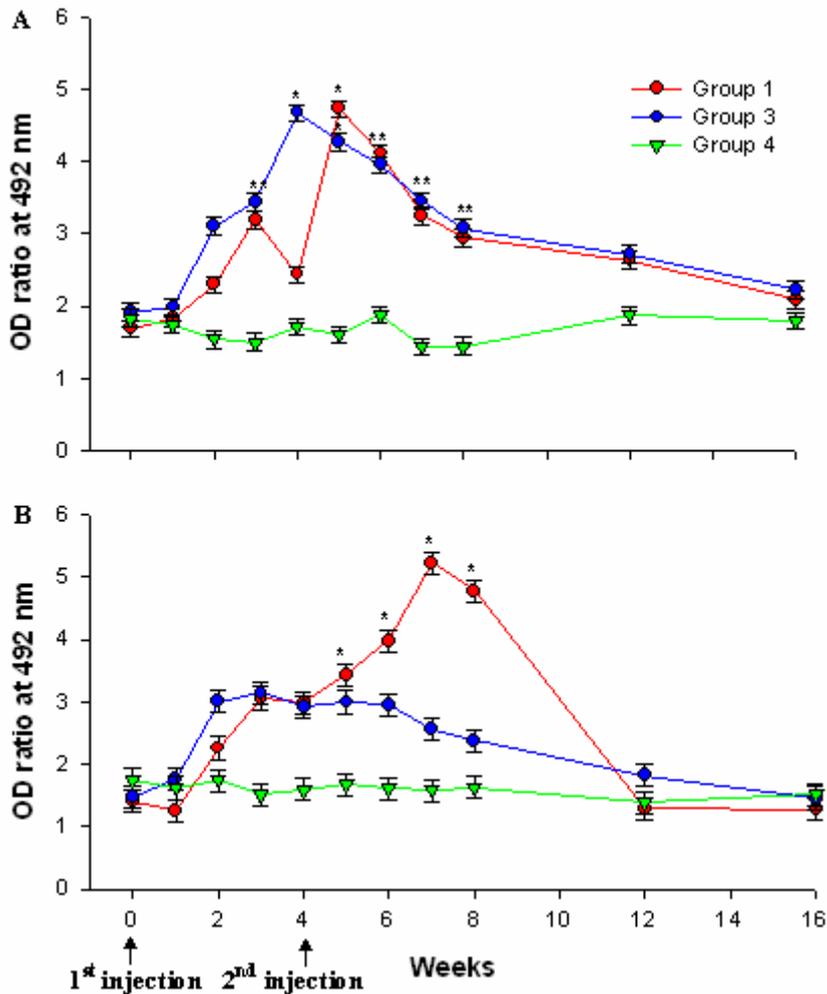


Figure 10. Specific IgG1 (A) and IgG2 (B) response observed by injection of constructs against recombinant MIC3. Group 1, pROP1/CD154+ pIgGRA7+pIgMIC3; Group 3, pROP1/CD154+ pIgGRA7+pIgMIC3 and pIgGRA1+pIgGRA6+pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p40).

7.3.2.4 Antibody responses detected by anti-rGRA1

Ewes of vaccinated group 2 started to produce specific IgG1 response against rGRA1 two weeks after the 1st injection, and this IgG1 response became significantly ($p < 0.001$) stronger one week after the 2nd injection, reaching a peak (OD ratio of 7.4) at week 6, followed by a dramatically declined (Fig 11, A). The sheep of group 3 also produced a significantly ($p < 0.001$) higher level of specific anti-GRA1 IgG1 response, but only at two to four weeks after the 2nd injection, reaching a peak (OD ratio of 6) at week 7.

Significant differences ($p < 0.001$) were observed on IgG1 production between group 2 and group 4 from week 5 to 12, and between group 3 and group 4 from week 6 to 8.

Compared with the induced anti-GRAl IgG1 response, the level of anti-GRAl IgG2 responses were lower in both treated group 2 and 3 sheep (Fig 11, B). The immunized group 2 started to produce an IgG2 response two weeks after the 1st injection, and the level of response became a little higher after the booster (week 6 to 8). However, the treated group 3 stimulated only a very low level of IgG2 response two weeks after the 2nd injection. There was no significant difference in IgG2 production between groups.

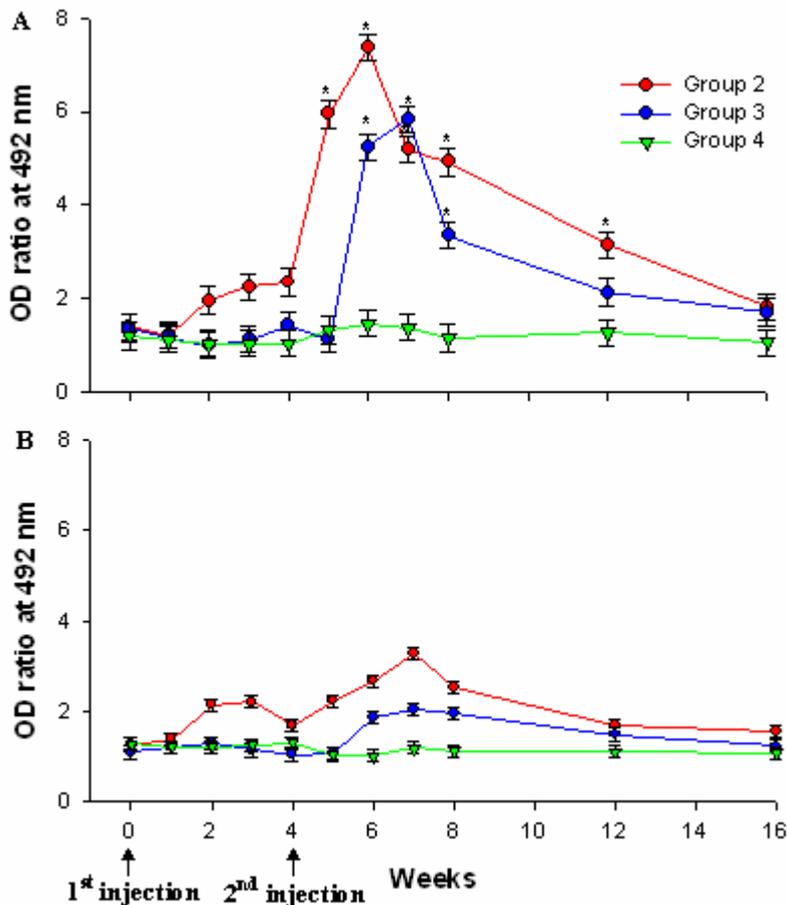


Figure 11. Specific IgG1 (A) and IgG2 (B) response observed by injection of constructs against recombinant GRAl. Group 2, pIgGRA1+ pIgGRA6+pIgMAG1; Group 3, pROP1/CD154+pIgGRA7+ pIgMIC3 and pIgGRA1+pIgGRA6+pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p41-42).

7.3.2.5 Antibody responses detected by anti-rGRA6

In DNA vaccinated group 2, two significant ($p < 0.001$) increase of specific anti-GRA6 IgG1 levels were observed at two weeks after the 1st injection and two weeks after the 2nd injection, and the level of IgG1 decreased dramatically after a peak (OD ratio of 8.5) at week 6 (Fig 12, A). In group 3, a significant ($p < 0.001$) increase in specific anti-GRA6 IgG1 levels was detected only at two to four weeks after the 2nd injection. Significant differences ($p < 0.001$) were observed in anti-GRA6 IgG1 production between group 2 and group 4 from week 2 to 8, and between group 3 and group 4 from week 6 to 8.

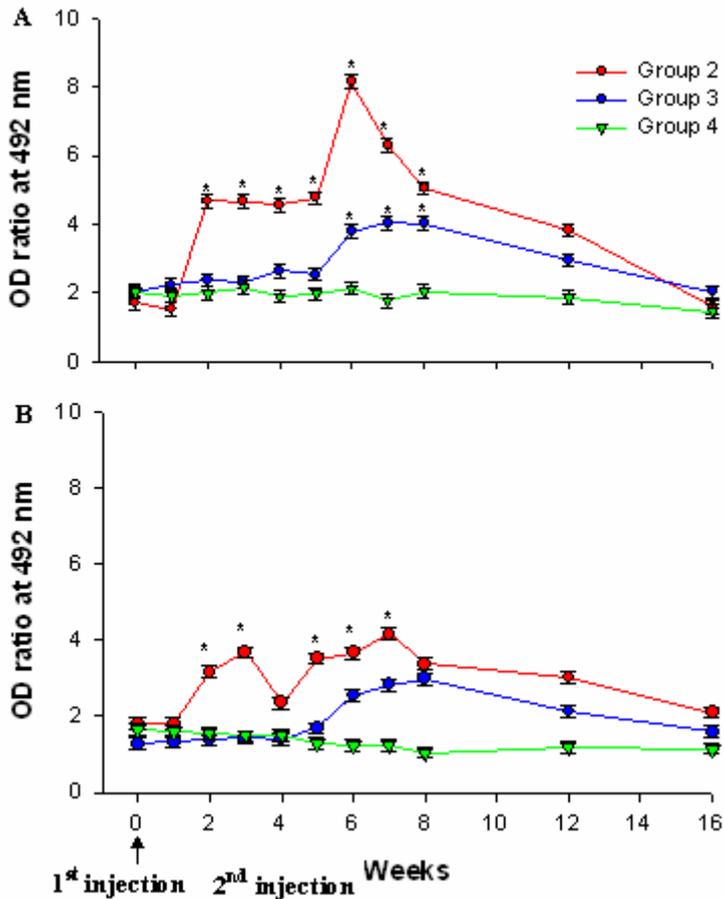


Figure 12. Specific IgG1 (A) and IgG2 (B) response observed by injection of constructs against recombinant GRA6. Group 2, pIgGRA1+ pIgGRA6+ pIgMAG1; Group 3, pROP1/CD154+ pIgGRA7+pIgMIC3 and pIgGRA1+pIgGRA6+pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p43-44).

The level of stimulated anti-GRA6 IgG2 responses in both treated group 2 and 3 were not as high as induced anti-GRA6 IgG1 levels in those groups (Fig 12, B). A higher ($p<0.001$) level of IgG2 antibody response in group 2 occurred from two weeks after 1st injection to three weeks after the 2nd injection, compared with that produced in group 3 and the control group. The group 3 had a higher anti-GRA6 IgG2 production only two weeks after the 2nd injection. The control group 4 remained low throughout the experiment.

7.3.2.6 Antibody responses detected by anti-rMAG1

Ewes of vaccinated group 2 started to stimulate specific IgG1 response against rMAG1 two weeks after the 1st injection. There was a further increase in the IgG1 response one week after the booster, and reaching a peak (OD ratio of 8.9) at week 7, followed by a dramatic decline (Fig 13, A). The sheep in group 3 also produced a significantly ($p<0.001$) higher level of specific anti-MAG1 IgG1 response, but only at two to four weeks after the 2nd injection, reaching a peak (OD ratio of 5) at week 7. Significant differences ($p<0.001$) were observed on IgG1 production between group 2 and group 4 from week 5 to 8, and between group 3 and group 4 from week 6 to 8.

Compared with IgG1 levels produced against rMAG1, the level of anti-MAG1 IgG2 responses were lower in both treated group 2 and 3 (Fig 13, B). Compared with control group, the immunized group 2 started to produce significant ($p<0.001$) high level of IgG2 response at two to three weeks after the 1st injection, and one to four weeks after the 2nd injection. While, the group 3 only stimulated a low level of IgG2 response starting two weeks after the 2nd injection.

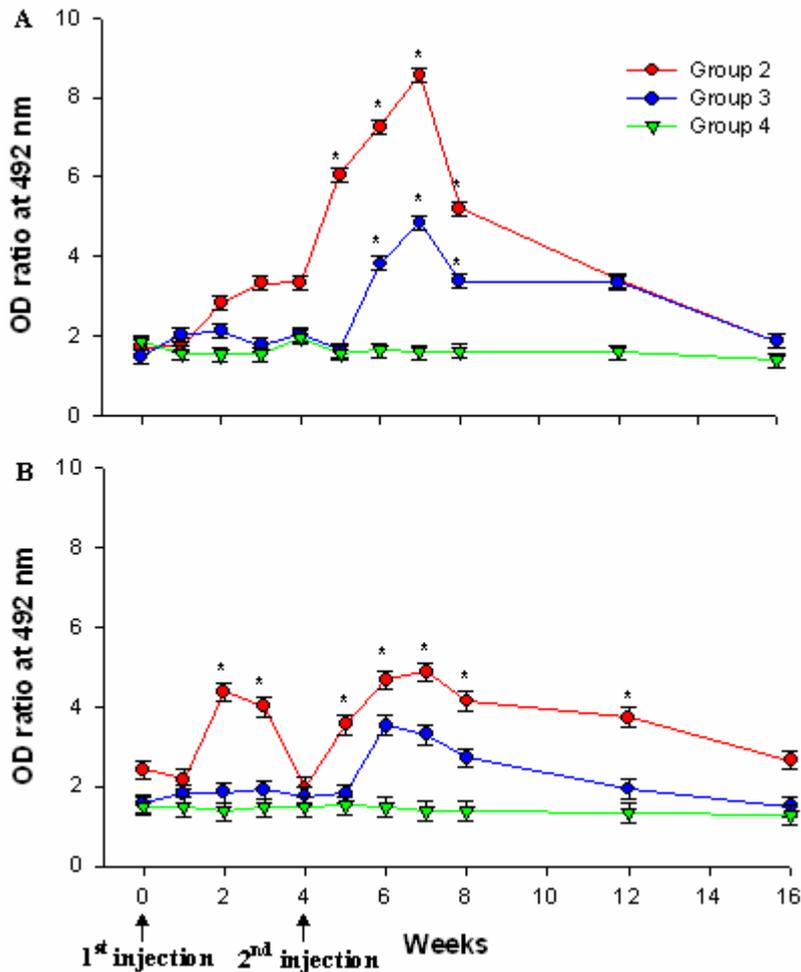


Figure 13. Specific IgG1 (A) and IgG2 (B) response observed by injection of constructs against recombinant MAG1. Group 2, pIgGRA1+pIgGRA6 +pIgMAG1; Group 3, pROP1/CD154+pIgGRA7+pIgMIC3 and pIgGRA1+pIgGRA6+pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p44).

7.3.3 Antibody responses against *T. gondii* native antigens (TA)

One week after the 1st injection, small increases of IgG1 levels against native *T. gondii* antigen were found in all three plasmid DNA vaccinated groups (1, 2, and 3), followed by a small decrease (Fig 14, A). After the 2nd injection, the IgG1 levels in all the tested groups increased dramatically, reaching peaks at week 6 or 7: the highest value was from group 1, followed by group 2 and then group 3. Significant ($P < 0.001$) differences

were found in the levels of anti-native *T. gondii* IgG1 between treated groups 1 and 2 and control after the 2nd injection (week 6 and 7).

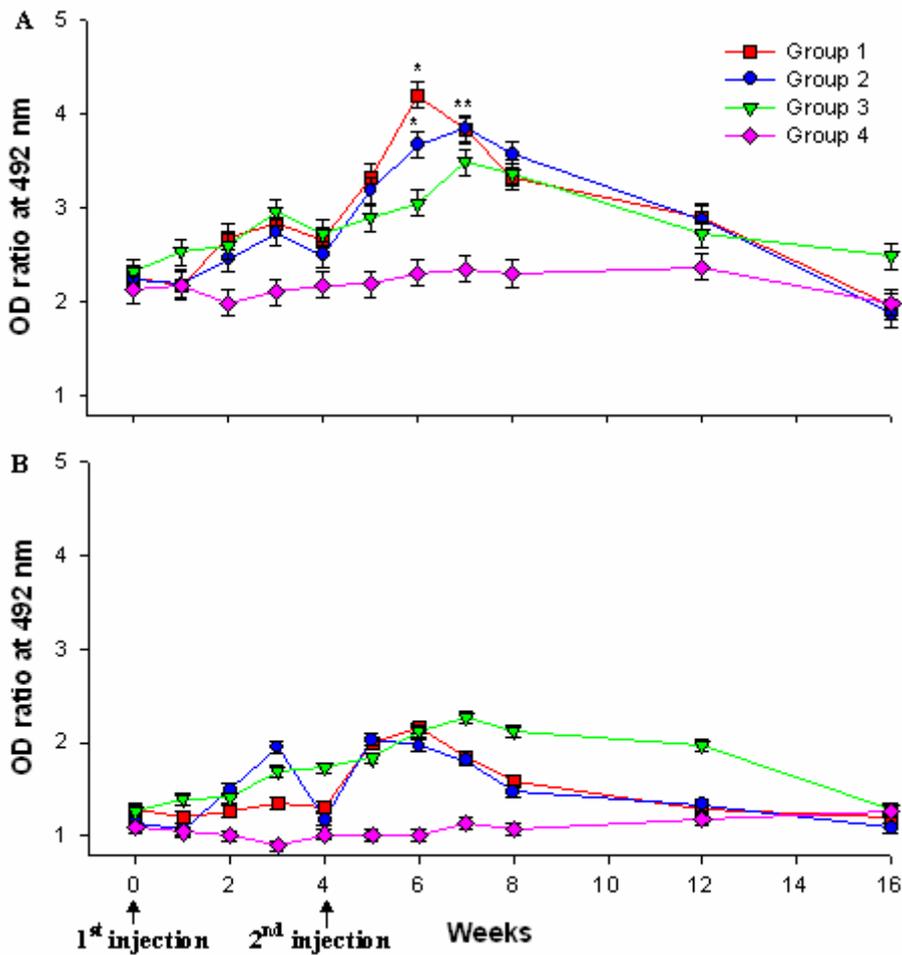


Figure 14. Specific IgG1 (A) and IgG2 (B) response observed by injection of constructs against TA antigen. Group 1, pROP1/CD154+pIgGRA7+pIgMIC3; Group 2, pIgGRA1+pIgGRA6+pIgMAG1; Group 3, pROP1/CD154+pIgGRA7+pIgMIC3 and pIgGRA1+pIgGRA6 +pIgMAG1; Group 4, PBS. Statistically significant differences ($P < 0.001$, marked with an asterisk*) were observed between some groups. Data shown is the mean values \pm S.E.M of 40 sheep. Additional details are in the text (p45-47).

The sheep from treated group 3 produced the highest anti-native *T. gondii* IgG2 levels among the tested groups (Fig 14, B). The ewes from treated group 2 produced higher IgG2 levels than that from group 1 before the 2nd injection, but they had similar IgG2 levels after that. The IgG2 antibodies response against *T. gondii* protein was also

observed between the treated groups and control group 4, especially after the second injection.

8. Discussion

The injections of the ewes with all the DNA plasmids encoding *T. gondii* peptides showed antibody responses. The important finding was that both of the immunisations with the combination of plasmids pVAXROP1/CD154+ pVAXIgGRA7+pVAXMIC3 produced significantly ($P<0.001$) high level of specific anti-ROP1 IgG1 antibody in the serum.

The results have advanced our understanding of the use of DNA vaccines against *T. gondii* in sheep. Data from the experiment have supported the hypothesis that sheep respond serologically, with variable levels of antibody responses, to mixed DNA constructs encoding for all the tested *T. gondii* recombinant antigens (ROP1, GRA7, MIC3, GRA1, GRA6 and MAG1) and the native *T. gondii* antigen. The results also indicate that there were changes in antibody levels over time, with a significant ($P<0.001$) increase after immunization.

Most studies on mice have found that injection of individual plasmids encoding *T. gondii* antigens give at least a partial protection from its infection. A few experiments working on DNA cocktail vaccinations (injected plasmid DNA of mixed *T. gondii* antigen) have been reported to enhance the protection against toxoplasmosis in a mouse model (Fachado et al., 2003) and in a pig experiment (Jongert et al., 2007b). Fachado et al. (2003) found that immunization with a mixture of plasmids expressing SAG1 and ROP2 induced both increased humoral and cellular immune responses, in comparison with vaccination of plasmids expressing either SAG1 or ROP2 alone. In another experiment, a GRA1-GRA7 DNA vaccine cocktail was able to elicit a strong humoral immune response against *T. gondii* pig infection (Jongert et al., 2007b). Jongert, et al.

(2007a) using mice also reported that a two-gene (GRA1 and GRA7) cocktail of DNA vaccine elicited higher endpoint titers than using a three-gene cocktail (GRA1, GRA7 and ROP2) or single-gene injection. However, a reverse result (Sedegah et al. 2004) has found that there was a significant suppression of immune responses when the plasmids were pooled in a cocktail and injected into a single site. In my study, groups of three-gene vaccines were injected into separated sites of sheep and variable levels of IgG antibody responses were produced. This is the first study working on sheep and the first investigation on immunizing with several separate plasmids DNA into one sheep.

8.1 Antibody responses detected in immunized sheep from group 1

We found that the sheep in group 1 had induced significant ($P < 0.001$) levels of anti-ROP1 specific IgG1 after both injections, and a strong anti-GRA7 specific IgG1 response was also developed after the 2nd injection (Fig 5A), suggesting there is a strong humoral immunity response from this injection. Specific anti-ROP1 and anti-GRA7 responses in IgG2 were also observed, but at a low level (Fig 5B). Unlike the ROP1 and GRA7 antigens, this combination induced similar levels of both IgG1 and IgG2 against MIC3 antigen, but still lower than the anti-ROP1 and anti-GRA7 IgG1 levels.

Previous studies using mice have reported that individual plasmids DNA encoding ROP1 (Chen et al., 2003), GRA7 (Vercammen et al., 2000) and MIC3 (Bhopale, 2003; Ismael et al., 2003) induced strong antibody responses. Generally, following immunization of a DNA construct containing a *T. gondii* antigen into a sheep, the processed peptides of the antigen from a muscle cell will be exposed to APCs, together with MHC-I and MHC-II molecules, and these will stimulate the immune response. The antigen released from the cell will also stimulate B cells by binding to their

immunoglobulin-like receptors, and thus a humoral immune response will be elicited. This would be demonstrated by the presence of IgG1 and IgG2 antibodies in immunized sheep serum, and the absence of IgG antibodies in control sheep, which had only PBS injection. In this experiment, each sheep was injected with three separate plasmid peptides in different spots, and developed different levels of antibody responses. An explanation for this may be that after injection, the major accepted body tissue, muscle cells are unequally assembled to take those three different antigenic peptides; one is more than the others. According to our data (Fig 5A), the injections elicited the highest anti-ROP1 IgG1 levels, suggesting that most muscle cells may take plasmid DNA encoding ROP1/CD154 peptide to be transcribed and translated into proteins, which are then released to bind and stimulate B cells to produce antibodies. Another reason could be that the gene product was a fusion of CD154 and ROP1. Manoj et al. (2003) injected sheep by using CD154 to construct bovine herpesvirus 1 glycoprotein plasmid DNA and had significantly enhanced immune responses. This indicated that using CD154 to target plasmid-expressed Ag could significantly enhance immune responses induced by this vaccine.

Jongert et al. (2007a) showed that pGRA7 seemed to have a positive influence on titers against the antigen and had immuno-dominant role on stimulating humoral responses after combination of either a two-gene (pGRA1 and pGRA7) or a three-gene (pGRA1, pGRA7 and pROP2) cocktail of DNA vaccines. In the current study, the level of specific anti-GRA7 IgG1 showed the second highest response in the group 1 sheep after injection and anti-MIC3 IgG1 response was even lower than that of GRA7 (Fig 6A). This suggested that in this combination (ROP1/CD154, GRA7 and MIC3), pIgGRA7

and pIgMIC3 in sheep did not play dominant role in stimulating a humoral immune response.

Compared with the IgG1 response that was induced by the vaccination in group 1, specific anti-ROP1 IgG2 response was lower (Fig 5B). IgG1 antibody production is induced by Th2 cells, while IgG2 is induced by Th1 cells. The predominant protective immunity of either Th1 or Th2 cells is reflected by Th1/Th2 ratio or IgG2/IgG1 ratio. The IgG2 antibody production against *T. gondii* MIC3 antigen in the current study showed the highest level. The anti-MIC3 of IgG2 level was higher than that of IgG1, but both of them were lower than the IgG1 produced by anti-ROP1 and anti-GRA7. The results have been found in a mice study that obtained a very high level of IgG2a antibody titers from intramuscular vaccination with an immature form of MIC3 protein (Ismael et al., 2003).

8.2 Antibody responses detected in immunized sheep from group 2

The present study demonstrated that sheep immunized with a mixture of plasmids DNA encoding GRA1, GRA6 and MAG1 genes were all able to elicit a strong humoral immune response characterized by the production of high levels of both IgG1 and IgG2 antibodies against *T. gondii* recombinant antigens GRA1, GRA6 and MAG1 (Fig 6). The anti-GRA6 IgG1 level raised after the 1st injection, and the IgG1 anti-GRA1, GRA6 and MAG1 were all significantly stimulated post booster. The increased production of IgG2 antibodies against the above three recombinant antigens were also observed.

Induced humoral immune responses against GRA1 (Kato et al., 2005), GRA6 (Lecordier et al., 2000) and MAG1 (Chen et al., 2001) antigens have been demonstrated

after DNA vaccines encoding any of these genes. Bivas-Benita et al. (2003) and Kato et al. (2005) found that immunization of mice with GRA1 plasmid DNA vaccine produced a high level of both IgG1 and IgG2 anti-GRA1 antibodies. Lecordier et al. (2000) showed that the N-terminal hydrophilic part of GRA6 was recognized by positive IgG human sera in an immunoblot. Another experiment suggested that GRA6 complemented by SAG1 expression reached a higher sensitivity for detection of IgM antibody to *T. gondii* (Li et al., 2003). Nielsen et al. (2005) indicated that the MAG1/BAG1 DNA vaccines induced the synthesis of specific anti-MAG1 IgG2a antibody in about 80% of immunized mice after oral challenge with *T. gondii*. These findings supported the result in the current study.

8.3 Antibody responses detected in immunized sheep from group 3

This study also found that sheep in group 3 stimulated strong humoral immune responses characterized by increased ($P < 0.001$) anti-ROP1 IgG1 antibody production after the 1st injection and high levels of anti-GRA1, GRA6 and MAG1 IgG1 responses after 2nd injection (Fig 7). This suggested that the elicited immune response may be due to the dominant expression of ROP1/CD154 gene inside the sheep. A possible reason is that antigenic competition occurred when sheep received a mixture of the six plasmids. Antigenic competition may occur between T cells if they respond to different peptides presented on the same antigen-presenting cell (Kedl et al., 2000). They also suggested that T cells that responded to the same peptide-MHC competed with each other by lowering the amount of ligand with which the cells could react (Kedl et al., 2002). As a result, the activation of high-affinity cells was favoured.

8.4 Comparison of antibody responses detected in immunized sheep from group 3 with group 1 or 2 and group 4

The current study compared antibody responses against individual *T. gondii* antigens between sheep of treated group 3 with group 1 or 2 and group 4. No antibody response was detected in any of sheep sera in the control group 4, as they were only injected with PBS instead of vaccination. Except for the IgG1 response against MIC3 in treated group 3 sheep being slightly stronger than that in group 1, the group 1 sheep produced both higher IgG1 and IgG2 antibodies against ROP1, GRA7 and MIC3 antigens after DNA injections than group 3 sheep (Fig 8, 9 and 10). Compared with the treated group 3 and group 2 sheep, the induction of both IgG1 and IgG2 antibodies responses were all stronger in treated group 2 sheep than from those in group 3 (Fig11, 12 and 13). This may be explained by the immune response being dose-dependent. The sheep in group 3 only received half the amount (1 mg) of each DNA plasmid compared with the sheep in group 1 or 2 which were injected with 2 mg of the same DNA plasmid. Thus, two injections with the same DNA plasmid would provide better immune response than the single injection.

8.5 Antibody responses against native *T. gondii* antigen in all experiment groups

Like the antibody levels against recombinant antigens, there was also an enhancement of both IgG1 and IgG2 levels against native tachyzoite antigen being engaged after the 2nd injection in all three treated groups, but not in control group (Fig 14). This suggests that all three combinations of plasmid DNA vaccinations can stimulate humoral immunity in sheep.

8.6 Use of CpG ODN and Liposomes

Previous studies have found that synthetic oligodeoxynucleotides containing a CpG motif activate APCs (e.g. B cells) through a toll-like receptor (TLR-9). This induces cell signaling and thus activates a pro-inflammatory cytokine response, which enhances both humoral and cellular immunity (Krieg et al., 1996; Boggs et al., 1997). The role of CpG ODN used as adjuvant in vaccination against *T. gondii* infection remains controversial. Kringel et al. (2004) and El-Malky et al. (2005) showed that CpG ODN was an effective adjuvant for use in vaccination against toxoplasmosis in pigs and mice. However, Sedegah et al. (2004) stated that CpG ODN had only a limited role in the protection against *T. gondii* infection, and Spencer et al. (2004) even found that CpG adjuvantation appeared to not enhance survival after mice had been challenged with tachyzoites. Co-delivered liposomes with plasmid DNA have been reported to provide protection by inducing CTL responses and B cells to produce antibodies (Gregoriadis et al., 2002). In the current study, the three plasmid groups were all received CpG ODN and liposomes. The trial design did not include plasmid preparations without CpG or liposomes, so efficiency of either adjuvant was not tested directly.

9 Conclusions

In this study, injections at different sites with individual components from the three-gene combination of pROP1/CD154 + pGRA7 + pMAG1 gave the highest production of antibodies, suggesting the potential vaccine candidates for the development of protection against *T. gondii* infection in sheep. In order to improve the efficacy of DNA vaccination, some different gene combination or injection dose difference could be investigated. Different vaccination approach also could be used in the future, such as using a jet gun, which is able to enhance the efficacy of transfection and provide more

options for vaccine delivery, such as subcutaneous and oral. The design of future experiments could also add the groups without liposome or without CpG in order to decrease cost of the vaccine.

10 References

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