Toxoplasma gondii: DNA vaccination with genes encoding antigens MIC2, M2AP, AMA1 and BAG1 and evaluation of their immunogenic potential

George Dautu[a], Biscah Munyaka[a,b], Gabriella Carmen[a,c], Guohong Zhang[a], Yoshitaka Omata[a], Xuan Xuenan[a] and Makoto Igarashi[a*]

[a]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

[b]Kenya Medical Research Institute (KEMRI), P.o.Box 54840-00200 Nairobi, Kenya

[c]Servicio Nacional de salud y Calidad Animal, 595-(21)-584-496 San Lorenzo-Paraguay

dDepartment of Basic Veterinary Science, School of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

[*]Corresponding Author Makoto Igarashi

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

Tel: +81-155-49-5802, Fax: +81-155-49-5643

E-mail: makoto@obihiro.ac.jp

Abstract

A combination of antigenic regions of microneme proteins have been previously reported as being protective against chronic toxoplasmosis. In this work, we evaluated immune responses induced by immunizing BALB/c and C57BL/6 mice intradermally with plasmid DNA encoding the protein sequences of *Toxoplasma gondii* AMA1, MIC2, M2AP and BAG1. Mice immunized with the *AMA1* gene developed high levels of serum IgG2a and c antibodies as well as cellular immune responses associated with IFN-γ synthesis suggesting a modulated Th1-type of response. Immunization with the *AMA1* gene resulted in a partial but significant protection against the acute phase of toxoplasmosis compared to *MIC2*, *M2AP* and *BAG1* genes. Therefore, the *AMA1* gene appears to generate a strong specific immune response and also provides effective protection against toxoplasmosis more than the *MIC2*, *M2AP* and *BAG1* genes.

Index Descriptors and Abbreviations: Toxoplasma gondii; DNA immunization; DNA, deoxyribonucleic acid; MIC2, microneme protein-2; M2AP, MIC2 associated protein; AMA1, apical membrane antigen-1; BAG1, bradyzoite antigen-1; IgG, immunoglobulin G

1. **Introduction**

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. The parasite infects most species of domestic animals, birds and humans in most parts of the world. It is estimated that up to 500 million people worldwide are infected with *T. gondii* (Denkers and Gazzinelli, 1998). Usually, *T. gondii* infection of an immunocompetent individual is clinically asymptomatic. However, in immunocompromised individuals and pregnant women, infection with the parasite can cause severe complications (Hughes, 1985). In immunocompromised patients (such as in AIDS patients), the reactivation of encysted bradyzoites into actively replicating and cytolytic tachyzoites can lead to fatal toxoplasmic encephalitis (Dzierszinski et al., 1999; Luft and Remington, 1992). In domestic animals, especially sheep and goats, the parasite can cause abortions and stillbirth resulting in significant losses to the livestock industry (Buxton, 1998). Furthermore, the tissue cysts of *T. gondii* in meat of infected livestock are an important source of infection for humans (Buffolano et al., 1996). The devastating effect of toxoplasmosis in both humans and domestic animals makes it one of the diseases of great economic importance in many parts of the world. Therefore a vaccine which will provide sterile immunity against *T. gondii* is urgently required. The only vaccine developed to date is the live attenuated one in which live tachyzoites of strain S48 were used as commercial vaccine for ovine toxoplasmosis (Buxton and Innes, 1995). Despite the use of these vaccines in veterinary medicine, their use in human medicine is not accepted as the risk of reversion to a pathogenic form is high (Ogra et al., 1991).

Significant progress has been made in the identification of vaccine candidates which can induce protective immunity against *T. gondii* infection. Most work has been focused on surface antigens of tachyzoites (Bhapole, 2003), including SAG1 (30 kDa), SAG2 (22 kDa) and SAG3 (43kDa). Among these surface antigens, SAG1 is one of the most promising vaccine candidates. Interesting work has also been done on excretory secretory antigens (ESA) of *T. gondii* and these ESA (GRA1, GRA4 and GRA7) have been shown to play an important role in the stimulation of the protective immune system in mice (Cesbron-Delauw and Capron, 1993) and it was shown that they are expressed by both stages (tachyzoite and bradyzoite stage). In recent years, microneme proteins which are responsible for adhesion and invasion (Beghetto et al., 2005; Soldati et al., 2001; Tomley and Soldati, 2001) have been implicated as vaccine candidates. The micronemal protein MIC3, a potent adhesin of *T. gondii*, was recently shown to be a suitable vaccine candidate (Ismael et al., 2003). A combination of antigenic regions of *T. gondii* microneme protein (MIC2, MIC4, M2AP and AMA1) was also shown to induce protective immunity against chronic toxoplasmosis in mice (Beghetto et al., 2005) and a more recent report shows that immunization with MIC1 and MIC4 induces protective immunity in mice (Lourenço et al., 2006).

In the present study, we were interested in evaluating the immune responses induced by immunizing mice with full length genes of *T. gondii AMA1*, *MIC2*, *M2AP* and *BAG1* using a gene gun. For our experiments, two strains of mice (BALB/c [H-2^d] and C57BL/6 [H-2^b]) with different major histocompatibility haplotypes and different susceptibilities to *T. gondii*-induced infection were used (Brown and Mcleod,

1990). We analyzed the subclass of generated antibodies and cytokines in mice immunized with the above genes as an indicator of the type of immune response induced. Finally, we compared the immune response induced and protection conferred by DNA immunization using plasmids encoding the protein sequences of AMA1, MIC2, M2AP and BAG1 after oral challenge with cysts of the *T.gondii* Beverley strain.

2. **Materials and methods**

2.1. *Animals*

Six to eight weeks old female BALB/c (a resistant strain) (H-2^d) and C57BL/6 mice (a susceptible strain) (H-2^b) used in this experiment were purchased from CLEA, Japan.

2.2. *Parasites*

Two *Toxoplasma gondii* strains (RH and Beverley) were used for this study. *T. gondii* RH strain was maintained in our laboratory through serial passage in Vero cells grown in modified Eagles medium (Sigma-Aldrich, UK) supplemented with 5% fetal calf serum (FCS). *T. gondii* (Beverley strain) cysts used for challenging the mice were obtained from the brains of orally infected ICR mice (CLEA, Japan) and maintained by monthly passage.

2.3. *Preparation of T. gondii lysate*

T. gondii lysate was prepared from RH strain tachyzoites as previously described (Fatoohi et al., 2002) with minor modifications. Briefly, the obtained tachyzoites were washed with phosphate-buffered saline (PBS) and adjusted to $1x10^8$ tachyzoites/ml of PBS. The parasites were disrupted by three freeze-thaw cycles and the crude extract was clarified by centrifugation at 2,500 x *g* for 15 minutes. The

resulting soluble fraction was filtered through a Millex GV 0.22-μm filter unit (Millipore, Bedford, MA 01730 U.S.A). The protein concentration in the supernatant was measured using a Coomassie protein assay reagent kit using bovine serum albumin (BSA) as the standard (Pierce, Rockford, USA) and kept at -80°C until use.

2.4. *Plasmid construction*

T. gondii MIC2, M2AP, AMA1 and BAG1 cDNAs containing entire coding regions were amplified by PCR from the *T. gondii* RH strain tachyzoite cDNA using the following specific primers whose restriction sites are underlined. MIC2: 5'-TTGAATTCCCATGAGACTCCAACGCGAAG-3' (forward) and 5'-TTCTCGAGCTACTCCATCCATCCACATATCAC-3' (reverse); M2AP: 5'-TTGAATTCCCATGAAACTCGCTGCCGTG-3 (forward) and 5'-TTGTCGACTTACGCCTCATCGTCACTCG-3' (reverse); AMA1: 5'-GCGAATTCATGGGGCTCGTGGGCGTA-3' (forward) and 5'-TTCTCGAGCTAGTAATCCCCCTCGAC-3' (reverse); BAG1: 5'-TTGGATCCATGGCGCCGTCAGCATCGCAT-3' (forward) and 5'-TTCTCGAGCTACTTCACGCTGATTTGTTG-3 (reverse). The amplified cDNAs were inserted into the eukaryotic expression vector pcDNA6-V5-HisA (Invitrogen, USA) by double digestion with appropriate restriction enzymes and ligation was finally done using T4 DNA ligase (Invitrogen, USA). The resulting plasmids were named pMIC2, pM2AP, pAMA1 and pBAG1. Plasmids were then purified from transformed *Escherichia coli* (*E. coli*) DH5α cells by anion-exchange chromatography (EndoFree plasmid giga kit, Qiagen Sciences, Maryland, USA) as per manufacturer's instructions. The purified plasmids were dissolved in sterile

endotoxin-free TE buffer and kept at -20ºC until use. The integrity of the plasmids was determined by agarose gel electrophoresis after digestion with appropriate restriction enzymes. The DNA concentration was determined by measuring the optical density at 260 nm (OD_{260}) using an Ultrospec 2100 spectrophotometer (Amersham Biosciences, England). The 260/280 OD ratios were between 1.80 and 2.00 for the purified DNA and this indicated that the preparations were free of major contaminants.

2.5. *Production of recombinant MIC2, M2AP, AMA1 and BAG1 proteins*

T. gondii MIC2, M2AP, AMA1 and BAG1 cDNA containing entire coding sequences were subcloned in the pGEX6P-2 vector (Pharmacia) as described above. Resulting plasmids were expressed in *E. coli* strain BL21 (DE3) plysS cells as glutathione *S*-transferase (GST) fused proteins herein referred to as recombinant GST-MIC2, M2AP, AMA1 and BAG1. The fusion proteins were produced by IPTG induction and affinity purified by glutathione sepharose beads (Amersham Biosciences, Uppsala, Sweden). The eluted fractions were dialysed against phosphate buffered saline (PBS) and the amount of recombinant proteins was evaluated using both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Coomassie protein assay reagent kit. The recombinant proteins were finally kept at -80ºC until use.

2.6. *Expression of pMIC2, pM2AP, pAMA1 and pBAG1 in mammalian cells*

The human embryonic kidney derived 293T cells were transfected with

pMIC2, pM2AP, pAMA1, pBAG1 or an empty vector (control plasmid) using the calcium precipitation method as previously described (Sambrook and Russell, 2001). Briefly, the 293T cells were maintained *in vitro* with minimum essential medium (MEM) supplemented with 10% fetal calf serum. A day before transfection, the 293T cells were transferred in a 6-well tissue culture plate (3 x 10^5 cells/well). Eight micrograms of plasmid DNA were dissolved in 100 µl distilled H_2O, and a further 100µl of 0.5 M $CaCl_2$ was added and the resulting 200 µl mixture was incubated at room temperature for 1 hour. After 1 hour incubation, 200µl of HNP buffer (50 mM HEPES, pH7.1, 280 mM NaCl, 1.6 mM NaH_2PO_4) was added to the previously prepared 200 µl DNA mixture and a further 1 hour incubation was performed. The resulting 400 µl of solution containing DNA precipitates was added to each well and incubated for 6 hours at 37ºC in a 5% CO_2 incubator. After incubation, cells were rinsed with culture medium and fresh culture medium was added to the rinsed cells which were finally incubated for 48 hours in a 5% CO_2 incubator.

2.7. *Western blot analysis*

Cells were harvested 48 hours after transfection, and lysed with RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 0.1% Triton-X100, 1% Nonidet P-40). The lysates of transfected cells and *T. gondii* tachyzoites (RH strain) were dissolved in SDS-PAGE sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue), heated at 95°C for 5 minutes and separated on a 10% polyacrylamide gel. The separated proteins were probed with anti-MIC2, M2AP, AMA1 and BAG1 polyclonal antibodies raised in mice at a dilution ratio of 1:100 in 1x PBS containing 3%

skimmed milk (PBS-M). Bound antibodies were detected using sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences) diluted in 1:1000 in PBS-M. Peroxidase activity was revealed by using a substrate mixture of 0.25mg/ml diaminobenzidine tetrahydrochloride (Sigma), 100mM Tris, pH7.5 and 0.05% H_2O_2. Molecular mass standards (SeeBlue plus2 Pre-stained standard, Invitrogen) were used.

2.8. *Gene gun DNA immunization*

Gene gun immunization was done as previously described (Mohamed et al., 2003; Saito et al., 2001). Briefly, plasmid encoding MIC2, M2AP, AMA1 and BAG1 genes were affixed onto gold particles (1.6µm diameter) at a rate of 2µg DNA per 1 mg of gold by the addition of 1M $CaCl_2$ in the presence of 0.05 M spermidine. Plasmid DNA-coated gold particles were loaded onto Gold-Coat tubing in the presence of polyvinylpyrrolide (360,000 MW), as an adhesive, at a concentration of 0.05 mg/ml. Plasmid DNA-coated gold particles were accelerated into the shaved abdomen of mice using a Helios Gene Gun at a helium discharge pressure of 400 psi. Each mouse received two shots per each immunization, delivering approximately 2 µg DNA per mouse. Immunizations were done three times with a 2 weeks interval in between. Five to eight mice were used for each experimental group.

2.9. *Assessment of the humoral response to DNA immunization*

Serum IgG antibody responses to *T. gondii* MIC2, M2AP, AMA1 and BAG1 were measured using Western blot analysis and enzyme-linked immunosorbent assay (ELISA) as follows.

(i) *Western blotting*

Western blotting was carried out as described above. The *T. gondii* antigen was separated on a 10% polyacrylamide gel under reducing conditions.

(ii) *ELISA*

Levels of specific IgG antibodies in mice serum samples were determined as previously described (Ismeal et al., 2003) with slight modifications. In brief, the 96 flat-bottom wells of micro-titer plates (Maxisorp; Nunc, Denmark) were coated overnight at 4°C with *T. gondii* recombinant proteins (GST-MIC2, M2AP, AMA1, and BAG1) at 5 µg/ml in 50 mM sodium carbonate buffer (pH 9.6). The plates were washed with PBS containing 0.05% Tween 20 (PBS-T), pH 7.4. Non-specific binding sites were blocked with PBS-M for 1 hour at 37°C. The plates were then washed once with PBS-T and subsequently 50 µl of mice sera diluted 1:100 in PBS-M was added and incubated at 37ºC for 1 hour. After incubation, the plates were washed six times with PBS-T. The bound antibodies were detected by incubating at 37°C for 1 hour with sheep anti-mouse IgG-conjugated with horseradish peroxidase (Amersham Biosciences) diluted at a ratio of 1:2000 in PBS-M. Finally the plates were washed six times with PBS-T and the bound peroxidase enzyme activity was revealed by adding 100 µl/well of ABTS substrate i.e., 3 mg 2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (Sigma), in ABTS buffer (0.1M citric acid monohydrate, 0.2 M disodium hydrogen phosphate), pH 4.0 and 1µl H_2O_2 per 10 ml of buffer . Absorbances at 415 nm in each well were measured using a MTP-500 microplate reader (Corona Electrical, Japan).

2.10. *Determination of the anti-MIC2, M2AP, AMA1, BAG1 IgG subclass*

The anti-MIC2, M2AP, AMA1 and BAG1 subclasses were determined by ELISA as described above. The goat anti-mouse IgG1, IgG2a, IgG2b and IgG2c conjugated with horseradish peroxidase (Bethyl Laboratories, Inc), were used at a concentration of 1:2000 in PBS-M. The level of the IgG2a subclass was not evaluated in C57BL/6 mice, because previous reports indicate that the IgG2a gene is deleted in C57BL/6 mice (Desolme et al., 2000; Jouvin-Marche et al., 1989; Martin and Lew, 1998). However, as an indicator of Th1 response in C57BL/6 mice sera, the level of IgG2c subclass was evaluated.

2.11. *Splenocyte stimulation and cytokine quantification*

Two weeks after the last immunization, spleen cells were harvested and hemolysed in lysis buffer (0.83% NH_4Cl, 0.01 M Tris-HCl pH 7.2). Cells were washed with PBS, and then plated into 96 well microplates at $3x10^5$/200 µl/well in RPMI-1640 medium (Sigma) supplemented with 5% FCS. The cells were stimulated by adding 10µg/ml of Concanavalin A (ConA) as the positive control, 10µg/ml of recombinant protein (GST-MIC2, M2AP, AMA1 and BAG1), or culture medium alone and incubated at 37ºC in a 5% CO_2 incubator and splenocyte supernatant was collected after 24 hours for IL-4 and 96 hours for IFN-γ. The IL-4 and IFN-γ were evaluated using a commercial ELISA kit according to the manufacturer's instructions (Biosource International, USA). Cytokine concentrations were determined by making reference to the standard curves constructed with known amounts of mouse recombinant IL-4 or IFN-γ. Spleen cells from pcDNA immunized mice were used as controls and were stimulated the same way as described above. The sensitivity limits of the assays were 5 pg/ml for IL-4 and 1 pg/ml for IFN-γ.

2.12. *Challenge infection*

C57BL/6 and BALB/c mice were infected orally with 20 cysts of the *T. gondii* Beverley strain 2 weeks after the last immunization. Mortality was monitored for a period of 30 days after challenge. The experiments were conducted in accordance with the standards relating to the care and management of experimental animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan).

2.13. *Statistical analysis*

Levels of significance for the differences between groups of mice were determined by the Mann-Whitney test. Statistical analysis was carried out using Prism 3 software (Graph Pad, San Diego, Calif.).

3. RESULTS

3.1. *Expression of MIC2, M2AP, AMA1 and BAG1 in mammalian cells*

In order to examine expression of recombinant antigens in mammalian cells, human embryonic kidney cells (293T cells) were transfected with pMIC2, pM2AP, pAMA1, pBAG1 or an empty vector for 48 hours. The protein extracts were then analyzed by SDS-PAGE and Western blotting (Fig. 1A, B, C and D). In Fig. 1A, a

band at about 115 kDa was recognized by mouse anti MIC2 polyclonal sera in protein extracts of cells transfected with pMIC2 (Fig. 1A, lane 2) and was slightly lower than that observed in *T. gondii* tachyzoite lysates (Fig. 1A, lane 3). In Fig. 1B, a band at about 48 kDa was recognized by mouse anti M2AP polyclonal sera in protein extracts of cells transfected with pM2AP (Fig. 1B, lane 2) and was slightly higher and different from that observed in *T. gondii* tachyzoite lysate which had two bands at about 43 kDa and 40 kDa, respectively (Fig. 1B, lane 3). In Fig.1C, a band at about 70 kDa was recognized by mouse anti AMA1 polyclonal antibody in the protein extract of cells transfected with pAMA1 (Fig. 1C, lane 2) and was higher than that observed in the *T. gondii* tachyzoite lysate at about 67 kDa (Fig.1C, lane 3). In Fig.1D, a band at about 30 kDa was recognized by mouse anti BAG1 polyclonal sera in protein extracts of cells transfected with pBAG1 (Fig. 1D, lane 2). The BAG1 antibody did not react with *T. gondii* lysate of tachyzoite (Fig. 1D, lane 3) and this could be because BAG1 is specifically expressed in the bradyzoite stage (Bohne et al., 1995) and not in the tachyzoite stage. In all cases, no bands were observed in the protein extract of cells transfected with the empty vector (Fig. 1A, B, C and D, lane 1). These results indicate that pMIC2, pM2AP, pAMA1 and pBAG1 appeared to direct the synthesis of antigenic MIC2, M2AP, AMA1 and BAG1 proteins in mammalian cells. However, we noticed some molecular mass differences between the proteins produced in the 293T mammalian cells and in *T. gondii* lysate (MIC2, M2AP and AMA1). These differences could be due to some post-translational modification.

3.2. *Humoral immune responses induced by DNA immunization using gene gun*

Sera from immunized C57BL/6 and BALB/c mice were collected 7 days after

the last immunization and analyzed by Western blot for specific anti-MIC2, M2AP, AMA1 and BAG1 responses. Mice immunized with the empty vector were included as negative controls (Fig.2A, B, C, D and E). In the Western blot analysis, all tested sera from mice immunized with pMIC2 reacted strongly with a protein band at about 115 kDa (Fig. 2B). Mice immunized with pM2AP reacted with a double protein band at expected molecular masses of 43 kDa and 40 kDa, respectively (Fig. 2C). Sera from mice immunized with pAMA1 reacted strongly with a single protein band at about 67 kDa as expected (Fig. 2D). Mice immunized with pBAG1 reacted with a single protein band at 30 kDa (Fig. 2E). In contrast, the sera from mice immunized with the empty vector could not detect 115 kDa (MIC2), 43/40 kDa (M2AP), 67 kDa (AMA1) or 30 kDa (BAG1) proteins (Fig. 2A). These sets of data show that DNA immunization with the gene gun induces the production of specific antibodies that are able to recognize the endogenous *T. gondii* MIC2, M2AP and AMA1 proteins and recombinant BAG1 protein expressed in 293T cells. Similar results were obtained from sera of BALB/c mice immunized with pMIC2, pM2AP, pAMA1 and pBAG1 (data not shown).

To determine the levels of antibody titers, all sera from BALB/c and C57BL/6 mice were tested by ELISA using specific recombinant proteins (Fig. 3A and B). Empty vector (pcDNA) sera from both BALB/c and C57BL/6 mice were also tested by ELISA using recombinant MIC2, M2AP, AMA1 and BAG1. High titers were observed in groups immunized with pAMA1 followed by pMIC2, pBAG1 and then pM2AP. In contrast, mice immunized with the empty vector did not produce antibodies against MIC2, M2AP, AMA1 and BAG1.

3.3. *Specific IgG subclass induced by DNA vaccination*

In order to determine whether a Th1 or Th2 response was elicited in immunized mice, the distribution of sera IgG subclass (IgG1, IgG2a, IgG2b and IgG2c) was analyzed using corresponding recombinant proteins as coating antigens i.e., GST-MIC2, M2AP, AMA1 and BAG1. Empty vector immunized mice sera were used as negative controls and were tested against each of the above recombinant proteins (Fig. 3 A and B). In sera of BALB/c mice immunized with pAMA1, higher levels of IgG2a and IgG2b were detected than in the sera of pMIC2, pM2AP, pBAG1 or empty vector immunized mice. In contrast, a low amount of IgG1 was detected in the sera of pAMA1 immunized mice when compared to pMIC2, pM2AP, or pBAG1. High levels of IgG1 were detected in the sera of pM2AP immunized mice with slightly high levels being detected in the sera of pMIC2 and pBAG1 immunized mice (Fig. 3A). In the C57BL/6 mice, higher levels of IgG2c and IgG2b were detected in the sera of pAMA1-immunized mice than those immunized by pMIC2, pM2AP or pBAG1. On the other hand, low levels of IgG1 were detected in the sera of mice immunized with pAMA1 and pMIC2 and slightly higher levels were detected in the sera of pM2AP and pBAG1 immunized mice (Fig. 3B). Our data indicate that the *AMA1* gene generates a specific Th1 type of response in both BALB/c and C57BL/6 as revealed by the high ratio of IgG2a to IgG1 in BALB/c or IgG2c to IgG1 in C57BL/6 (data not shown). The data further show that in pMIC2-, pM2AP- and pBAG1-immunized BALB/c mice, a mixed Th1/Th2 type of response was generated as revealed by a less polarized IgG2a/IgG1 ratio (data not shown). A similar situation was observed in C57BL/6 mice immunized with pM2AP and pBAG1, respectively. However, in the C57BL/6 mice immunized with pMIC2, a Th1 type of response was generated as revealed by a high IgG2c to IgG1 ratio (data not shown).

3.4. *Cellular responses*

The supernatant of splenocytes cultured from mice immunized with pMIC2, pM2AP, pAMA1 and pBAG1 were harvested at different times after restimulation by specific recombinant proteins (GST-MIC2, M2AP, AMA1 and BAG1) and assessed for the production of IL-4 and IFN-γ activities (Table 1A and B). Large amounts of IFN-γ were detected in the restimulated splenocyte cultures of pAMA1 immunized mice. The IFN-γ detected in restimulated splenocyte cultures of pMIC2, pM2AP and pBAG1 immunized mice were low compared to pAMA1 immunized mice. On the other hand, no specific release of IL-4 was seen from any culture supernatants further confirming the results of the IgG subclass.

3.5. *Protection of mice against challenge with T. gondii following DNA vaccination*

Groups of C57BL/6 mice (8 per group) and BALB/c mice (5 per group) were immunized with pMIC2, pM2AP, pAMA1 or pBAG1. Mice immunized with the empty vector served as a control. Two weeks after the last immunization, mice were orally challenged with cysts of *T. gondii* Beverley strain. Mortality was checked daily for one month (Fig. 4). From our results, DNA immunization of BALB/c with pAMA1 using gene gun significantly increased the survival rate by 60% [n =5] in comparison with pMIC2 (P=0.013), pM2AP (P=0.0024), pBAG1 (P=0.0038) or the empty vector control (P=0.0058). In pMIC2 immunized group, the survival rate was 40% (n = 5), in comparison with pM2AP (P=0.010), pBAG1 (P=0.010) or the control group (P=0.015). In the pM2AP and pBAG1 immunized groups, 20% (n = 5) survival was recorded and there was no statistical significant difference between the

two groups or in comparison with the empty vector control ($P > 0.05$, Fig. 4A). In the C57BL/6 mice, pAMA1 immunized groups had a survival rate of 37.5% (n = 8), in comparison with pM2AP (P=0.0091), pBAG1 (P=0.0327) or the control group (P=0.0038). Similar results were obtained with pMIC2 (37.5% survival was recorded [n=8], P=0.0064) in comparison with the control group or pM2AP (P=0.0151). In pBAG1 immunized mice, 12.5% survived ([n = 8], [P = 0.014]) in comparison with the control group or pM2AP (P=0.0238). None of the mice from pM2AP, or control groups survived the infection (Fig. 4B).

4. **Discussion**

DNA immunization has been shown to be the most effective way of inducing specific humoral and cellular immune responses in a number of vertebrate host species (Donnelly et al., 1994; Huygen et al., 1996, Vercammen et al., 2000). Mohamed et al., 2003 reported that the route of plasmid DNA delivery also plays a part in achieving good responses. They showed that gene gun delivery of plasmid DNA was more effective than either the intramascular or intraperitoneal routes.

In this study, we compared the effectiveness of immune responses induced by gene gun DNA vaccination using different *T. gondii* antigens (AMA1, MIC2, M2AP and BAG1). These antigens (AMA1, MIC2 and M2AP) are secreted by *T. gondii* apical organelles called micronemes. Secretion from these organelles form part of the parasite invasion machinery (Striepen et al., 2001; Carruthers and Sibley, 1997) whereas BAG1 is a small heat-shock protein which is specifically expressed in the bradyzoite (Di Cristina et al., 2004; Bohne et al., 1995). It promotes the differentiation of *T. gondii* from tachyzoites to bradyzoites and protects *T. gondii* from

environmental stress (Mun et al., 1999). It was previously shown to have some vaccine effects both in the acute and chronic stage (Mohamed et al., 2003).

We show that the *AMA1* gene induces a more specific Th1 type of response compared to the *MIC2*, *M2AP* and *BAG1* genes which induce a mixed Th1/Th2 type of response. We also show that the protective effect induced by the *AMA1* gene against *T. gondii* is better than that induced by the *MIC2*, *M2AP* and *BAG1* genes in the acute phase.

The current study further shows that the recombinant AMA1, MIC2, M2AP and BAG1 proteins produced *in vivo* by DNA vaccination using a gene gun were immunogenic; this is so because all of the C57BL/6 and BALB/c mice vaccinated intradermally with pAMA1, pMIC2, pM2AP and pBAG1 produced specific antibodies and were able to detect the endogenous *T. gondii* AMA1, MIC2, M2AP in *T. gondii* lysate and recombinant BAG1 (produced in 293T cells) on Western blots. ELISA results show that pAMA1-immunized BALB/c and C57BL/6 mice produced highly specific antibody titers followed by pMIC2, pBAG1 and pM2AP. Our results confirm that seroconversion can readily be obtained by intradermal DNA vaccination using a gene gun.

For naturally occurring *T. gondii* infection, it has been demonstrated that a Th1-biased response is required for effective protection (Denkers and Gazzinelli, 1998; Gazzinelli et al, 1993). This suggests that a good immunization protocol should be able to direct the T-helper cells towards a Th1 rather than a Th2 type of response. To determine the T-helper response of immunized mice, we evaluated the nature of the IgG subclass achieved during DNA immunization. In the BALB/c mice, a high ratio of IgG2a/IgG2b to IgG1 was exhibited by mice immunized with pAMA1. In the pMIC2-, pM2AP- and pBAG1-immunized groups; the ratio of IgG2a/IgG2b to IgG1

was less polarized as the sera IgG1 levels were consistently higher. In the C57BL/6 mice, a high ratio of IgG2c/IgG2b to IgG1 was exhibited in mice immunized with pAMA1 and pMIC2 whereas the IgG2c/IgG2b to IgG1 ratio was less polarized in pM2AP and pBAG1 immunized mice. In the current work, a mixed IgG1 and IgG2a/b response to DNA immunization was observed and similar observations on other *toxoplasma* genes were previously reported (Angus et al., 2000; Leyva et al., 2001; Boyle et al., 1997; Haddad et al., 1998; Nass et al., 1998). From the cytokine results, a large amount of IFN-γ was produced by splenocytes of mice immunized with pAMA1. Detectable but low amounts of IFN-γ were produced by splenocytes of mice immunized by pMIC2, pM2AP and pBAG1. We were also interested in finding out if IL-4, which plays a major role in controlling the development of cell-mediated immunity (Le Gros et al., 1990; Swain et al., 1990; Quelle et al.,1995; Vercammen et al , 2000) was produced by recombinant proteins (AMA1, MIC2, M2AP and BAG1) stimulated splenocytes from vaccinated mice. However, no significant amount of IL-4 was produced by the vaccinated groups. These findings are similar to those seen in *T. gondii* infection which shows that INF-γ is the central cytokine in resistance against *T. gondii* during both the early and late stages of infection (Suzuki et al., 1988). We therefore suspect that the significant protective effects induced by *AMA1* gene immunization may be attributed to the enhancement of INF-γ producing cells.

It is well known that several factors can influence the type of immune response obtained by DNA vaccination. It has been reported that plasmid DNA contains certain sequences that are found in bacterial, but not in mammalian DNA. These sequences comprise non-methylated CpG dinucleotides with surrounding characteristic motifs, which have immunostimulatory effects (Stevenson and Rosenberg, 2001; Klinman et al., 1996; Sato et al., 1996). It has been further reported

that both the level of protein expression and the differential cellular localization of the DNA-expressed antigen can influence not only the subclass of the antibody generated (Leyva et al., 2001) but also the strength of the immune response induced (Svanholm et al., 1999, Torres et al, 1999). We therefore suggest that the effectiveness of the *AMA1* gene in comparison with the *MIC2*, *M2AP*, or *BAG1* genes could be attributed to these factors.

We evaluated the protection induced by pAMA1, pMIC2, and pM2AP and pBAG1 DNA vaccination by orally infecting vaccinated mice (C57BL/6 and BALB/c) with 20 cysts of the avirulent Beverley strain. In BALB/c mice, 60% and 40% survival was observed in mice immunized with pAMA1 and pMIC2, respectively whereas pM2AP and pBAG1 groups demonstrated a 20% survival which was similar to that of the control. In C57BL/6 mice, pAMA1- and pMIC2-immunized mice, a 37.5% survival was recorded. A 12.5% survival was recorded in pBAG1-immunized mice whereas pM2AP immunized mice died at the same time as the control mice. From our results, a higher mortality was observed in C57BL/6 than in BALB/c mice and this could be related to the particular course of the disease in this strain. Previous reports on inflammatory mediator production indicate that T-cell derived cytokines may promote pathological changes during *T. gondii* infection of C57BL/6 mice and the major finding to mice succumbing to oral *T. gondii* infection is the inflammation of the ileum due to INF-γ produced by $CD4^+$ lymphocyte, predominantly of the α/β type (Liesenfeld et al., 1996; Vercammen et al., 2000). The high mortality in C57BL/6 mice could be due to an uncontrolled pathological Th1-type of response. Despite the difference in mortality, a partial but most significant degree of protection was obtained in the mice (C57BL/6 and BALB/c) vaccinated with pAMA1 followed by pMIC2 and pBAG1. No significant protection in the mice

immunized with pM2AP was observed. We were also interested to see the protection conferred by *BAG1* gene. From our results, some degree of protection was observed in the acute phase although the protective effect was not significant.

In conclusion, we have demonstrated that immunization with the AMA1 gene generates a more specific Th1 type of response and also offers a significant degree of protection in both strains of mice (BALB/c and C57BL/6) in comparison with *MIC2*, *M2AP* and *BAG1* genes.

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Figure Legends

Fig. 1. Expression of recombinant antigens in mammalian cells. Immunoblot analysis of 293T cells transfected with empty vector (A, B, C, D, lane 1, negative control), pMIC2 (A, lane 2), pM2AP (B, lane 2), pAMA1 (C, lane 2) or pBAG1 (D, lane 2) compared with *T. gondii* tachyzoite lysate (A, B, C, D, lane 3, positive control), probed with sera from mice immunized with recombinant GST-MIC2 (A), M2AP (B), AMA1 (C) or BAG1(D). The position of molecular mass standard is shown on the left side.

Fig. 2. Detection of specific anti-MIC2, M2AP, AMA1 and BAG1 IgG antibodies in sera of C57BL/6 mice immunized with pMIC2, pM2AP, pAMA1 and pBAG1 by Western blotting using *T. gondii* antigen. Reactivities of sera from mice immunized with empty plasmid (A), pMIC2 (B), pM2AP (C), pAMA1 (D) and pBAG1 (E) with their corresponding molecular masses at 115, 43/40, 67 and 30 kDa respectively. Results from one of the three similar experiments are shown.

Fig. 3. Determination of specific anti-MIC2, pM2AP, pAMA1 and pBAG1 total IgG and IgG subclass profiles in the sera of BALB/c (A) or C57BL/6 (B) mice. Sera were collected 1 week after the last immunization and tested by ELISA using corresponding recombinant proteins (MIC2, M2AP, AMA1 and BAG1). Control pcDNA sera were tested similarly with the above named recombinant proteins. Results are expressed as mean OD_{415} nm ± SD coming from single serum sample

determination and represent one of the three similar experiments.

Fig. 4. Protection of BALB/c (A) and C57BL/6 (B) mice, respectively against *Toxoplasma gondii*. The mice were immunized with pMIC2, pM2AP, pAMA1 and pBAG1. Mice immunized with the empty vector were included as a negative control. The mice were orally infected with 20 cysts of the avirulent *T. gondii* Beverley strain two weeks after the last immunization and observed daily for mortality. There were 5 – 8 mice in each group. Results from one of the three similar experiments are shown.

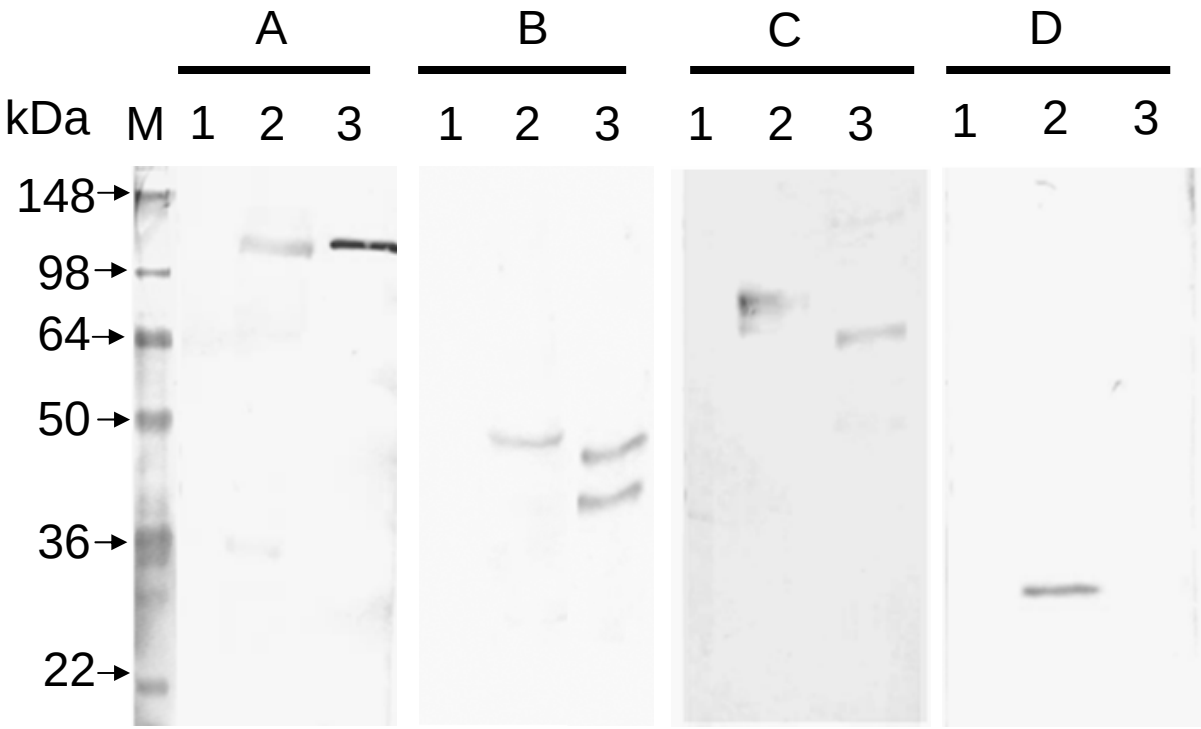


Figure 1

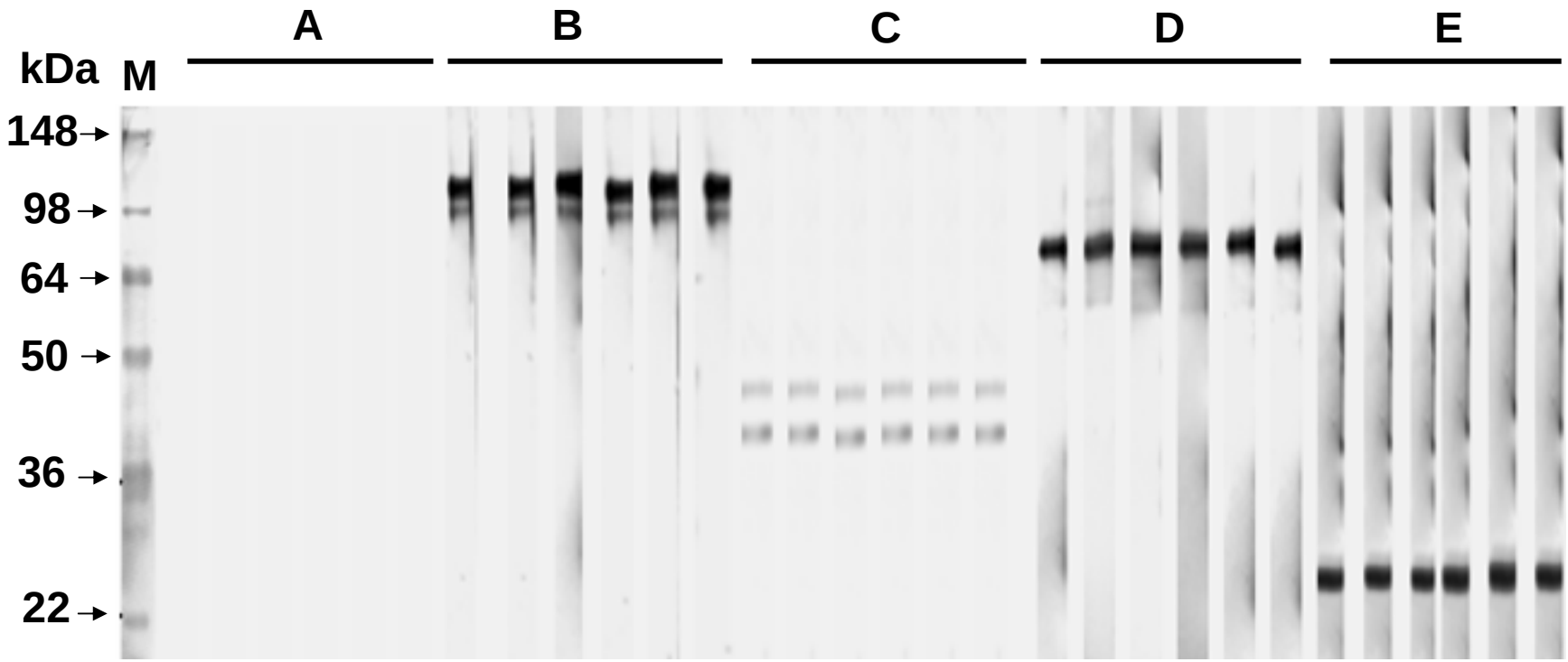


Figure 2

A

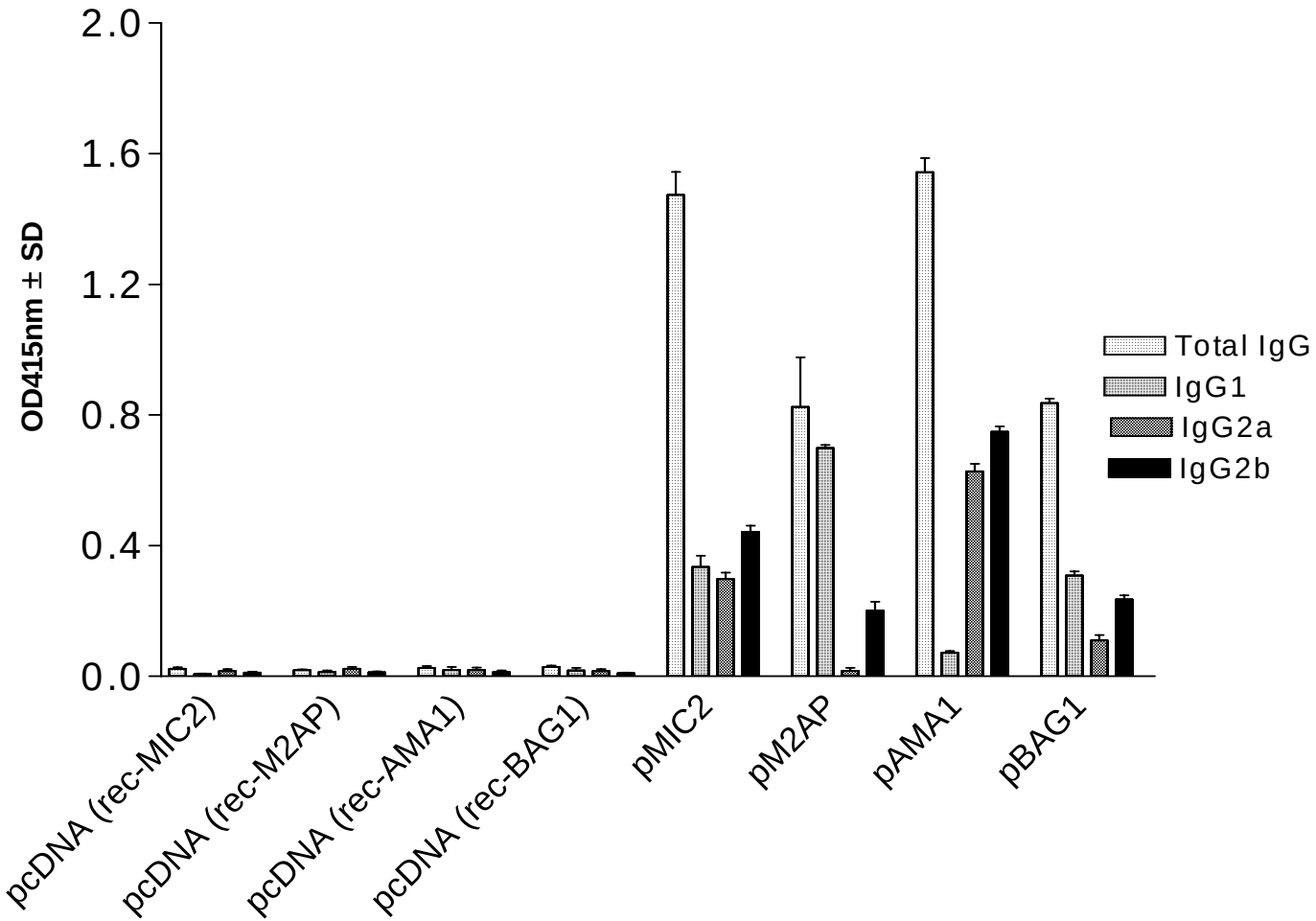

2.0
1.6
1.2
0.8
0.4
0.0
OD415nm ± SD
Total IgG
IgG1
IgG2a
IgG2b
pcDNA (rec-MIC2)
pcDNA (rec-M2AP)
pcDNA (rec-AMA1)
pcDNA (rec-BAG1)
pMIC2
pM2AP
pAMA1
pBAG1

Figure 3A

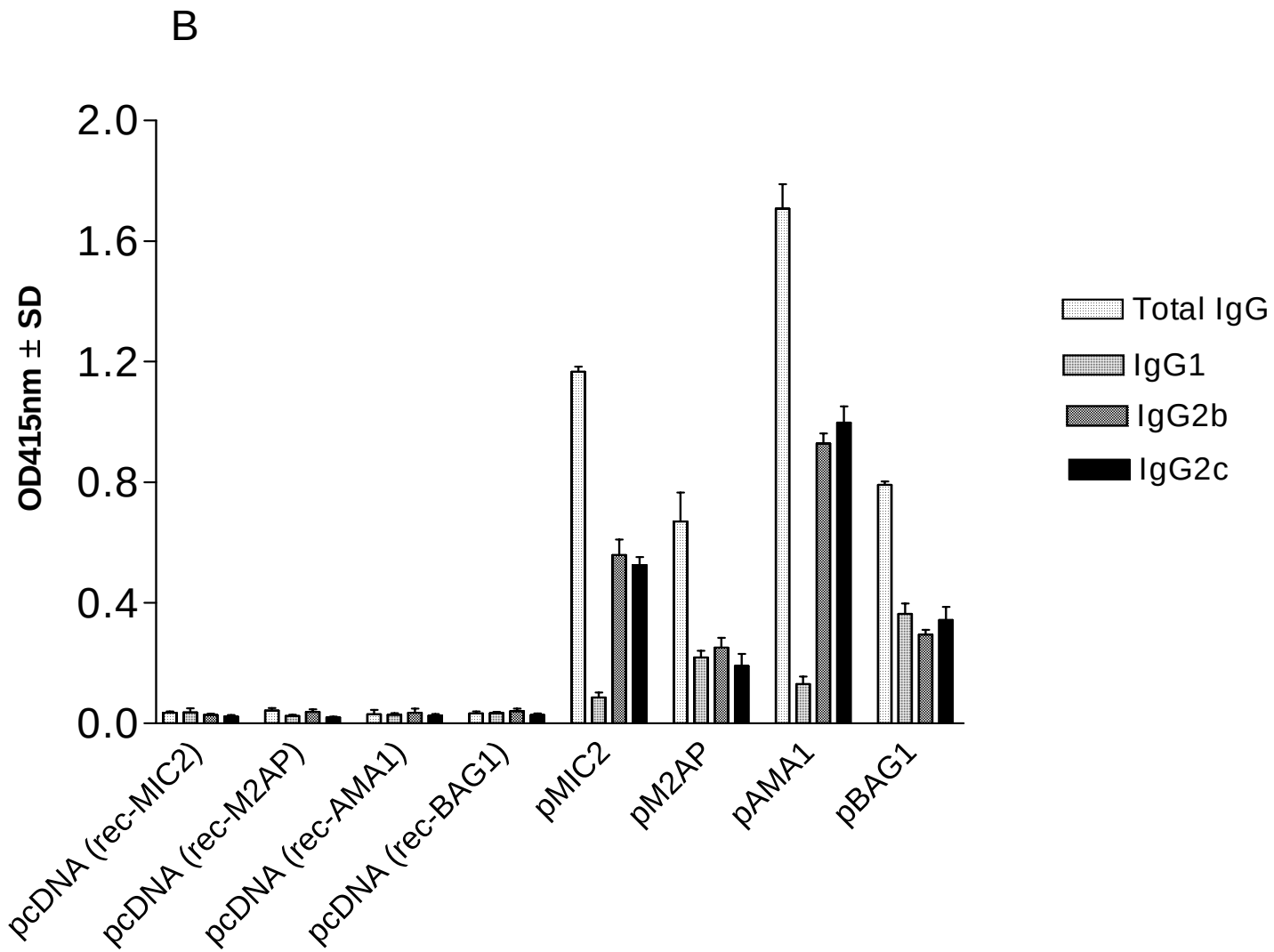

B
2.0
1.6
1.2
0.8
0.4
0.0
OD415nm ± SD
Total IgG
IgG1
IgG2b
IgG2c
pcDNA (rec-MIC2)
pcDNA (rec-M2AP)
pcDNA (rec-AMA1)
pcDNA (rec-BAG1)
pMIC2
pM2AP
pAMA1
pBAG1

Figure 3B

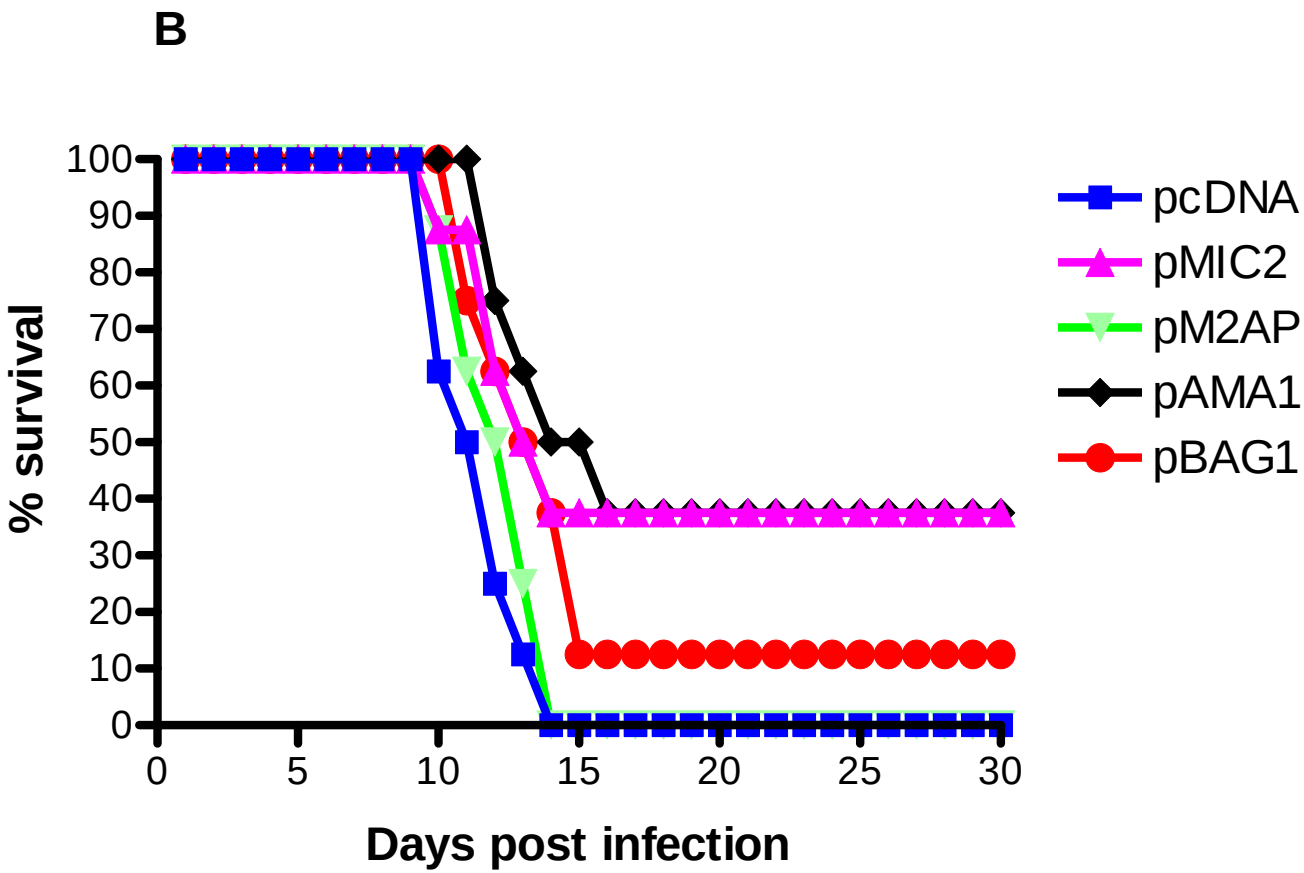
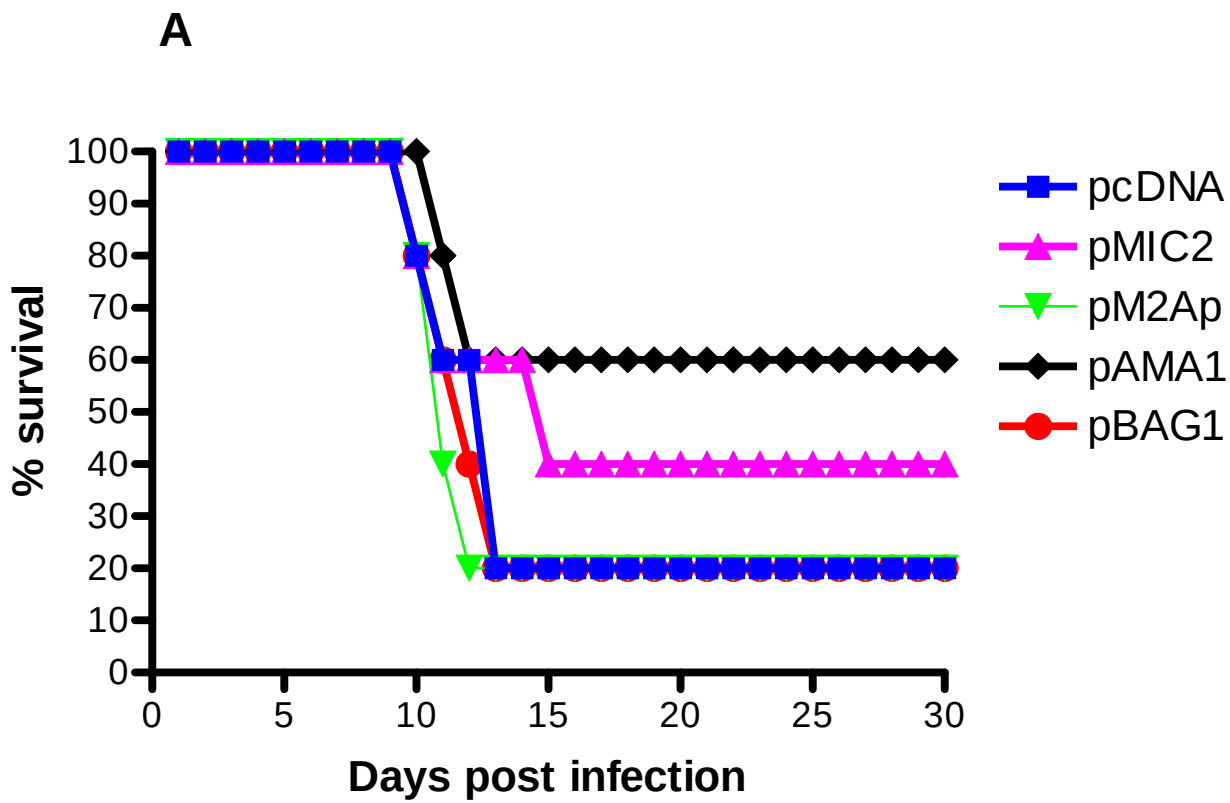

B
100
90
80
70
60
50
40
30
20
10
0
% survival
0
5
10
15
20
25
30
Days post infection
pcDNA
pMIC2
pM2AP
pAMA1
pBAG1

Figure 4B


A
100
90
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% survival
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5
10
15
20
25
30
Days post infection
pcDNA
pMIC2
pM2Ap
pAMA1
pBAG1

Figure 4A

TABLE 1A. Cytokine production by splenocytes of immunized BALB/c mice after stimulation with recombinant proteins

Immunization regime	Stimulus	Cytokine production (pg/ml) mean ± SD	
		IFN-γ	IL-4
Empty vector	recombinant-MIC2	12.70 ± 5.0	6.42 ± 3.6
Empty vector	recombinant-M2AP	9.97 ± 3.7	5.83 ± 1.7
Empty vector	recombinant-AMA1	10.50 ± 2.3	6.73 ± 2.0
Empty vector	recombinant-BAG1	10.96 ± 2.8	6.50 ± 2.3
pMIC2	medium	13.20 ± 0.5	< 5
pMIC2	recombinant-MIC2	55.89 ± 8.5	7.35 ± 4.0
pM2AP	medium	9.39 ± 1.5	< 5
pM2AP	recombinant-M2AP	23.55 ± 2.9	8.03 ± 3.1
pAMA1	medium	11.47 ± 0.7	< 5
pAMA1	recombinant-AMA1	146.3 ± 9.6	6.76 ± 1.5
pBAG1	medium	10.1 ± 1.1	< 5
pBAG1	recombinant-BAG1	24.92 ± 3.3	7.89 ± 2.4

[a]Cell-free supernatants were harvested and assayed for IL-4 activity at 24 hours, and IFN-γ activity at 96 hours.

TABLE 1B. Cytokine production by splenocytes of immunized C57BL/6 mice after stimulation with recombinant proteins

Immunization regime	Stimulus	Cytokine production (pg/ml) mean ± SD	
		IFN-γ	IL-4
Empty vector	recombinant-MIC2	12.30 ± 1.3	14.0 ± 1.6
Empty vector	recombinant-M2AP	11.64 ± 2.0	12.9 ± 1.7
Empty vector	recombinant-AMA1	12.06 ± 1.6	13.5 ± 1.3
Empty vector	recombinant-BAG1	11.52 ± 2.3	13.2 ± 2.1
pMIC2	medium	12.95 ± 2.0	< 5
pMIC2	recombinant-MIC2	35.00 ± 5.0	16.0 ± .05
pM2AP	medium	11.72 ± 1.1	< 5
pM2AP	recombinant-M2AP	29.00 ± .05	14.6 ± 1.2
pAMA1	medium	12.80 ± 1.0	< 5
pAMA1	recombinant-AMA1	126.0 ± 33	18.2 ± 5.0
pBAG1	medium	11.25 ± 0.8	< 5
pBAG1	recombinant-BAG1	35.00 ± 10	15.0 ± 1.3

[a]Cell-free supernatants were harversted and assayed for IL-4 activity at 24 hours and IFN-γ activity at 96 hours.