Vaccine (2008) **26**, 1832–1845

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The protective efficacy aganist *Schistosoma japonicum* infection by immunization with DNA vaccine and levamisole as adjuvant in mice

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Received 7 June 2007; received in revised form 16 January 2008; accepted 30 January 2008
Available online 22 February 2008

KEYWORDS
Immune response;
Schistosoma japonicum;
DNA vaccine;
Levamisole;
Gramulomas formation

Summary Levamisole (LMS) as an adjuvant enhances cell-mediated immunity in DNA vaccination; we investigated the efficacy and liver immunopathology alleviation of a DNA vaccine, VR1012-SjGST-32, in a LMS formulation in the murine challenge model. Compared to controls, the VR1012-SjGST-32 plus LMS can reduce worm and egg burdens, as well as, immunopathological complications associated chronic inflammation significantly in liver, which were apparently associated with Th1-type response. Together, these results suggest that the LMS as a potential Schistosome DNA vaccine adjuvant can enhance both worm killing and disease prevention, which is possibly mediated through the induction of a strong Th1-dominant environment in immunized mice.

Abbreviations: Sj, *Schistosoma japonicum*; SjGST-32, SjGST26 fused with 32 kDa asparaginyl proteinase; CFSE, carboxyfluorescein diacetate succinimidyl ester; TMB, tetramethyl benzidine.

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doi:10.1016/j.vaccine.2008.01.056

Introduction

Schistosomiasis is a chronic parasitic disease that affects more than 200 million people in 74 countries worldwide, mostly in developing countries, causing approximately 20,000 deaths per year [1]. Schistosomes are also important pathogens for several domestic animal species and cause economic losses in endemic areas. The disease is associated with daily production of eggs by adult worms. The eggs that fail to escape the body are deposited into the liver, intestine, and genitourinary tract, where they stimulate a strong inflammatory reaction and granuloma formation that eventually leads to death [2]. Currently, Schistosomiasis control strategy is mainly based on the treatment of infected individuals by chemotherapy. However, drug treatment does not prevent individuals from reinfection that is constantly observed in individuals living in endemic area. Moreover, the development of parasite resistance to the drugs being used in mass chemotherapy has already been reported [3—5]. Thus the development of a vaccine is apparently the only practical measure for disease control. The use of irradiated cercariae for vaccination is the best animal model described hitherto, leading up to 90% or more protection against challenge infection [6]. However, culturing of the parasitic pathogen in large amounts for the purpose of vaccine preparation is completely impractical. Hence, the identification of relevant immunogens and their preparation by synthetic or recombinant DNA technologies are imperative for the development of an anti-Schistosome vaccine [7]. Several studies are in progress testing different anti-Schistosomiasis vaccine candidates and different vaccination protocols [8—10]. Many researchers agree that a vaccine aimed at reducing worm burden and/or egg production would be the most effective and cost-efficient way to control Schistosomiasis. It has been determined that a vaccine resulting in at least 40% reduction in worm burden would significantly reduce morbidity and transmission rates [3,11]. However, DNA vaccination alone is limited in that it often generates only weak immune response, particularly the cellular response. Various approaches have been developed recently in order to improve its vaccine potency, particularly with the respect to choice of a better adjuvant. Adjuvants are believed to function as a depot for prolonged antigen release, as a non-specific stimulus for the immune system, or both. Recently, it has also been realized that different adjuvants as ligands can preferentially directly interact different type of toll-like receptors (TLR) on antigen presenting cells that monitor any pathogenic invasion and in turn to active the host's immune responses [11—13].

Levamisole (LMS), a synthetic and soluble phenylimidazolthiazole, has been widely used as a pesticide for domestic animals over 30 years and also used in several human clinical trials as an anti-cancer drug [14]. Our laboratory has recently reported that LMS injected with a DNA vaccine against the foot mouth disease virus (FMDV) stimulated cellular immune responses in conjunction with a strong production of IFN-γ [13]. Furthermore, we have also demonstrated that LMS has been used effectively for the killed viral vaccines [11]. Early studies with experimental animal models established that vaccination with SjGST-32 which is a fusion protein of SjGST and Sj asparaginyl endopeptidase induced partial protection against cercarial challenge [9,15].

Our results demonstrate that LMS has a potent adjuvant activity to enhance the protective immunity induced by VR1012-SjGST-32 and leads to the reduction of liver pathology as a result of the induction of a Th1 response.

Materials and methods

Mice and parasites

Female C57BL/6 mice (H-2^b), 6—8 weeks of age, were purchased from The Animal Institute of the Chinese Medical Academy (Beijing, China), housed under a 12-h light cycle, and fed with pathogen-free food and water. The life cycle of a Chinese strain of *Schistosoma japonicum* was maintained in a laboratory of the Hunan Institute of Schistosomiasis Control. The parasites were originally collected in *Oncomelania hupensis* snails from Guichi County, Hunan Province, China. Cercariae of *S. japonicum* was maintained routinely in *O. hupensis* snails in the same laboratory and prepared by exposing infected snails to light for 1 h to induce shedding. Cercarial numbers and viability were examined under a dissective microscope prior to infection.

Fluorescent dye and antibodies

Fluorescent-labeled anti-mouse monoclonal antibodies including, anti-IL4-PE, anti-IFN-γ-FITC, anti-CD4-FITC, anti-CD4-PE, anti-IL10-PE and isotype controls were purchased from eBiosciences (CA, USA).

Parasite antigens and DNA vaccines

The VR1012-SjGST-32 gene consists of fused SjGST and Sj-32 genes was described previously [15]. Briefly, the SjGST coding sequence was obtained by PCR amplification using plasmid pMD-18-T-SjGST as the template and the primers containing PstI and BamHI restriction sites. The PCR product harboring the SjGST gene was cloned into the PstI and BamHI sites of 4.9 kb VR1012 plasmid (Vical Inc., San Diego, CA) downstream from the human cytomegalovirus promoter and designated as the VR1012-SjGST. Using the BamHI and EcoRI digested Sj32 gene from the pET28a plasmid, was then subcloned into VR1012-SjGST at same sites to yield the VR1012-SjGST-32, where the SjGST and Sj32 genes formed one open reading frame. Constructs and its empty vector were extracted by alkaline lysis, purified by PEG8000 precipitation and diluted in saline solution. The open reading frame of the fused gene was subcloned into the pGEX-3X-1 (Invitrogen Inc., USA), expressed in *Escherichia coli* (BL21 (DE3) pLysS) and purified by glutathione—sepharose chromatography.

Immunization schedule

Mice were randomly divided into five groups (nine per group), and immunized intramuscularly on days 0, 14, and 28 with either VR1012-SjGST-32 alone or formulated in 1% LMS as an adjuvant, empty VR1012 vector was used as a

control. The mice were bled before and 2 weeks after each immunization.

Challenge infection

Two weeks after the final boost, mice were challenged percutaneously with 30 ± 1 of cercariae for 20 min by a wet glass lid method as documented previously [16]. On day 42 following the challenge, adult worms were perfused from hepatic portal system and also manually removed from mesenteric veins after mice were sacrificed. The recovered adult worms from each mouse were individually identified as male, female and total worm burdens/animal. The livers and spleens were collected and weighed. Eggs of parasites were recovered from each mouse liver and counted as described previously [16,17]. The formula to calculate worm and egg reduction rates were as follows. Worm reduction rate (%) = [(average number of recovered worms of control group − average number of recovered worms of experimental group)/average number of recovered worms of control group] × 100. Egg reduction rate (%) = [(average number of eggs/g liver tissue in control group − average number of eggs/g liver tissue in experimental group)/average number of eggs/g liver tissue of control group] × 100.

Analysis of specific antibody responses by ELISA

Collected serum samples were diluted and used to determine total IgG and IgG isotype responses by ELISA. Ninety-six microtiter plates were coated overnight at 4 °C with recombinant SjGST-32 antigen at 10 μg/100 μl per well. Wells were subsequently blocked with 3% of BSA-PBST for 2 h at 37 °C and serial dilutions of sera sample were added. As secondary antibodies, 100 μl of horseradish peroxidase labeled goat anti-mouse IgG, IgG1, IgG2a, IgG2b (Sigma, St. Louis) were added and incubated at 37 °C for 1 h. The dilutions for these antibodies were at 1:3000 for IgG, IgG1, IgG2a, and IgG2b. After five washes with PBST, 10 mg of TMB tablet (Sigma, St. Louis) were dissolved in 0.025 M phosphate—citrate buffer and 50 μl of the resulting solution were added to each well. The reaction was stopped by addition of 0.2 M of H_2SO_4 and the resulting OD at 450 nm/620 nm was analyzed with a plate reader (Magellan, Tecan Austria GmbH). The antibody titers of total IgG, IgG1, IgG2a and IgG2b isotypes were defined as the highest dilution that gave an above 2:1 ratio between testing serum and the naive negative control.

T-cell proliferation assays-CSFE staining

Mice were sacrificed on day 7 after the third immunization, single suspension lymphocytes isolated from immunized mice were labeled with CFSE (Molecular Probes, OR) as described previously [18]. To evaluate their proliferation activities, T cells were cultured in 96-well microtiter plates with a triplicate at 2×10^5 cells per well in RPMI-1640 medium with 10% of FBS and stimulated 10 μg/ml rSjGST-32 as a specific antigen. 5 μg/ml of Con A as a positive control, The CFSE labeled cells were incubated at 37 °C with 5% CO_2 for 48 h and collected for the flow cytometry analysis on a FACSCalibur analyzer (BD Biosciences, USA).

Flow cytometry and intracellular cytokine staining

T cells were isolated on day 7 after the third immunization as described previously [19]. Briefly, T cells at 0.5×10^6 cells per 20 μl were stimulated in 96-well plates with recombinant antigens (10 μg/ml) and anti-CD28 (5 μg/ml) mAb for 6 h at 37 °C and 5% CO_2. Monensin (2 μg/ml) was added for the last 4 h and the cells were washed three times with PBS/10% FCS. Cells were blocked with 1 μl of Fcγ mAb (0.5 μg/ml) for 30 min at 4 °C and fixed with 4% of paraformaldehyde at 4 °C for 15 min. The cells were permeablized with 0.1% saponin at 4 °C for 10 min, immunostained with isotype controls, or double stained with anti-CD4-FITC and anti-IL4-PE, or anti-CD10-PE and anti-IFN-γ-FITC for 30 min at 4 °C. The cells were analyzed using FACSCalibur and Cellquest Pro software (BD Biosciences, USA).

Histopathological examination for granulomas and fibrosis in liver

After 6 weeks of the challenge, animals were sacrificed and their livers were removed. The extent of pathological changes in the liver was assessed macroscopically on the day of sacrifice. The granulomas and fibrosis in the liver were examined microscopically. Briefly, the ventral median lobe of the liver was fixed in 4% phosphate-buffered paraformaldehyde, pH 7.4, dehydrated and embedded. Serial sections were prepared and stained with haematoxylin—eosin (H&E) to reveal granuloma and with Martius Scarlet Blue to exhibit fibrosis in the liver [20]. The sizes of non-confluent granulomas formed around a single egg containing a mature miracidium were analyzed and recorded by a computer-assisted image analysis device (Leitz, Dresden, Germany). All granulomas were measured in two liver sections selected to be sufficiently distant from each other to ensure that a granuloma was not measured twice.

Immunohistochemical analysis

Liver samples were obtained from challenged animals and fixed by a solution containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2 at room temperature for 48 h. The serial tissue sections at 5-μm thick were obtained after embedded in paraffin. Antigen retrieval was performed by autoclaving the sections (10 min at 120 °C) in 0.1 M sodium citrate buffer (pH 6.0). Subsequently, sections were incubated in 10% normal goat serum in PBS for 30 min to block non-specific binding sites before reacted with the rabbit anti-mouse against iNOS, Bcl-2 and Bax antibodies (Victoria, BC, Canada) at 1:50 dilutions in PBS for 2 h. The slides were further incubated with sheep anti-rabbit IgG conjugated with avidin (Burlingame, CA, USA) for 1 h and followed by a incubation of the biotinylated peroxidase (Victoria, BC, Canada) for an additional 1 h. Staining was visualized by the addition of 3,3-diaminobenzidin (DAB, Sigma, St. Louis, MO, USA) for 15 min and counterstained with haematoxylin mounted with neutral balsam. The numbers of positive cells were semi-quantitatively analyzed under a light microscope at 40× magnifications. The total visual areas (TVA) were traced ran-

Table 1 Primer sequences and conditions for RT-PCR for target genes

Target genes	Primers	Parameters of PCR reactions	Production size (bp)
HPRT	5′-GTTGGATACAGGCCAGACTTTGTTG, 3′-GAGGGTAGGCTGGCCTATGGCT	94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s	352
IFN-γ	5′-CATTGAAAGCCTAGAAAGTCTG, 3′-TGACTCCTTTTCCGCTTCCTGAG	94 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s	320
TNF-α	5′-TGTCTGATGCAGCAGGTGG, 3′-AAGACAGGGCTCTCCAGAC	94 °C 40 s, 56 °C 30 s and 72 °C 40 s	300
IL-4	5′-GAAAGAGACCTTGACACAGCTG, 3′-GAACTCTTGCAGGTAATCCAGG	94 °C for 30 s, 54 °C for 30 s and 72 °C for 40 s	360
IL-10	5′-CCAGTTTTACCTGGTAGAAGTGATG, 3′-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA	94 °C for 40 s, 60 °C for 30 s and 72 °C for 40 s	440
IL-12	5′-ATGGCCATGTGGGAGCTGGAG, 3′-TTTGGTGCTTCACACTTCAGG	94 °C for 40 s, 55 °C for 30 s and 72 °C for 40 s	309
IL-5	5′-GAAAGAGACCTTGACACAGCTG, 3′-GAACTCTTGCAGGTAATCCAGG	94 °C for 40 s, 55 °C for 30 s and 72 °C for 40 s	277
TGF-β	5′-CCTCCCCCATGCCGCCCTCG, 3′-CCAGGAATTGTTGCTATATTTCTG	94 °C for 40 s, 57 °C for 30 s and 72 °C for 40 s	545

domly and the total areas (TPA) of the positive cells in the TVA were determined using an image analyzer (high-speed color image analyzer SP500; Olympus, Tokyo, Japan). The results were expressed as a percentage of TPA/TVA.

RT-PCR detection of cytokine mRNAs in the liver

The pattern of cytokine mRNA expressions in the granulomatous livers after challenge was determined by the standard procedure of reverse transcription-PCR. Liver samples were homogenized in 1 ml of Trizol (Sigma—Aldrich, USA) in a tissue grinder (Omni International, Waterbury, USA), and total RNA was isolated as recommended by the manufacturer. cDNA was synthesized with AMV reverse transcriptase and Oligo(dT)18 primer. The PCR reaction was performed with optimized primers specific for hypoxanthine phosphoribosyl transferase (HPRT), a housekeeping gene as an internal control, or for cytokine genes [21]. The sequences of the primers and conditions for PCRs are listed in Table 1. The PCR products were resolved on 2% agarose gels and visualized by ethidium bromide (EtBr) staining under UV light. The cytokines were quantified and analyzed with the Bio-Rad Image software (Quantity One 4.2.0).

Statistics

Data were subjected to Student's *t*-test to calculate the levels of significance of differences between the groups, where $*p < 0.05$, $**p < 0.01$.

Results

Determination of antigen-specific antibody responses and their isotypes

To examine the effect of LMS on antibody response induced by VR1012-SjGST-32 vaccine, sera of each group were collected and analyzed against rSjGST-32 antigen by ELISA. Two weeks after the last immunization and prior to the challenge, the mean titer of anti-SjGST-32 antibody in the LMS as the adjuvant group was significantly higher than that in the group immunized with VR1012-SjGST-32 alone ($p < 0.05$). The relatively low variability in response among individuals depicted by the error bars was found to be consistent among the groups (Fig. 1). To dissect the antibody response affected by the LMS, isotypes of IgG1, IgG2a and IgG2b were analyzed. As shown in Fig. 1, the mice immunized with VR1012-SjGST-32 + LMS induced significant higher level of IgG2a ($p < 0.01$) and IgG2b ($p < 0.05$) when compared with

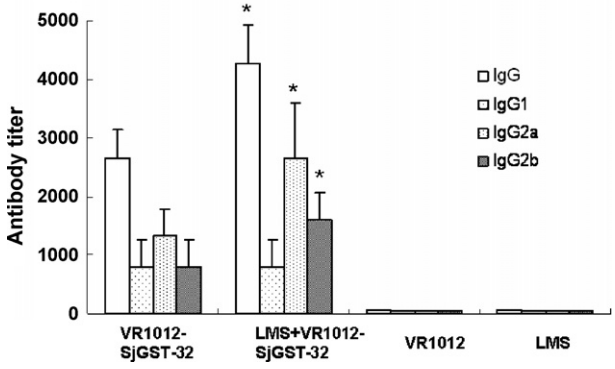

Figure 1 Effects of the LMS as adjuvant on the level of antibodies. C57BL/6 mice in each group ($n = 9$) were immunized with VR1012-SjGST-32, VR1012-SjGST-32 plus LMS, vector control and LMS alone three times at bi-week intervals as indicated on the *x*-axis. Serum samples were collected and diluted for detecting by ELISA on day 14 after the final boost. rSjGST-32 antigen at 2 μg/ml was coated on each well in 96-well plate. Titers of total IgG, IgG1, IgG2a and IgG2b were determined as indicated on the *y*-axis. The assays were performed in triplicate. Values and bars represent the mean and S.D. Statistically significant compared with the VR1012-SjGST-32, where $*p < 0.05$; $**p < 0.01$ using paired Student's *t*-test.

the same IgG isotype response in mice given VR1012-SjGST-32 alone. However, there was no difference in IgG1 between groups immunized with VR1012-SjGST-32 with or without LMS. It suggests that mice immunized with VR1012-SjGST-32 + LMS were able to elicit antibody responses that favor the Th1-type response.

The adjuvant effect of LMS on T-cell proliferative response

To further determine whether the adjuvant LMS influences cell-mediated immunity, single cell suspensions of lymphocytes were prepared from the spleen of the immunized mice on day 7 after the last immunization to perform the T-cell proliferation assay. Fig. 2A and B shows that the highest proliferation response was achieved from the T cells isolated from spleens of the animals injected with the VR1012-SjGST-32 + LMS and followed by the VR1012-SjGST-32 alone. This result suggests that LMS as an adjuvant can serve to enhance an antigen-specific T-cell response.

The adjuvant effect of LMS on cytokine expressions

Cytokines play a pivotal role in the modulation of immune responses upon infection or immunization [22—24]. To examine the profile of antigen-specific cytokine production in $CD4^+$ T cells, we examined the level of IFN-γ (Th1 type) and IL-4 (Th2 type) production of CD4 T cells by intracellular

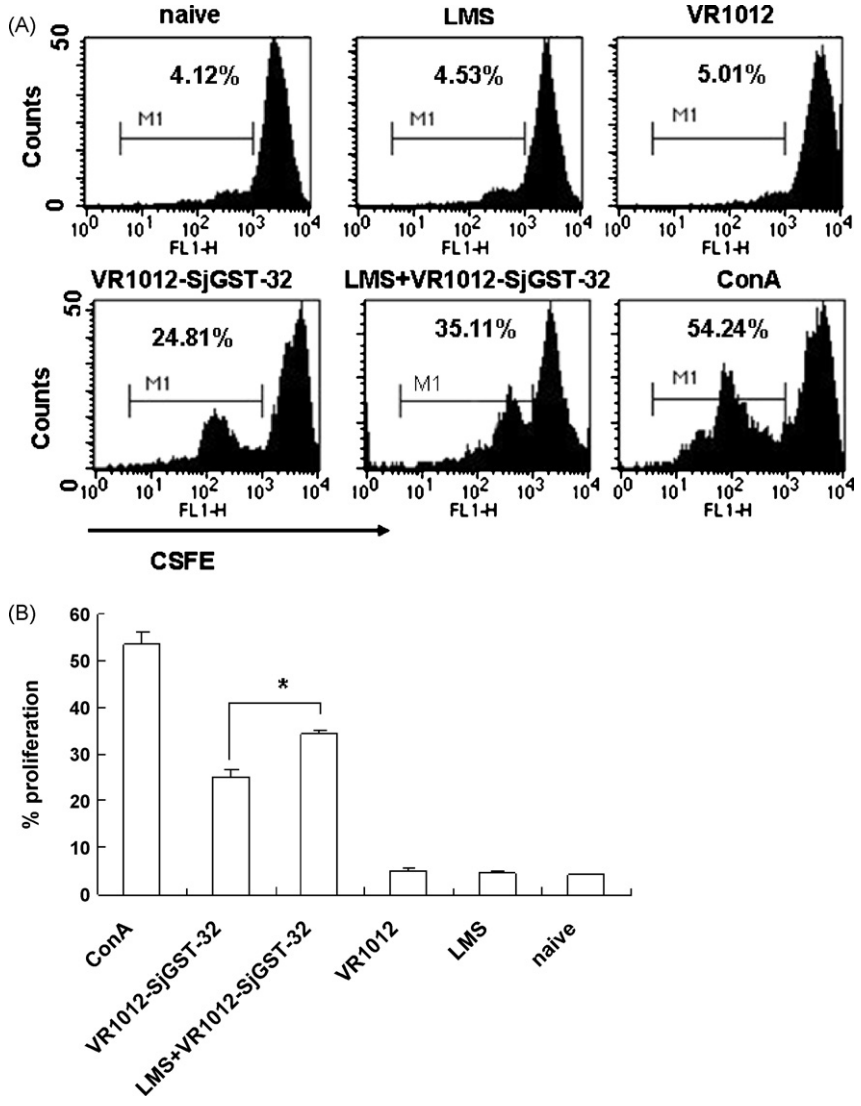

Figure 2 Analysis of T-cell proliferation in response. T cells of the immunized mice were isolated on day 7 after the last immunization and labeled with CFSE. These CFSE-positive cells were stimulated in vitro with the 10 μg/ml of rSjGST-32 as the specific antigen for each group and 5 μg/ml of Con A as a positive control. The proliferation responses were analyzed in FACScalibur by setting the gate to the CFSE positive cells indicated as the M1 after 48 h stimulation: (A) the proliferative percentages from naive, LMS, VR1012 vector, VR1012-SjGST-32 and VR1012-SjGST-32 + LMS immunized groups, respectively; (B) the graphic results of the proliferation from each group are sum from three independent experiments. Values and bars represent the mean and S.D. Statistically significant compared with the VR1012-SjGST-32. *$p < 0.05$ using paired Student's t-test.

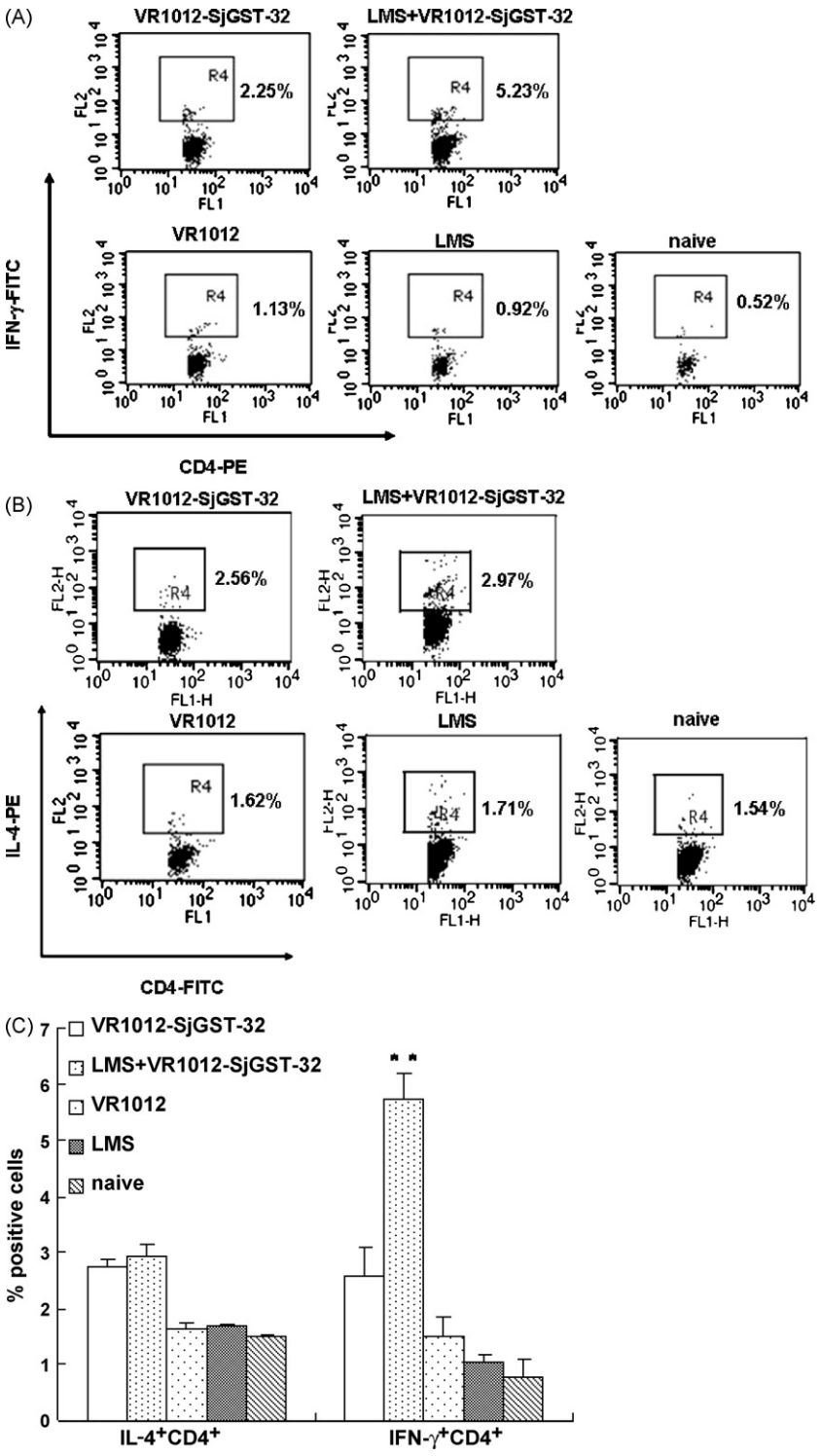

Figure 3 Analysis of production of antigen-specific cytokines by intracellular staining and FACS analysis. T cells isolated and purified from spleen of C57BL/6 mice after the final boost were stimulated with rSjGST-32 for 6 h in vitro culture. Intracellular stainings for IFN-γ in $CD4^+$ (A) and IL-4 in $CD4^+$ (B) cells was performed. The percentages of positive cells were showed in each dot-plot in the gated area. C is a representation of the percentage of intracellular cytokines of each group. The data shown are representative of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ using paired Student's *t*-test.

staining with fluorescent-labeled specific antibodies. Total T cells were isolated 7 days after the last immunization and re-stimulated in culture with the rSjGST-32 antigen and double stained with anti-CD4 plus anti-IL4 or anti-CD4 plus anti-IFN-γ and analyzed by FACS. As shown in Fig. 3A, mice immunized with VR1012-SjGST-32 + LMS showed a higher percentage of IFN-γ in $CD4^+$ T cells after the immunizations, whereas the percentage of IL-4 in $CD4^+$ T cells showed no difference

when compared with VR1012-SjGST-32 alone (Fig. 3B). The result suggests that LMS as an adjuvant can stimulate production of aTh1 type of cytokine which in turn enhance an antigen-specific Th1 response.

Determination of protective efficacy against challenge infection

Worm reduction

To determine the efficacy of the protective response derived from DNA vaccine+LMS, each mouse was challenged with 30 of *S. japonicum* cercariae on day 14 after the last immunization, sacrificed and then perfused on day 45 after the challenge. The number of recovered worms from each animal was counted and calculated. As depicted in Table 2, the average number of recovered adult worms showed a significant reduction of 25.14% in the VR1012-SjGST-32 group compared to the control group. The addition of LMS as an adjuvant dramatically enhanced the protection mediated by VR1012-SjGST-32 by up to 38.15% compared to the control group. There was significant difference between the VR1012-SjGST-32 immunized groups with and without LMS ($p < 0.05$).

Hepatic egg reduction

The numbers of hepatic eggs per gram of liver tissue per group are presented in Table 2. Using analysis of variance, the eggs per gram of liver in the group immunized with VR1012-SjGST-32 alone were reduced by 39.6% compared to the control mice. The addition of LMS in the VR1012-SjGST-32 was lead to a significant reduction a of 49.8% in hepatic egg burdens ($p < 0.05$) compared to the control mice. There was significant difference between the immunized group with VR1012-SjGST-32 + LMS and the immunized group without LMS ($p < 0.05$). Thus, vaccination of mice with VR1012-SjGST-32 + LMS can significantly reduce both worm and hepatic egg burdens.

Anti-hepatic egg granuloma formation and granuloma fibrosis effects

As liver is a major organ affected during Schistosome infection in mice, we determined how extensive the livers in immunized mice would be affected by examining the histopathologic changes after the challenge. Macroscopically, the livers from mice immunized with VR1012-SjGST-32 + LMS exhibited less inflammation appearances compared with livers from VR1012-SjGST-32 alone or the control group. We measured and analyzed differences in hepatic granuloma formations among the different immunized groups using a NYD-image analysis system. We observed that egg-induced granulomas after the challenge were smaller in size, lower in number and significantly smaller in the mean areas of non-confluent granulomas in the livers from the mice immunized with VR1012-SjGST-32 + LMS compared with other groups (Table 2, Fig. 4).

As fibrosis is a sequel to egg granuloma formation during Schistosomiasis, collagen depositions and collagenous materials (blue stain) surrounding the Schistosome eggs within the liver were significant reduced in VR1012-SjGST-32 + LMS immunized mice after being stained by Martius Scarlet Blue

Table 2 Immunization and protection

Groups	Female worms	Male worms	Total worms	Reduction (%)	Egg number/liver (10^4)	Reduction (%)	Liver weight (g)	Spleen weight (g)	Mean diameter (μm)	Granuloma size ($\times 10^3$ μm^2)
Control	10.63 ± 1.50	11.0 ± 1.19	21.6 ± 2.65	–	4.77 ± 0.85	–	1.63 ± 0.13	0.41 ± 0.08	71.33 ± 17.67	3.39 ± 1.36
LMS	9.50 ± 1.69	10.0 ± 1.93	19.5 ± 3.53	9.83	4.45 ± 1.03	6.55	1.47 ± 0.27	0.36 ± 0.08	60.07 ± 16.53	2.89 ± 1.86
VR1012-SjGST-32	8.13 ± 0.99	8.00 ± 1.19	16.12 ± 2.66	25.40	2.80 ± 0.95	39.58	1.34 ± 0.15	0.29 ± 0.09	47.25 ± 17.78	2.07 ± 1.43
LMS+VR1012-SjGST-32	6.50 ± 2.62	6.88 ± 2.41	13.38 ± 4.6*	38.15	2.40 ± 0.5*	49.84	1.19 ± 0.2*	0.28 ± 0.10	36.03 ± 6.79*	1.04 ± 0.36*
VR1012	9.86 ± 2.34	10.4 ± 2.22	20.2 ± 4.49	6.35	4.40 ± 0.87	7.73	1.68 ± 0.14	0.38 ± 0.07	61.09 ± 22.23	2.43 ± 1.23

Note: (1) C57B/6 mice were vaccinated intramuscularly three times bi-weekly interval, challenged with 30 of *S. japonicum* cercariae on day 42 and perfused on day 85; (2) gross tissue weight of mice was measured; (3) worm and egg burdens and reduction rates were counted and calculated as described in the ''Materials and methods'' section; (4) *$p < 0.05$ as significant change.

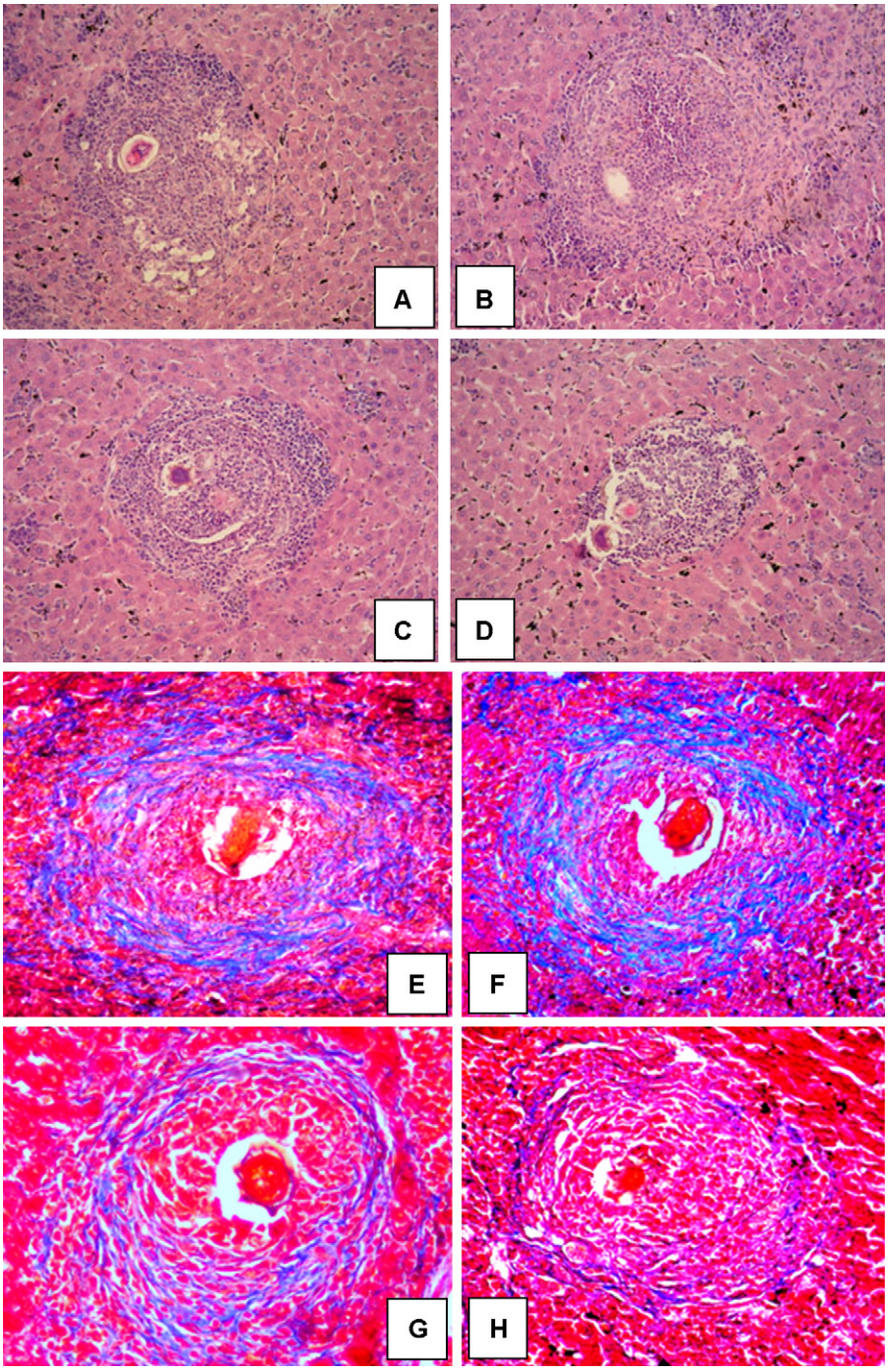

Figure 4 Liver pathology and collagen deposition after *S. japonicum* infected controls and the vaccinated mice. Liver sections taken from the C57BL/6 mice 6 weeks after challenge infection were stained by H&E to reveal the hepatic histological changes and by Martius Scarlet Blue to examine the collagen depositions (blue stains). The areas show hepatic granulomas in each representative section: (A and E) infection control showed larger size of granuloma and extensive collagen depositions; (B and F) mice with LMS alone also showed larger size of granuloma and more collagen depositions; (C and G) mice immunized with VR1012-SjGST-32 showed smaller size of granuloma and few collagen depositions; (D and H) mice immunized with LMS plus VR1012-SjGST-32 showed smallest size of granuloma and fewer collagen depositions.

compared with those in other groups (Fig. 4E—H). These results suggest that the LMS is an effective potential adjuvant in reducing collagen depositions and fibrosis induced by the Schistosome egg, thereby lowering liver pathogenesis resulting from the Schistosome infection.

Expressions of iNOS, Bcl-2 and Bax in liver

Evidence suggests that the expression of iNOS is reciprocally correlated with the development of fibrosis and hepatic damage [25]; therefore, we examined its expression

and location in liver among the groups by immunohistochemical analysis. We observed that the level of iNOS was increased in hepatic cells within and surrounding the granulomas in the group immunized with VR1012-SjGST-32 + LMS, and decreased in other groups (Fig. 5A—E), suggesting that induction of high level of iNOS expression by the addition of LMS in the VR1012-SjGST-32 vaccination may lead to the less hepatic fibrosis and less live damage.

Next, we sought the correlation of the hepatic fibrosis and pathogenesis observed in the challenge animals with their hepatic cell apoptosis by examining the expressions and locations of Bcl-2, and Bax by immunohistochemical analysis with specific antibodies. We observed that the Bcl-2 was highly expressed in hepatic cells within and surrounded the granulomas in the group immunized with VR1012-SjGST-32 + LMS, but noted that Bcl-2 was low and less sporadic in

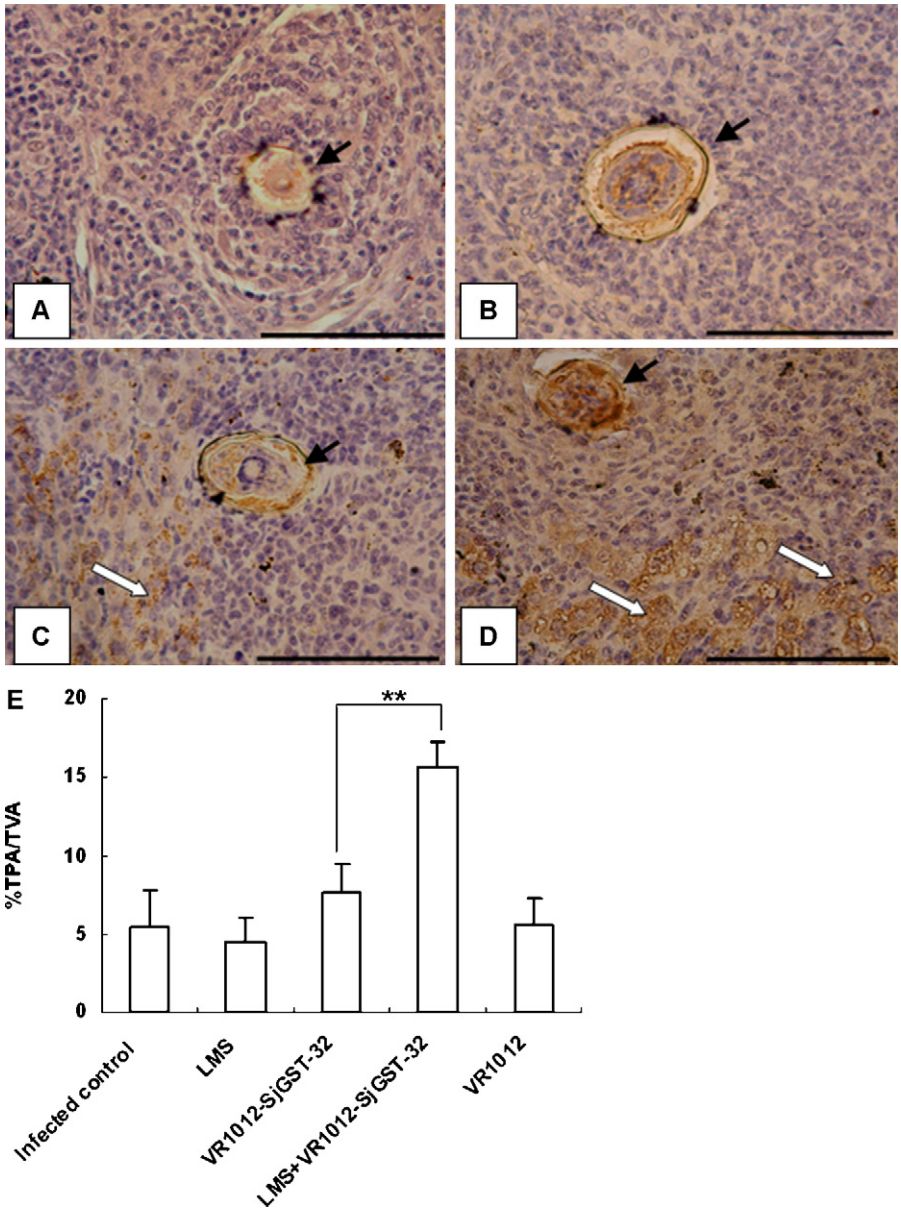

Figure 5 Analysis of expressions of iNOS and Bcl-2 in liver of C57BL/6 mice. The liver sections were obtained from mice 6 weeks after the challenge infection and reacted with anti-iNOS (A—E) or anti-Bcl-2 (F—J) monoclonal antibody. The positive reactions were determined by reacting with the secondary antibody conjugated with the biotinylated peroxidase and colorimetric reaction. The arrows indicate the positive cells and arrowheads show *S. japonicum* egg. (A and F) infection control, positive signal was hardly observed around the egg in the liver; (B and G) mice injected with LMS alone, few positives; (C and H) mice immunized with VR1012-SjGST-32, few positives; (D and I) mice immunized with LMS adjuvant plus VR1012-SjGST-32, showed numerous specific cells around the matured,visual egg. The expressions of iNOS and Bcl-2 were examined under a light microscope at 40× magnifications to show the positive cells (all scale bars indicate 150 μm in size). Semi-quantitative analysis of expression of iNOS (E) and Bcl-2 (J) on the sections is indicated as the percentage of TPA/TVA as described in the ''Materials and methods'' section. $*p < 0.05$ and $**p < 0.01$ when compared with mice immunized with VR1012-SjGST-32.

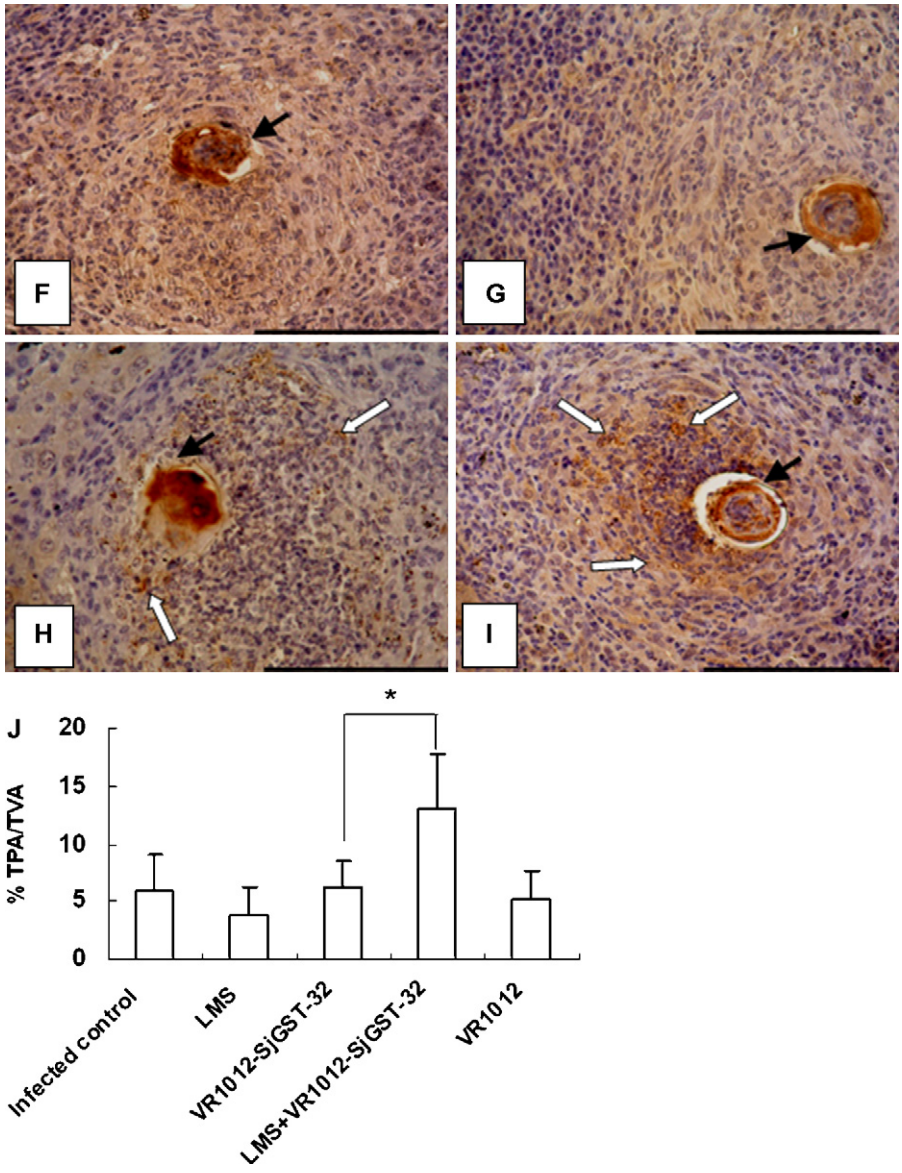

Figure 5 (*Continued*).

liver around the granulomas in other groups (Fig. 5F—J). In contrast, the expression of Bax was low in the group immunized with VR1012-SjGST-32 + LMS and higher in the other groups-LMS (data not shown). The results suggest that apoptotic induction by Schistomiasis in liver can be suppressed by DNA vaccination with LMS, which may be correlated with lower liver of hepatic fibrosis and pathogenesis in this adjuvant group.

Cytokine expression profile after the challenge

The Schistosome infection is generally thought to induce a strong Th2 response in animals. To examine the profile of the antigen-specific cytokine productions in $CD4^+$ T cells after the challenge, we examined the levels of IFN-γ (Th1 type) and IL-4 and IL-10 (Th2 type) productions of splenic T cells by the intracellular staining as described above. Total lymphocytes were isolated 42 days after the challenge, restimulated in culture with the rSjGST-32 antigen, double stained with respective antibodies and analyzed by FACS. As shown in Fig. 6A—D, we observed significant reductions of IL-4 and IL-10 and an enhanced production of IFN-γ in $CD4^+$ T cells in mice immunized with VR1012-SjGST-32 plus LMS after the challenge. This suggests that the LMS as an adjuvant can up-regulate the Th1-type response that correspond with the down-regulation of the Th2 cytokine expressions observed after the challenge.

Expressions of cytokines in liver

To understand what factor(s) may contribute to less liver pathology, we attempted to analyze the cytokine profiles of the livers of mice. Total RNA was extracted from liver of each group after the challenge and RT-PCR was used to analyze the level of cytokine expressions, such as IL-12, TNF-α and IFN-γ for the Th1-type response, IL-4 and IL-5

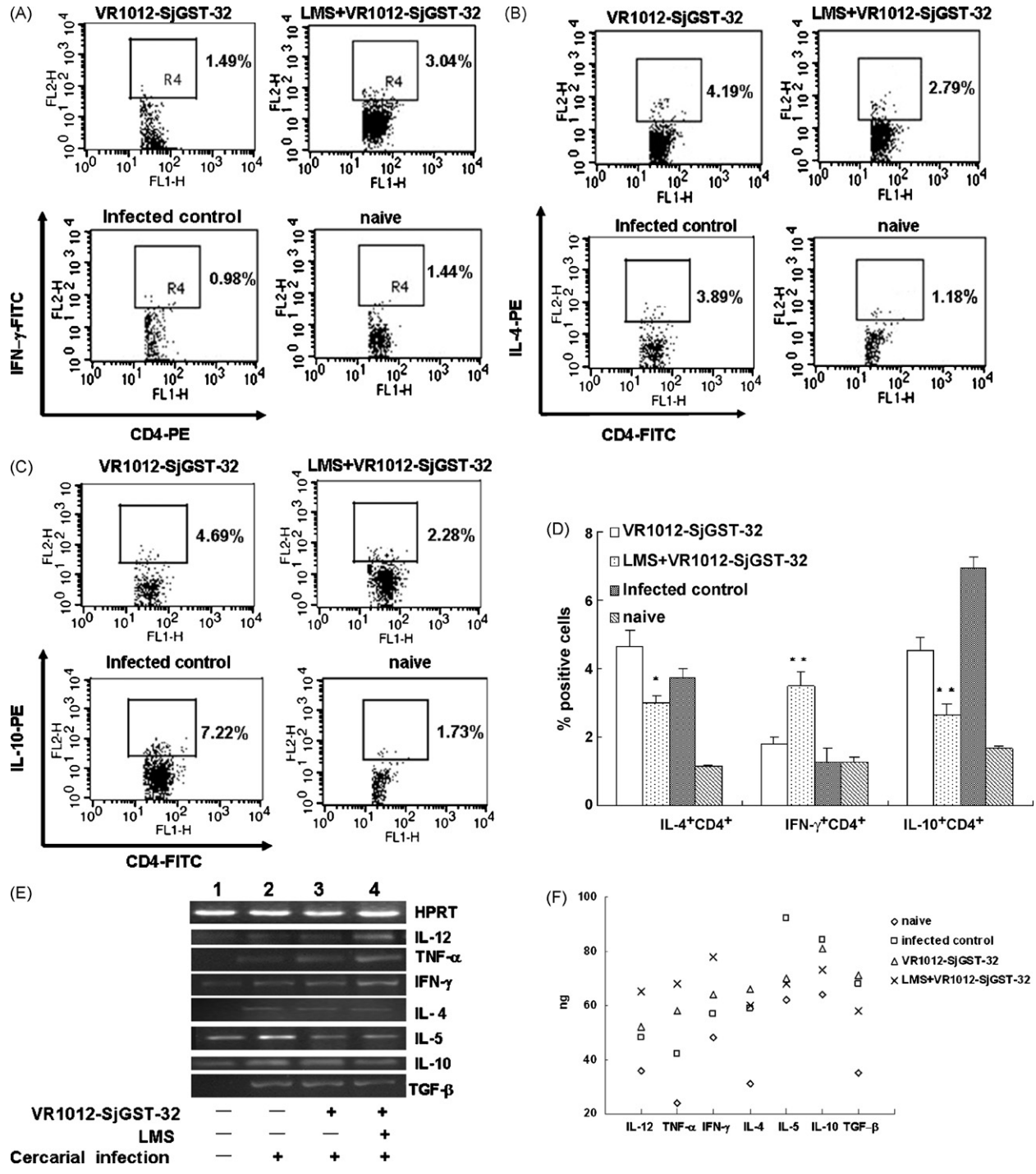

Figure 6 Analysis of antigen-specific cytokine profiles by FACS and RT-PCR after the challenge infection. T cells isolated from the spleen of C57BL/6 mice after the cercarial challenge were stimulated with rSjGST-32 as the specific antigen for 6 h in culture. Intracellular staining cells were performed as described in the ''Materials and methods'' section. The percentage of cytokine-positive cells is shown in the dot-plot and the gate was set on $CD4^+$ T cells or events: (A) percent of IFN-γ^+ $CD4^+$ T cells in total T cells after the challenge; (B) percent of IL-4^+CD4^+ T cells in total T cells after the challenge; (C) percent of IL-10^+CD4^+ T cells in total T cells after the challenge; (D) a representation of the percentage of intracellular cytokines of each group from three independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ when compared with mice immunized with VR1012-SjGST-32 group using paired Student's *t*-test. For RT-PCR analysis, total RNA was isolated from the livers of immunized or naive mice (six per group) after 6 weeks of the challenge infection; (E) the level of gene expressions for IL-12, TNF-α, IFN-γ, IL-4, IL-5, IL-10 and TGF-β were determined after RT-PCR followed by the band intensity seen under UV light after electrophoresis on 1.5% agarose gel. The level of HPRT expression, a housekeeping gene, was used to serve as an internal control; (F) a relative intensity of cytokines to the known quantity of DNA markers was quantified with the Bio-Rad Image software (Quantity One 4.2.0) and was shown in the graphical representation.

for the Th2 type response, and IL-10 and TGF-β for anti-inflammatory response. As shown in Fig. 6E and F, the level of mRNAs for IL-12, TNF-α and IFN-γ in the liver induced in the group immunized with VR1012-SjGST-32 + LMS was higher than in the group immunized with VR1012-SjGST-32 alone or in the control; whereas the expressions of IL-4, IL-10 and TGF-β were lower in the VR1012-SjGST-32 + LMS group compared with other groups. The level of mRNA for IL-5, a relevant cytokine in granuloma formation, was elevated in the control group but not affected in the immunized groups, suggesting anti-granuloma formation from the DNA immunizations. The results suggest that using LMS as adjuvant in DNA vaccination can up-regulate local Th1, down-regulate Th2 types of cytokines and showed little affect on the anti-inflammatory cytokines after challenge. The preferred Th1-type response induced by DNA vaccine+LMS observed in this study is apparently correlated reciprocally with the granuloma formations and with hepatic fibrosis, and this reciprocal behavior is consistent with other previous reports [26,27].

Discussion

LMS, commonly used as a pesticide, has a potent Th1 stimulation capacity if it is used with DNA vaccine as we have previously documented [13,28,29]. The present study demonstrates that administration of DNA vaccine encoding the fusion product of SjGST-32 formulated with LMS conferred partial protection in terms of worm burden and egg counts in the liver of mice following the parasitic challenge. Such protection apparently associated with a Th1-type response as demonstrated by high levels of IFN-γ and low level of IL-4 and IL-10 production in $CD4^+$ T cells, as well as by increased IgG2a, IgG2b and diminished IgG1. Strikingly, Schistosomiasis induced liver pathogenesis such as the granuloma formation, collagen deposition and fibrosis was marked suppressed in the DNA vaccine+LMS group. This milder liver immunopathology was concurrent with locally high levels of expression of Th1 type of cytokines, iNOS and Bcl-2 and low level of expression of Th2 cytokines and Bax in mice liver, suggesting an inhibition of egg induced apoptosis and immunopathology in liver by immunization of DNA vaccine+LMS.

Several lines of evidence suggest that cell-mediated immunity involving IFN-γ-activated effector cells is the major mechanism of protective immunity in the mouse challenge model [12,30,31]. Moreover, in vitro studies have demonstrated that Schistosomula, the presumed target of immune response, can be killed by macrophages or endothelial cells that have been activated via the combinations of several cytokines including IFN-γ and IL-12 [32]. Therefore, Th1-type of immune response seems to be important in the induction of resistance against Shistosomiasis in the mouse. In the present study, we clearly showed that Schistosome DNA vaccine encoding the SjGST-32 plus LMS as the adjuvant stimulated the Th1-type response in our animals.

Fibrosis development in granulomas is associated excessive hepatic collagen depositions during the granulomatous inflammation in murine Schistosomiasis. A variety of molecules stimulate the differentiation of stellate cells into myofibroblasts that secrete extracellular matrix proteins, including collagens, fibronectin, and glycosaminoglycans. The precise mechanisms of the formation of granulomas and fibrosis in the liver remain to be illustrated [33,34]. Several studies reported that granulomas formations and fibrosis were regulated by cytokines produced by macrophages and lymphocytes [35]. In murine Shcistosomiasis, a strong Th2-type cytokine response dominated the immunopathogenic response of the host to the parasites eggs, that the reduction in fibrosis has been proposed to result from both reduced expression of collagen-induced cytokines namely TGF-β and IL-4, and a corresponding increase in cytokines, as IFN-γ and TNF-α [36]. It has been shown that the elevated IFN-γ, TNF-α, IL-12 secretion and the absence of IL-4, IL-10 and TGF-β by liver cells can in fact regulate granulomas and collagen formation. Indeed, administration of recombinant IFN-γ to Schistosome infected mice has been shown to reduce collagen deposition [37,38]. Additionally, TGF-β has been shown to be elevated within the liver, and is associated with fibrosis and liver collagen synthesis during Schistosome infection [39,40]. Egg induced granulomas and fibrosis formations in livers were significantly reduced in the VR1012-SjGST-32 + LMS group (Fig. 4). Thus it is consistent that overall liver inflammation was reduced and is concurrently associated with the increased expressions of Th1-type cytokines in the liver as demonstrated in this study. Much lower levels of apoptosis in the hepatic cells were also associated with the significant reduced expression of hepatic collagens surrounding the granulomas in the group of VR1012-SjGST-32 + LMS (Fig. 4). By contrast, the liver granulomas in the control groups lacking LMS including the VR1012-SjGST-32 immunization exhibited at high levels which was reduced significantly in the livers secreting IL-4, IL-10, and TGF-β (Fig. 6E and F). Thus the relatively lower amounts of TGF-β and IL-10 in the liver of mice from the cytokine group adjuvant with LMS will also have significant implication for liver granulomas and fibrosis, since IL-10 has been shown previously to play a crucial role in murine Schistosome infection [41].

In addition, NO production in lung can mediate parasite elimination and is considered as one of the major killing mechanisms against lung stage Schistosomulae [26,42]. It has been also reported that mice treated with aminoguanidine, a selective inhibitor of iNOS expression, developed severe morbidity and increased hepatic damage in murine Schistosomiasis [25]. We demonstrated that higher level of iNOS induced in the group immunized with VR1012-SjGST-32 + LMS might indeed contribute to the reductions noted worm and egg burden observed in this adjuvant group (Table 2) and its hepatic pathology (Fig. 5). Thus vaccine plus LMS could ease the immunized hosts from development of severe hepatic damage by the activation of iNOS expression and by a systemic shift to a Th1-dominant response.

Numerous large clusters of apoptotic cells were observed to appear in the spleen and in inflammatory infiltrations around eggs in the liver 6 weeks after *S. mansoni* infection previously reported [43,44]. Bcl-2 is an anti-apoptotic protein residing in the mitochondria outer membrane and has been shown to prevent the activation of caspase 9 and 3. Bax is a pro-apoptotic protein in the Bcl-2 family can induce mitochondria damage and initiate the caspase-dependent cell death [11,45,46]. We have measured the expressions of Bcl-2 and Bax in liver in the different vaccination groups

after challenge. In light of the observed increase in Bcl-2 expression and decreased in Bax expression, our findings suggests that apoptosis of hepatic cells was down-regulated in the group vaccinated with VR1012-SjGST-32 + LMS and up-regulated in the other groups (Fig. 5).

Although previous studies have showed that the treatment with levamisole hydrochloride embrocating on the infection day or 1 day before infection resulted in 100% worm reduction rates [47,48], the administration of 1% LMS as adjuvant used in this study did not directly influence the protection since the use 1% LMS alone did not provide any protection (Table 2). Rather, 1% LMS directly influenced immune responses when it combined with VR1012-SjGST-32 vaccine and result in 37.15% worm reduction rates and 49.5% egg reduction rate. Generating a protective memory response is a key factor for the successful vaccine development. Although immunized mice were partially protected from the challenge in 2 weeks after the immunization, it would be interested to determine if such protection can be last for longer period of time under the current protocol or an optimal immunization schedule may be needed.

Collectively, our data demonstrates that the immunization with VR1012-SjGST-32 + LMS-induced Th1-type immune responses in mice, which effectively suppressed the development of Schistosome egg granulomas, reduces collagen formations and fibrosis in mice. The use of LMS as adjuvant may present a feasible to rational strategy to minimize the morbidity induced by Schistosoma infection.

Acknowledgments

This work was supported in part by the WHO Special Programme for Research and Training in Tropical Diseases (T16/181/720, ID No. A70040) and CAU Research Initiative Fund to BW. We would like to express our gratitude to Dr. Richard X. Ascione for his editing and thanks Dr. Jane Q.L. Yu and Mr. Zhonghuai He for their technique assistances.

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