Construction and immunogenicity of pseudotype baculovirus expressing *Toxoplasma gondii* SAG1 protein in BALB/c mice model

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** Summary**

*Toxoplasma gondii* is a protozoan parasite causing toxoplasmosis to almost one-third of population all over the world. One of the most efficient ways to control this disease is immunization. However, so far, there is no effective vaccine available against this pathogen. Recently, a baculovirus pseudotype with vesicular stomatitis virus G protein (Bac-VSV–G) was found to efficiently transduce and express transgenes on mammalian cells, so it was considered as an excellent expressing vector. In this study, the value of Bac-VSV–G in delivering *T. gondii* antigen was investigated. *T. gondii* SAG1 gene was cloned into Bac-VSV–G, and recombinant baculovirus BV-G-SAG1 was obtained. Indirect immunofluorescence test showed BV-G-SAG1 was efficiently transduced and expressed in pig kidney cells. Then BALB/c mice were immunized with BV-G-SAG1 at different doses (1 × 10⁸, 1 × 10⁹, and 1 × 10¹⁰ PFU/mouse) and challenged with *T. gondii* RH strain tachyzoites after immunization. The levels of specific *T. gondii* antibody, interferon-γ (IFN-γ), IL-4, IL-10 expression and release, and the survival rate of treated mice were evaluated. Compared with the mice immunized with DNA vaccine (pcDNA/SAG1) encoding the same gene, BV-G-SAG1 induced higher levels of specific *T. gondii* antibody and (IFN-γ) expression with dose-dependent manner and the survival rate of mice with BV-G-SAG1 was significantly improved. These results indicated that pseudotype baculovirus-mediated gene delivery can be utilized as an alternative strategy to develop new generation of vaccines against *T. gondii* infection.

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1. Introduction

*Toxoplasma gondii* is an obligate intracellular protozoan parasite. As a significant human and animal pathogen, it was estimated to infect about one-third of the world’s human population and almost all warm-blooded mammals which was frequently associated with congenital infection and abortion [1,2]. In addition, *T. gondii* is also an opportunistic pathogen. In the immunocompromised individuals, especially one with HIV/AIDS, *T. gondii* is responsible for the development of a variety of clinical syndromes, the most frequent of which is toxoplastic encephalitis (TE) [3,4]. Epidemiologically, *T. gondii* is an important food-borne parasite, humans were usually infected through parasite contaminated meat, especially from pigs and lambs, and oocysts of *T. gondii* are shed by cats into the environment [1,5]. Thus, development of effective and safe ways for controlling *T. gondii* infection in these animals which play important roles in disease transmission is crucial to human health, and vaccine is one of those ways [6]. Till now, there have been several types of vaccines against toxoplasmosis developed and tested for their immunological effects in animal models, but few of them have been licensed for use, principally due to biosafety concerns or poor efficacy [6–10]. Therefore, developing a cheap and effective vaccine against *T. gondii* in animals is an important goal for scientists worldwide.

Under the control of the mammalian promoters (CMV), the baculovirus with vesicular stomatitis virus G protein (Bac-VSV–G) has been used as a novel vector to transfer and express foreign genes in mammalian cells for vaccine development [11–13]. Those not only owing to striking features of baculovirus, including: (i) cytopathic effects are not produced in mammalian cells; (ii) recombinant baculoviruses are easily constructed and can produce high titers [10¹⁰ plaque-forming units (PFU)/ml]; (iii) the DNA insert capacity is very large (˃30 kb) [14–16], but also because Bac-VSV–G-CMV can
extend the host range and increase the transduction efficiency in mammalian cells [17]. Direct vaccination with recombinant pseudotype baculovirus inducing high-level humoral and cell-mediated immunity against various antigens have been demonstrated in many pathogens, such as influenza virus HA [18], porcine reproductive and respiratory syndrome virus (PRRSV) [19], Japanese encephalitis virus (JEV) [20], porcine circovirus type 2 (PCV2) [21], and Plasmodium falciparum [22].

In *T. gondii*, the major immunodominant surface antigen (TgSAG1) has been proved to be an ideal candidate vaccine for this parasite. Various forms of TgSAG1 including purified natural TgSAG1, recombinant TgSAG1, TgSAG1-derived peptides [23–26], nucleic acid vaccines encoding TgSAG1 [24], as well as pseudovirus expressing TgSAG1 [5] had been investigated for their immunogenicity. Although most of those vaccines can induce significant humoral and cellular immune responses in animal models against lethal challenge with *T. gondii*, only few of them have been licensed for use [6,7]. Therefore, the aim of this work was to assess the immunogenic properties and protective value of a recombinant pseudotype baculovirus encoding *T. gondii* SAG1 protein against virulent challenge with *T. gondii* RH strain in a BALB/c mouse model.

2. Materials and methods

2.1. Parasites and cell

Tachyzoites of the highly virulent *T. gondii* (RH) strain (obtained from National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China) were harvested from the peritoneal fluid of BALB/c mice after infected 5–6 days by injecting 1 ml of 0.1 M phosphate buffered saline (PBS, pH 7.2) as described [27]. The exudate was separated by low speed centrifugation (100 × g for 5 min) at 4 °C to remove the cellular debris. The parasites in the supernatant were precipitated by centrifugation at 600 × g for 10 min and then washed in 0.1 M PBS, pH 7.2, counted in a hemocytometer and adjusted to 5 × 10^5 parasite/ml.

Pig kidney cells (PK-15) were grown and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 μg/ml penicillin.

2.2. Construction of recombinant baculoviruses

To construct recombinant baculovirus, several vectors and baculovirus were employed in this study, including BV-G, BV-G-EGFP and BV-G-CMV. BV-G is a baculovirus transfer vector in which the VSV–G gene is under the control of the polyhedrin promoter of pFastBac1 (Invitrogen) [19]; BV-G-EGFP is a pseudotype baculovirus expressing EGFP and is driven by the CMV-IE promoter. BV-G-CMV is another pseudotype baculovirus without heterologous genes downstream from the CMV-IE promoter. To generate recombinant baculovirus BV-G-SAG1, the DNA fragment containing the *T. gondii* SAG1 expression cassette [CMV-SAG1-BGH poly(A)] was released from pcDNA–SAG1 plasmid (constructed by National Key Laboratory of Agricultural Microbiology and the SAG1 sequence was the same with GenBank accession number AV217784) and inserted into BV-G, resulting in the recombinant transfer plasmid BV-G-SAG1 (Fig. 1). The recombinant baculovirus was subsequently generated using the Bac-to-Bac System (Invitrogen) following the manufacturer’s instructions. Recombinant baculovirus was further amplified by propagation in Sf-9 cells. Virus purification was performed as described previously [28] and purified virus was resuspended in PBS (pH 7.2). The virus titer was determined by the BacPAK Rapid Titer assay (Clontech, Palo Alto, CA, USA) in Sf-9 cells.

2.3. Baculovirus transduction and indirect immunofluorescence assay (IFA)

Baculovirus transduction was performed as described previously [21]. At 36 h post-transduction, cells were processed for indirect immunofluorescence assay (IFA) with a rabbit anti-*T. gondii* polyclonal antibody (obtained from National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China). Fluorescent images were examined under a Nikon microscope.

2.4. Animal experiments

72 five to six weeks old female BALB/c mice were divided randomly into six groups (twelve mice per group). Each of three groups of mice was injected intramuscularly with 100 μl of PBS containing 1 × 10^8, 1 × 10^7, or 1 × 10^6 PFU of BV-G-SAG1, respectively. Each of other three groups was injected intramuscularly with 100 μl of PBS containing 1 × 10^8 PFU of BV-G-EGFP, 100 μl of PBS containing 100 μg of pcDNA–SAG1 or 100 μl of PBS on day 0 and on day 21, respectively. Blood samples were collected for serological tests on day 0, day 21 and day 42. On day 42, the splenocytes of four mice per group were isolated for IFN-γ, IL-10 and IL-4 analysis.

2.5. Serological tests

*T. gondii*-specific humoral immune response was evaluated by ELISA for determination of the IgG titer against *T. gondii* lysate antigen (TLA) as previously described [5]. All sera were used in twofold dilutions starting at a dilution of 1/40. Endpoint titers were defined as the dilution where the optical density (OD_{450}) exceeded the cut-off value. The cut-off value was calculated from preimmune sera (day 0) at a 1/40 dilution, with cut-off value = mean OD_{450} + 3 × S.D. OD_{450}.

2.6. Lymphocyte proliferation assay

Splenocyte suspensions were collected from immunized mice at 42nd day after immunization, and lymphocyte proliferation assay was tested as previously described [5]. In brief, 2 × 10^6 cells/well was cultured in 96-well plates in triplicate in RPMI-1640 medium. The culture was stimulated with 10 μg/ml TLA, respectively, for 48 h at 37 °C in 5% CO_2, then 10 ml of 5 mg/ml methyl thiazolyl tetrazolium (MTT, Sigma) was added to each well, and incubated for 4 h. Thereafter, the contents of all wells were discarded, and 100 ml dimethylsulfoxide (DMSO) was added to every well until the
3. Results

3.1. Construction and identification of recombinant baculovirus BV-G-SAG1

The recombinant baculovirus BV-G-SAG1 was constructed following the manufacturer’s instructions of the Bac-to-Bac System (Invitrogen). The correct structure of recombinant baculovirus BV-G-SAG1 was confirmed by restriction digestion, PCR and sequence analysis (data not shown).

3.2. Expression of recombinant baculovirus BV-G-SAG1 in PK-15 cells

To investigate whether the pseudotype baculovirus BV-G-SAG1 can express the SAG1 protein in mammalian cells, PK-15 cells were transduced with BV-G-SAG1, and subjected to IFA at 36 h post-transduction. As shown in Fig. 2A, the expressed SAG1 proteins (with bright fluorescence) could be detected by T. gondii-specific antibody in BV-G-SAG1-transduced cells, but not in cells transduced with BV-G-CMV (Fig. 2B). Strong antibody responses in immunized mice

To investigate the immunogenicity of BV-G-SAG1 in vivo, BALB/c mice were immunized i.m. with various dosages (1 × 10^8, 1 × 10^9, and 1 × 10^10 PFU/mouse) of BV-G-SAG1, 1 × 10^10 PFU/mouse of BV-G-EGFP, 100 μg pcDNA–SAG1 or PBS, respectively. Three weeks after primary immunization, all the mice immunized with BV-G-SAG1 or pcDNA–SAG1 developed detectable levels of IgG antibody, except for the group immunized with the BV-G-EGFP or PBS (Fig. 3). Following booster immunization, the mean titers of T. gondii antibodies increased greatly in mice immunized with BV-G-SAG1 and pcDNA–SAG1, and were significantly higher than the groups vaccinated with BV-G-EGFP or PBS (P<0.01).

2.8. Mouse protection test (MPT)

42 days post-immunization, eight mice were selected randomly from each group and injected intraperitoneally with 1 × 10^3 tachyzoites of T. gondii (RH strain). Tachyzoites were prepared as described in Section 2.1, counted in a hemocytometer and adjusted to 5 × 10^3 parasite/ml with 0.1 M PBS (pH 7.2). Then the symptom was observed and the survival time was recorded. Survival was monitored daily for 18 days after challenge.

2.9. Statistical analysis

For evaluating the protective immune responses among the different groups, all groups’ mean values were compared by analysis of variance (ANOVA) and Student’s t-test using SPSS v.12.0 software. P-values of <0.05 were considered statistically significant [5].

3.4. Lymphocyte proliferation assay

The splenocytes from mice immunized with BV-G-SAG1, BV-G-EGFP, pcDNA–SAG1 or PBS were prepared to assess the proliferative immune responses to T. gondii lysate antigen (TLA). The splenocytes from mice immunized with BV-G-SAG1 showed a slightly stronger proliferative response to TSA (P<0.05), compared with that of mice immunized with PBS without any virus or BV-G-EGFP, (P<0.05) (Fig. 4). Mice immunized with 10^10 PFU of BV-G-SAG1 produced significantly stronger splenocyte proliferative response than mice received 10^9 or 10^8 PFU of BV-G-SAG1 (P<0.05), exhibiting evident dose-dependent pattern. Mice immunized with 10^8 PFU of BV-G-SAG1 produced splenocyte proliferative response was almost the same with pcDNA–SAG1-group.

Throughout this experiment, mice immunized with 10^10 PFU of BV-G-SAG1 produced significantly higher antibody titers than mice received 10^8 PFU of BV-G-SAG1 (P<0.05), exhibiting evident dose-dependent pattern, whereas mice immunized with pcDNA–SAG1 produced almost the same antibody titers with 10^8 PFU of BV-G-SAG1.
3.5. IFN-γ, IL-10 and IL-4 expression assay

The cell-mediated immunity produced in the immunized mice was indirectly evaluated by measuring the amount of cytokines released in the supernatants from the cultures of TLA-stimulated spleen cells. As shown in Fig. 5, mean IFN-γ production of 683.55, 400.15, and 200.30 pg/ml was detected in mice inoculated with $1 \times 10^{10}$, $1 \times 10^{9}$, and $1 \times 10^{8}$ PFU/mouse of BV-G-SAG1, respectively, revealing significantly higher level of IFN-γ in mice immunized with $1 \times 10^{10}$ and $1 \times 10^{9}$ PFU/mouse of BV-G-SAG1 than in those received pcDNA–SAG1 (200.05 pg/ml) or $1 \times 10^{8}$ PFU/mouse of BV-G-SAG1 (200.30 pg/ml). As expected, no significant production of IFN-γ was detected in PBS-inoculated mice (28.01 pg/ml). Interestingly, splenocytes harvested from baculovirus-injected BV-G-EGFP (125.25 pg/ml) mouses produced higher background of non-specific IFN-γ responses (Fig. 5), which was demonstrated from the results that significant production of IFN-γ was detected in BV-G-SAG1, pcDNA–SAG1 or BV-G-EGFP-inoculated mice, while not in PBS-inoculated mice, after in vitro restimulation with T. gondii SAG1 protein. In contrast, there were no significant difference in IL-4 and IL-10 between these groups ($P > 0.05$). This profile of cytokine secretion suggests that the cellular immune response induced by the heterologous prime-boost strategy was skewed to the Th1-type immunity.

3.6. Lethal challenge test in BALB/c mice

Three weeks after the last immunization, the mice were intraperitoneally infected with $10^9$ tachyzoites of the highly virulent T. gondii RH strain. As shown in Fig. 6, the survival time of the mice inoculated with $10^{10}$ PFU of BV-G-SAG1 (17.12 ± 1.12 days), $10^9$ PFU of BV-G-SAG1 (11.06 ± 1.08 days), $10^8$ PFU of BV-G-SAG1 (9.06 ± 0.49 days) and pcDNA–SAG1 (8.87 ± 0.69 days) were significantly longer than the BV-G-EGFP (3.68 ± 0.37 days) and PBS (3.81 ± 0.25 days) control groups ($P < 0.05$). Moreover, significantly stronger protection was obtained when mice were immunized with $10^{10}$ PFU of BV-G-SAG1, with a marked increase in survival time (17.12 ± 1.12 days, $P < 0.05$), and 50% survival rate were achieved after a lethal challenge whereas all mice in the control groups died within 4 days after challenge.

4. Discussion

In this study, a recombinant pseudotype baculovirus virus expressing TgSAG1 named as BV-G-SAG1 was developed. The recombinant BV-G-SAG1 induced a strong Th1-type cell-mediated immune response and provided higher protection against lethal challenge with T. gondii RH strain in a mouse model. Both humoral and cellular immune responses are important to evoke protective immunity against T. gondii infection [29]. Our data have clearly demonstrated that direct vaccination with BV-G-SAG1 could develop T. gondii-specific humoral immune responses. Furthermore, Th1 cytokines IFN-γ and Th2 cytokines IL-4 and IL-10 detection results showed: BV-G-SAG1 induce high-level expression of IFN-γ, but there were no significant difference in the levels of IL-4 and IL-10 expression between these groups ($P > 0.05$). These findings indicated that like pcDNA–SAG1 and rPRV/SAG1, BV-G-SAG1 induced an enhanced Th1-type response.

As a vector for protein overproduction in insect cells, although the baculovirus carrying an appropriate mammalian cell-active promoter has exhibited high transduction efficiency and high-level expression of heterologous proteins in mammalian cells in vitro [12], its gene transduction in vivo is easy to be inactivated by serum complement [30]. This property limits its application for gene therapy and vaccine. Recent studies have demonstrated that vesicular stomatitis virus envelope G protein VSV–G-modified baculovirus exhibits greater resistance to inactivation by animal sera complement and can enhance gene transfer efficiencies in mouse skeletal muscle [12,22,30,31], which suggested this new baculovirus has potential use for gene therapy and vaccine. Based on those studies, the pseudotype baculovirus virus pFastBac-VSV–G-modified baculovirus exhibits greater resistance to inactivation by animal sera complement and can enhance gene transfer efficiencies in mouse skeletal muscle not only significantly extend its in vivo gene transfer efficiency, but also create some new possibilities for using baculovirus as a potential vaccine vector.

Compare with DNA vaccine pcDNA–SAG1, BV-G-SAG1 that encode the same antigen exhibited better immunogenicity, as demonstrated by significantly higher ELISA antibodies, SI, IFN-γ production and survival rate. There are two possible explanations for the enhanced immunogenicity of recombinant pseudotype baculovirus: (i) higher transduction efficiency of pseudotype baculovirus may result in greater antigen expression, an increased chance of being taken up by antigen-presenting cells (APCs), and subsequently a higher efficacy in the induction of immune responses. For example, pseudotype baculovirus can enter mouse skeletal muscle cells with higher efficiency [17,28], in contrast,
only 1–2% of muscle cells can capture plasmid DNA after direct intramuscular DNA vaccination. (ii) More efficient antigen presentation results in more efficient immune responses.

The reason why significantly high IFN-γ production in mice immunized with a dose of $1 \times 10^6$ and $1 \times 10^7$ PFU of BV-G-SAG1 were observed compared with mice which received pcDNA–SAG1 could be explained as follows. Apart from the factors discussed above which could enhance humoral immune responses, the “adjuvant” effect of baculovirus should be considered as another important reason. Baculovirus has the ability to induce innate immune responses through the Toll-like receptor 9 (TLR9)/MyD88-dependent signaling pathway, resulting in the production of various cytokines, including tumor necrosis factor-alpha, interleukin-6, and interferon.[18,28,32,33]. In this study, a higher production in mice indicated the “adjuvant” effect of baculovirus further. The similar result has been observed in pseudotype baculovirus expressing PRRSV’s GPs and M protein [19], JEV’s E protein [20] and PCV2’s ORF2 protein [21].

In conclusion, a T. gondii recombinant baculovirus vaccine BV-G-SAG1 was successfully constructed. Compared to control groups, BV-G-SAG1 was able to elicit a significant humoral and cellular immune response and extend survival time in mice challenged with the lethal RH tachyzoites. The results demonstrated that recombinant baculovirus vaccine BV-G-SAG1 provide favourable efficacy together with the unique advantages of the pseudotype baculovirus, especially the relatively easy manipulation, lack of toxicity, and lack of pre-existing antibody against baculovirus in the host. It suggests that gene delivery mediated by the pseudotype baculovirus could be utilized as an alternative strategy in the development of a new generation of vaccines against T. gondii.

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