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ORIGINAL PAPER

Evaluation of the adjuvant effect of pidotimod on the immune protection induced by UV-attenuated *Toxoplasma gondii* in mouse models

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Abstract The current anti-*Toxoplasma gondii* drugs have many shortcomings and effective vaccines against *T. gondii* may contribute to the control of this pathogen. Pidotimod is a synthetic substance capable of stimulating both cellular and humoral immunity. To investigate the possible adjuvant effect of pidotimod on the immune response to *T. gondii* in Kunming mice induced by ultraviolet-attenuated *T. gondii* (UV-*T.g*), in this study, mice were immunized intraperitoneal (i.p.) with UV-*T.g* or UV-*T.g* co-administered with pidotimod (UV-*T.g*+PT). After infection or challenge by i.p. injection of 10^2 RH tachyzoites, the animal survival rate, parasite burden in peritoneal lavage fluids, liver histopathology, the level of serum anti-toxoplasma IgG antibody, and the mRNA expressions of IL-2, IFN-γ, and TNF-α from spleen analyzed using real-time PCR, were compared among different groups. The results showed that, compared with infected controls, infected mice treated with pidotimod had significantly increased survival rate and extended survival time, decreased parasite burden, improved liver histopathology, increased level of anti-toxoplasma IgG antibody, and increased mRNA expressions of Th1-type cytokine (IL-2, IFN-γ, and TNF-α) ($P<0.01$), while mice vaccinated with UV-*T.g* and then challenged had even significantly increased survival rate and extended survival time, decreased parasite burden, improved liver histopathology, and increased mRNA expressions of Th1-type cytokines (IL-2, IFN-γ, and TNF-α) ($P<0.01$); furthermore, vaccinated mice co-administered with pidotimod had even more lower parasite burden, milder liver histopathology, and higher levels of Th1-type cytokine and anti-toxoplasma IgG antibody ($P<0.01$). Our data demonstrated that pidotimod in vivo could promote strong and specific humoral and cellular immune response to *T. gondii* challenge infection when co-administered with UV-attenuated *T. gondii*. It suggests that pidotimod may have the potential to be used as an effective vaccine adjuvant.

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Introduction

Toxoplasma gondii is an obligate intracellular parasite, which chronically infects 30–50 % of human population

worldwide (Jackson and Hutchison 1989), causes serious public health problems and is of great economic importance worldwide. So far, treatment of this disease is difficult due to toxic effects of available drugs. In addition, when *T. gondii* encysts in the tissues, there are no drug treatments available to eliminate the parasite (Suzuki et al. 2010). Therefore, development of vaccines against *T. gondii* infection in humans is of high priority. *T. gondii* infection stimulates both humoral and cellular immune responses (Denkers and Gazzinelli 1998). It has been demonstrated that a T helper cell type 1 (Th1)-biased response is required for effective protection in naturally occurring *T. gondii* infection (Filisetti and Candolfi 2004). Dendritic cells are the main source of IL-12 during in vivo *T. gondii* infection (Liu et al. 2006). IFN-γ is the cytokine that plays a central role in protection against *T. gondii* (Subauste and Remington 1991). While antibody-specific response for the parasite antigens can also stimulate protection against *T. gondii* infection (Mineo et al. 1993). We and others have demonstrated that the complete immune protection induced by the temperature-sensitive mutant of RH *T. gondii* strain (ts-4) against acute toxoplasmosis is mediated by $CD4^+$ and $CD8^+$ IFN-γ producing T cells, B cells, and IL-10 cytokines (Gazzinelli et al. 1991; Lu et al. 2004; Lu et al. 2009). Therefore, the ideal vaccine to protect against toxoplasmosis in humans would basically elicit a protective Th1 immune response (Cong et al. 2011). Studies have proved that adjuvants play an important role in the efficacy of vaccines to increase the strength of an immune response and determine the type of immune response generated (El-Malky et al. 2005). Thus, high immunogenicity of a vaccine requires the necessary assistance of an effective adjuvant.

Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4carboxylic acid) is a synthetic substance with important immunomodulatory properties in vitro and in vivo (Coppi and Mailland 1990; Migliorati et al. 1992). Clinical studies showed that pidotimod is effective against infectious relapses in patients suffering from chronic bronchitis (Bisetti et al. 1994), and the drug is useful to increase the immune defense during infections such as chronic obstructive pulmonary disease (Benetti et al. 1994). Pidotimod can stimulate human dendritic cells to release high amounts of pro-inflammatory molecules such as monocyte chemoattractant protein-1 (MCP-1) and TNF-alpha (TNF-α) cytokines and to drive T cell proliferation and differentiation towards a Th1 phenotype; and pidotimod also can promote strong and specific humoral and cellular immune response when co-administered intranasal with a model antigen in mice (Giagulli et al. 2009). Therefore, this study was designed to evaluate the potential effect of pidotimod as an adjuvant on UV-attenuated *T. gondii* in inducing a Th1 response and its efficiency to protect mice against the highly virulent RH strain of *T. gondii*.

Materials and methods

Mice and parasites

Kunming (KM, outbred) mice, female, aged 6–8 weeks at the commencement of the study, were obtained from the Experiment Animal Center of Sun Yat-sen University (Guangzhou, China). All animals were bred under specific-pathogen-free conditions at Sun Yat-sen University, and had free access to a commercial basal diet and tap water ad libitum. Mice were infected or challenged intraperitoneal (i.p.) with the highly virulent RH strain of *T. gondii* maintained in our laboratory. RH tachyzoites were propagated by i.p. passage in KM mice at 4 or 5 day intervals. All experiments were performed in compliance with the requirements of the Animal Ethics Committee at Sun Yat-sen University.

UV-irradiation of parasites

For the preparation of UV-irradiation of RH tachyzoites of *T. gondii*, we tried different times, such as 20, 30, 40, 50, and 60 min of UV-irradiation, and then selected 60 min for this study, by which the tachyzoites could not cause mouse death but could elicit an immune response in vivo. That is, 2.0×10^5 purified *T. gondii* tachyzoites of RH strain were suspended in PBS and placed on a glass slides to a depth of ≤1 mm, and irradiated under ultraviolet light (UV; 2,539 Å) for 60 min. The irradiated tachyzoites were used for inoculation immediately after exposure, and each mouse was i.p. injected with 0.2 ml suspensions containing 10^5 irradiated parasites.

Drugs

Pidotimod (99.6 % purity) was obtained from the Wuzhong Medicine Co., Ltd. (Suzhou, China), which was milled and diluted in saline. Mice were administered with pidotimod at the concentration of 400 mg/day/kg by gavage.

Vaccination and challenge infection

A total of 128 KM mice were included in this study. Mice were divided into seven groups, consisting of 4–12 mice per group, and tests were performed twice. (1) naïve mice; (2) mice were immunized by i.p. injection of 10^5 UV-attenuated tachyzoites of RH strain (UV-*T.g*) for three times (Yang et al. 2010) with a 10-day interval; (3) mice were given pidotimod (UV-*T.g*+PT) from day 1 post UV-*T.g*-immunization, and all the above-mentioned mice were challenged by i.p. injection of 10^2 RH tachyzoites at days 31 post immunization; (4) primary infection of naive mice was performed by i.p. injection with 10^2 RH tachyzoites (infected controls) at the same

time; (5) the infected mice were treated with pidotimod (*T.g*+PT) from day 1 postinfection (p.i.); (6) uninfected mice were treated with pidotimod at the same time, and (7) UV-*T.g*-immunized mice without *T. gondii* challenge as control. Mice were monitored daily for disease signs and death after either infection or challenge.

Enumeration of *T. gondii* tachyzoite burden in mouse peritoneal lavage fluids

When mice were killed, prior to death after infection or challenge, 5 ml saline was injected into the intraperitoneal, the peritoneal lavage fluids were extracted, tachyzoites were purified and live tachyzoites were counted by Trypan blue staining. Tachyzoites were enumerated using manual counting with a hemocytometer.

Histopathological analysis

Mice were sacrificed by CO_2 asphyxiation, and livers were harvested and immediately fixed in 10 % buffered natural formaldehyde (Guangzhou Chemical Reagent Factory, China). Four-micrometer-thick sections (50- or 100-μm distance between sections) of the organ from each mouse, stained with hematoxylin and eosin (H&E) (Sigma-Aldrich), and histological changes were evaluated by semi-quantitative histopathologic analysis. Liver sections were analyzed for the numbers of inflammatory foci according to Cavalcanti et al. (2011), with modifications. In brief, we analyzed the number of inflammatory foci per field at a magnification of ×10 under a light microscopy counting ten fields of each section at 5–7 days for infected controls prior to death after infection, at 9–12 days for infected mice treated with pidotimod prior to death after infection, at 30 days for mice vaccinated with UV-*T.g* or UV-*T.g*+PT and then challenged with virulent RH tachyzoites post-challenge, and mice vaccinated with UV-*T.g* alone were sacrificed at the same time.

Measurement of cytokine mRNA expression by quantitative real-time PCR

Total RNA was extracted from about 100 mg of spleen sample of each mouse using RNA Extraction Kit (TaKaRa) according to the manufacturer's protocol. The quality of total RNA was analyzed by running 5 μl of each RNA sample on a 1.0 % agarose gel and visualizing with ethidium bromide. The quantity of total RNA was measured by the absorbance at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). First-strand cDNA was constructed from 1.0 μg of total RNA with oligo (dT) as primers using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa), following the manufacturer's protocol. cDNA was stored at −80 °C until use.

To determine spleen tissue IL-2, IFN-γ, and TNF-α mRNA levels, quantitative real-time PCR (qRT-PCR) was performed using SYBR Green QPCR Master Mix (TaKaRa) according to manufacturer's instructions. Primers were listed in Table 1. Briefly, the total 10 μl reaction mixture contained 5.0 μl of SYBR® Premix Ex Taq™ (2×), 0.5 μl of each primer (10 pM), 3.0 μl of dH_2O, and 1.0 μl of cDNA (0.2 μg/μl). Amplification were initially pre-denaturized for 30 s at 95 °C, followed by 43 cycles of 5 s at 95 °C, 20 s at 60 °C with a LightCycler® 480 instrument (Roche Diagnostics). Specific mRNA expression levels were normalized to the housekeeping gene, β-actin mRNA, and the results are expressed as the fold change compared to uninfected controls.

Levels of anti-toxoplasma IgG in serum

Mice were bled before death and sera were stored at −20 °C until use. Specific antibodies were detected and quantified by end-point dilution ELISA assay (in triplicate) on sera from individual animals. Purified RH parasites (5×10^4/well) were placed in plastic 96-well microtiter plates (Costar), dried overnight, blocked with 5 % bovine serum albumin–PBS, and washed in PBS (pH 7.2)–0.05 % Tween 20. Sera were added at twofold serial dilutions and incubated for 2 h at 37 °C. Plates were washed and supplemented with an anti-Mouse IgG (Fab specific)-peroxidase antibody (1/60,000; Sigma-Aldrich) for 1 h. After a wash step, tetramethyl benzidine substrate (Sigma-Aldrich) in H_2O_2 was used for development. The reaction was stopped 30 min later by the addition of 2 N H_2SO_4. OD values were measured at 450 nm with an automatic microplate reader (Sunrise, Tecan, Austria).

Statistical analysis

Data are presented as mean±SEM. Differences were analyzed by using the Student *t* test or Wilcoxon rank sum test. However, the survival rate was expressed as the percentage of live animals, and the differences were analyzed by using the Kaplan–Meier analysis (MedCalc Statistical Software). A value of $P<0.05$ was considered statistically significant.

Results

Increased survival rate

Survival rates in different groups of mice were shown in Fig. 1. Following infection with 100 virulent RH tachyzoites, all infected controls (12/12) died from 5 to 7 days, whereas infected mice treated with pidotimod survived much longer, and the survival time of the treated mice ranged from 9 to 12 days, in which more than 67 % of the mice (8/12)

Table 1 Primer sequences of mouse target cytokines and housekeeping genes used for Quantitative real-time polymerase chain reaction (qRT-PCR) assays

Genes	Primer sequence (5′→3′)		References
IFN-γ	Forward primer	GGAACTGGCAAAAGGATGGTGAC	Jones et al. 2010
	Reverse primer	GCTGGACCTGTGGGTTGTTGAC	
TNF-α	Forward primer	CCCTCACACTCAGATCATCTTCT	Zhao et al. 2011
	Reverse primer	GCTACGACGTGGGCTACAG	
IL-2	Forward primer	CCCAAGCAGGCCACAGAATTGAAA	Orsatti et al. 2010
	Reverse primer	AGTCAAATCCAGAACATGCCGCAG	
β-actin	Forward primer	TGGAATCCTGTGGCATCCATGAAAC	Jones et al. 2010
	Reverse primer	TAAAACGCAGCTCAGTAACAGTCCG	

survived by day 9 ($P<0.0001$). Compared with infected controls, mice vaccinated with UV-*T.g* and then challenged with virulent RH tachyzoites 30 days later, showed a significant higher survival rate and longer survival time compared with that of the infected controls, and the survival time ranged from 7 to over 30 days, in which 50 % of the mice (5/10) survived by day 30 ($P<0.0001$); mice vaccinated with UV-*T.g*+PT and then challenged with RH tachyzoites, the survival time ranged from 9 to over 30 days, in which 70 % of the animals (7/10) survived by day 30 ($P<0.0001$). However, the survival rates between the mice vaccinated with UV-*T.g* and then challenged and mice vaccinated with UV-*T.g*+PT and then challenged had no significant difference ($P>0.05$).

Decreased parasite burden

To characterize the difference among different groups of mice in response to RH infection or challenge, their peritoneal lavage fluids were examined for the parasite burden at various times when becoming moribund. Figure 2 showed that parasite burden in peritoneal lavages obtained from groups of four infected control mice and infected mice treated with pidotimod. Infected mice without treatment had high number of tachyzoites, while infected mice with pidotimod treatment had significant lower number of tachyzoites ($P<0.01$). Compared with infected mice with pidotimod treatment, there were significantly decreased numbers of parasites in mice immunized with UV-*T.g* and then challenged ($P<0.01$), and UV-*T.g*+PT and then challenged ($P<0.01$). Compared with UV-*T.g* vaccinated mice after challenge, there were even significantly decreased numbers of parasites in mice vaccinated with UV-*T.g*+PT after challenge ($P<0.01$).

Improved liver histopathology

The liver tissues from different groups were examined histologically when infected mice became moribund and the severity of inflammation and necrosis were scored. Figure 3 showed that the control sections of liver from naive mouse (Fig. 3a), uninfected mouse treated with pidotimod at 30 days (Fig. 3b), and mouse immunized with UV-*T. gondii* alone at 40 days post immunization (Fig. 3c), which were negative or almost negative for inflammation. After primary i.p. RH strain of *T. gondii* infection, severe damage (strong inflammation and necrosis) (Fig. 3d) and a great amount of RH tachyzoites (Fig. 3e) were observed in the liver tissues of infected mice at 7 days p.i., and the infiltration presented a predominance of neutrophils in a discrete intensity. In contrast, moderate damage (obvious inflammation and necrosis)

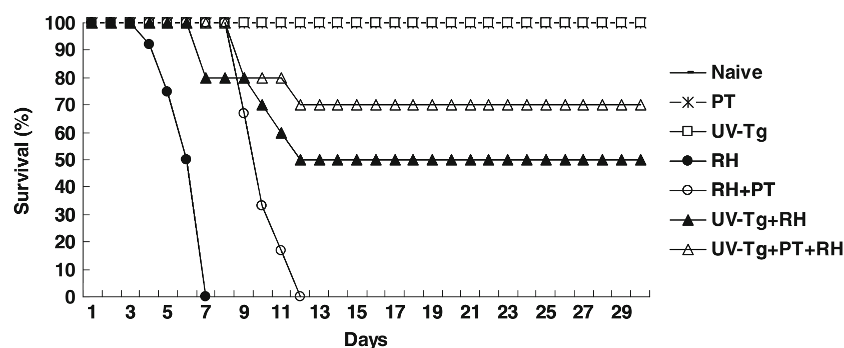

Fig. 1 Survival of naïve mice (*dash*, $n=4$); uninfected mice by pidotimod oral gavage (*asterisk*, $n=8$); UV-*T.g*-immunized mice without challenge (*open square*, $n=8$); mice infected by i.p. injection with 10^2 RH tachyzoites of *T. gondii* (*filled circle*, $n=12$), and infected mice with pidotimod treatment (*open circle*, $n=12$); mice vaccinated with UV-*T.g*, (*filled upright triangle*, $n=10$) or UV-*T.g*+pidotimod (*open upright triangle*, $n=10$) and then challenged by i.p. injection with 10^2 RH tachyzoites of *T. gondii*. The mice were monitored for survival on a daily basis until the termination of the experiment. The experiment was performed twice with similar results

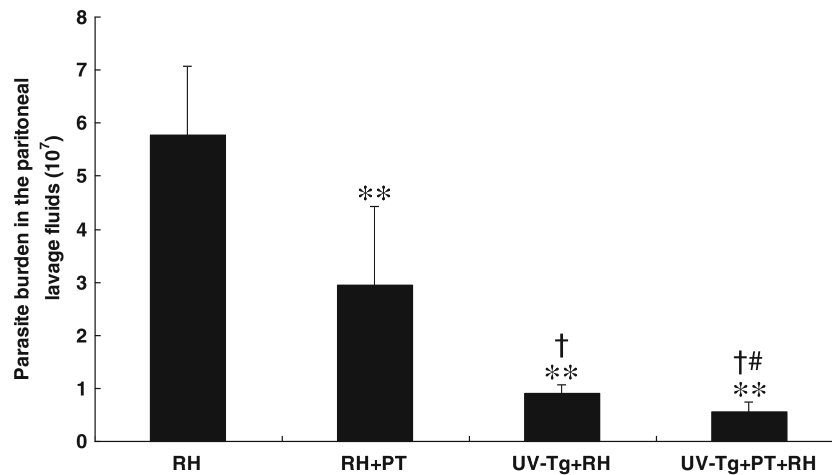

Fig. 2 Numbers of RH tachyzoites of *T. gondii* in peritoneal lavage fluids from infected or challenged mice at various times when becoming moribund after i.p. inoculation with 10^2 RH tachyzoites of *T. gondii*. Data are mean±SEM; $n=4$ mice per group, and the data are representative of two experiments. Symbols indicate statistically significant differences ($P<0.01$) for comparison with the uninfected control mice (*double asterisk*), the infected mice+pidotimod (*dagger*), and the UV-*T.g* vaccinated and then challenged mice (*number sign*)

(Fig. 3f) and some amount of RH tachyzoites (Fig. 3g) were observed in the liver tissues of infected mice with pidotimod treatment at 9 days p.i., and mild damage (mild inflammation) (Fig. 3h and i) were observed in the liver tissue of mice vaccinated with UV-*T.g* and then challenged with RH strain of *T. gondii* at 30 days post-challenge; whereas even milder histological evidence of liver inflammation (Fig. 3j and k) were observed in mice vaccinated with UV-*T.g*+PT and then challenged with the parasites at 30 days post-challenge.

Improved histological score

Quantitative analysis of the severity of inflammation and necrosis of liver sections (e.g., the number of inflammatory foci per field, three slides/animal) of different groups of mice was performed (Fig. 4). The results showed that a great number of large-sized inflammatory foci of neutrophil infiltrates were observed in the liver of infected control mice. In comparison, a significantly decreased number of medium-sized inflammatory foci of neutrophil infiltrates were observed in the infected mice with pidotimod treatment ($P<0.01$); however, there were significantly lower number of small-sized inflammatory foci in UV-*T.g*-immunized and then challenged mice ($P<0.01$) and even significantly lower number of small-sized inflammatory foci in UV-*T.g*+PT immunized and then challenged mice ($P<0.01$). UV-*T.g* alone could cause little or no inflammatory reaction at the early vaccinated stage (data not shown).

Enhanced Th1 mRNA cytokine responses in spleen

Cellular immunity was evaluated by measurement of cytokines IL-2, IFN-γ, and TNF-α in this study. As shown in Fig. 5, compared with naive control mice, mRNA levels of Th1 cytokine (IL-2, IFN-γ, and TNF-α) were significantly increased in uninfected mice treated with pidotimod ($P<0.01$). When compared with the infected controls, the infected mice treated with pidotimod produced significant higher levels of Th1 cytokine (IL-2, IFN-γ, and TNF-α) ($P<0.01$). When compared with mice immunized with UV-*T.g* alone, mice immunized with UV-*T.g* and then challenged with *T. gondii*, produced significant higher levels of Th1 cytokine (IL-2, IFN-γ, and TNF-α) ($P<0.01$); while compared with mice immunized with UV-*T.g* and then challenged with *T. gondii*, mice immunized with UV-*T.g*+PT and then challenged with *T. gondii* produced even significant higher levels of Th1 cytokine (IL-2, IFN-γ, and TNF-α) ($P<0.01$).

Increased serum *T. gondii*-specific IgG antibody response

Serum collected from each mouse was assessed for *T. gondii*-specific IgG antibody. As shown in Fig. 6, compared with uninfected controls, mice infected with *T. gondii* alone, infected mice with pidotimod treatment, mice immunized with UV-*T.g* alone, immunized with UV-*T.g* and then challenged with *T. gondii*, and immunized with UV-*T.g*+PT and then challenged with *T. gondii*, all produced significant higher titers of *T. gondii* antigen-specific IgG antibody ($P<0.01$). However, although uninfected mice treated with pidotimod did not induce increased antigen-specific IgG, infected mice with pidotimod treatment produced significantly increased *T. gondii*-specific IgG in comparison with mice infected alone ($P<0.01$). In addition, UV-*T.g*+PT immunized mice and then challenged with *T. gondii* produced significant higher titers of *T. gondii*-specific IgG antibody in comparison with mice immunized with UV-*T.g* and then challenged with *T. gondii* ($P<0.01$). It suggested that

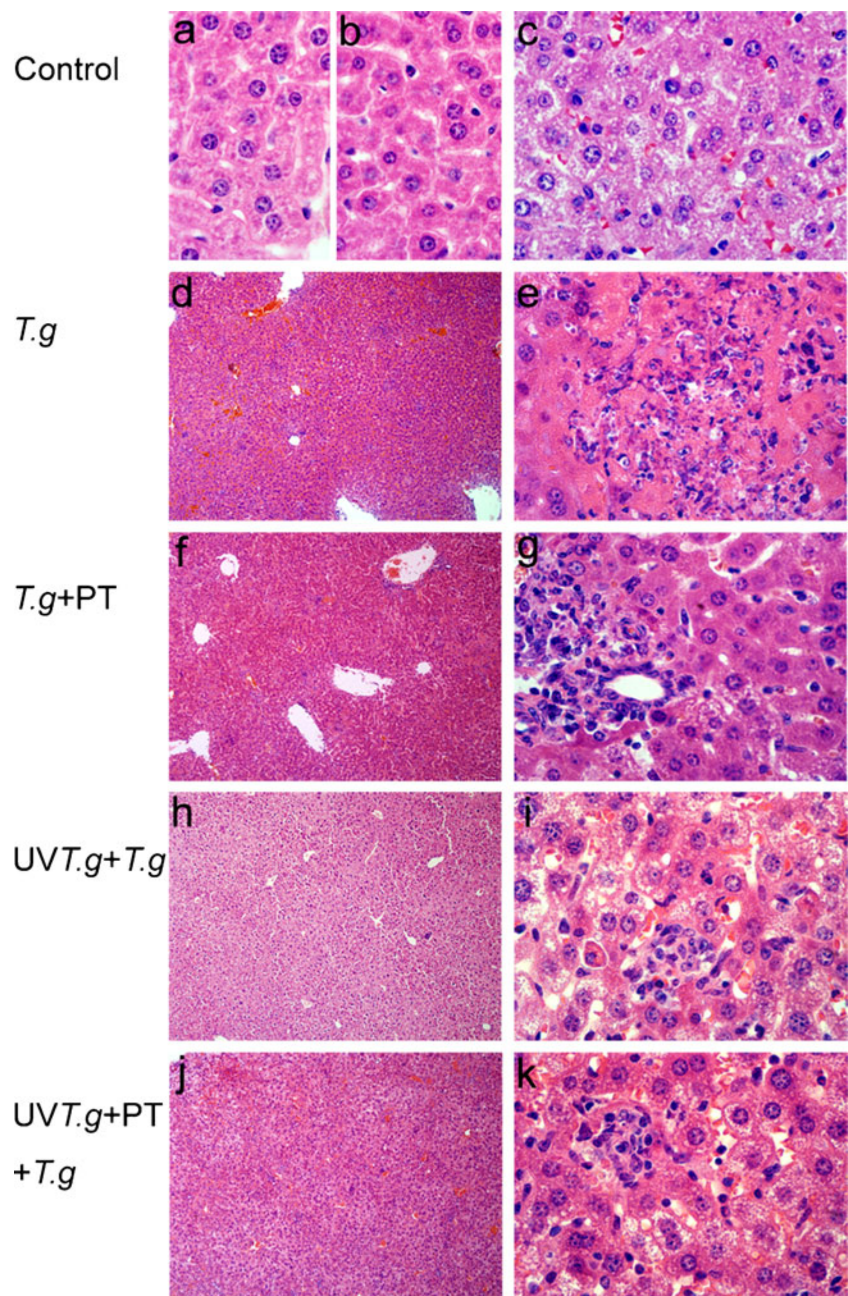

Fig. 3 Liver pathology. Panels: naive mouse (**a**); uninfected mouse treated with pidotimod at 30 days (**b**); mouse vaccinated with UV-*T.g* alone at 40 days post immunization (**c**); primarily i.p. infected mouse with 10^2 RH tachyzoites of *T. gondii* at 7 days p.i. (**d** and **e**); mouse infected with 10^2 RH tachyzoites of *T. gondii* and with pidotimod treatment at 9 days p.i. (**f** and **g**); UV-*T.g* vaccinated mouse and challenged with 10^2 RH tachyzoites at 30 days post-challenge (**h** and **i**), and UV-*T.g*+pidotimod vaccinated mouse and challenged with 10^2 RH tachyzoites at 30 days post-challenge (**j** and **k**). There were four mice per group, and data are representative of those from two experiments. Magnification, **d**, **f**, **h**, and **j** ×10, and **a**, **b**, **c**, **e**, **g**, **i**, and **k** ×100; H&E stain

pidotimod could stimulate specific humoral immunity in *T. gondii*-infected or -challenged murine host.

Discussion

Toxoplasmosis can lead to severe pathology in both humans and animals, and immunity to infection relies on the development of a strong cell-mediated immune response (Denkers and Gazzinelli 1998). Vaccination is a promising approach to protect animals and humans against pathogenic microorganisms, in particular intracellular parasites (Bunnell and Morgan 1998). Currently, curative medicines for the untreatable latent bradyzoite form of the parasite are not available, and a vaccine to prevent infection with this parasite is being sought (Hencken et al. 2010; Machado et al. 2010; Suzuki et al. 2010). So far only a few live, attenuated *T. gondii* strains used for immunization have been able to confer protective immunity and used in farm animals during these years (Innes et al. 2009). However, such a vaccine is not suitable for human use due to the potential reactivation to the pathogenic form. The main goal of immunization is the induction of a protective immune response against the pathogen, and successful vaccination strategies for *Toxoplasma* have relied on a live

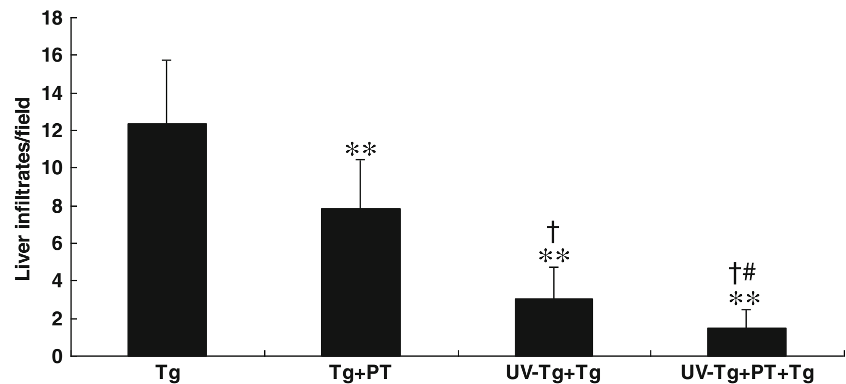

Fig. 4 Histological score analysis. Mice were killed at various times when becoming moribund, and the liver was obtained for histological analysis. Data are mean±SEM; n=4 mice per group. Symbols indicate statistically significant differences (P<0.01) for comparison with the uninfected control mice (*double asterisk*), the infected mice+pidotimod (*dagger*), and the UV-*T.g* vaccinated and then challenged mice (*number sign*)

vaccination approach using attenuated parasites which allows processing and presentation of antigen to the host immune system to stimulate appropriate cell-mediated immune responses. The effective immune response is mediated by $CD4^+$ and $CD8^+$ T cells and is associated with the production of IFN-γ (Gazzinelli et al. 1991; 1994). Pidotimod is a synthetic substance capable of modulating immune cell function, which has no clinical, hematological, or biochemical side-effects noted (La Mantia et al. 1999). Our previous study indicates that UV-attenuated tachyzoites of *T. gondii* may provide a novel tool for vaccine development (Yang et al. 2010). KM mice are the most widely used outbred colony in China, starting from Swiss mice brought to Kunming, China, from the Indian Haffkine Institute in 1944 (Shang et al. 2009). In the present study, we further evaluated the immune response induced by UV-attenuated *T. gondii* vaccine with pidotimod as adjuvant and then challenged with tachyzoites of highly virulent RH strain of *T. gondii* in KM mice. Our data demonstrated that the protective immunity of UV-attenuated *T. gondii* could be markedly enhanced by pidotimod co-administration. Our results indicate that co-administration of pidotimod can further enhance the potency of UV-*T. gondii* vaccine.

It has been reported that pretreatment of mice with irradiation-killed *Toxoplasma* induces resistance to challenge with virulent organisms (Bakal and in 't Veld 1979). Mice immunized with 200 Gy gamma-irradiated *T. gondii* RH tachyzoites, which lose their reproductive ability and maintain metabolic function, has extended murine survival time after subsequent tachyzoite challenge, and has minimal cerebral pathology after cyst challenge; the splenocyte stimulation by *T. gondii* antigen produce lymphoproliferation and cytokines (such as IL-10, IL-12, IFN-γ, and TNF-α) similar to those produced by chronic natural infection (Hiramoto et al. 2002). Similarly, in the current study, we found that the UV-attenuated tachyzoites of RH strain *T.*

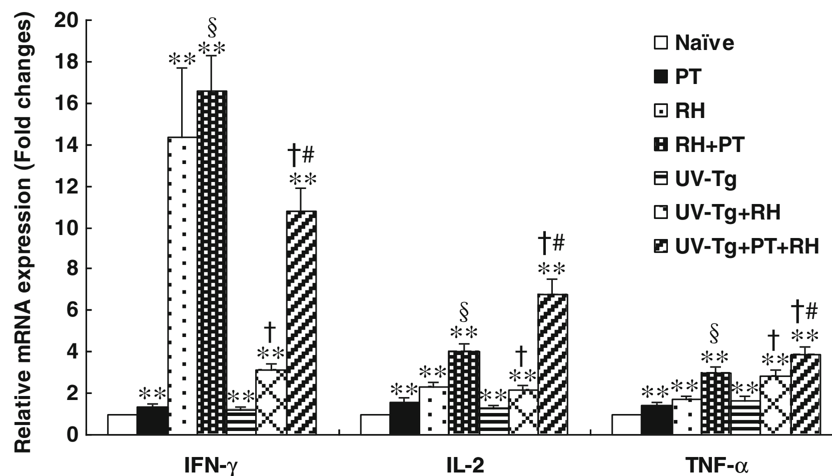

Fig. 5 Cytokine mRNA expression in spleens were analyzed by qRT-PCR. Mice were infected or challenged by i.p. injection with 10^2 RH tachyzoites, and were killed at various times when becoming moribund. Values are means from triplicate measurements, and the values are shown as a fold change to the uninfected controls. Symbols indicate statistically significant differences (P<0.01) for comparison with the uninfected control mice (*double asterisk*), the infected mice (*section symbol*), the UV-*T.g* vaccinated mice without challenge (*dagger*), and the UV-*T.g* vaccinated and then challenged mice (*number sign*). There were four mice per group, and data are representative of two separate experiments

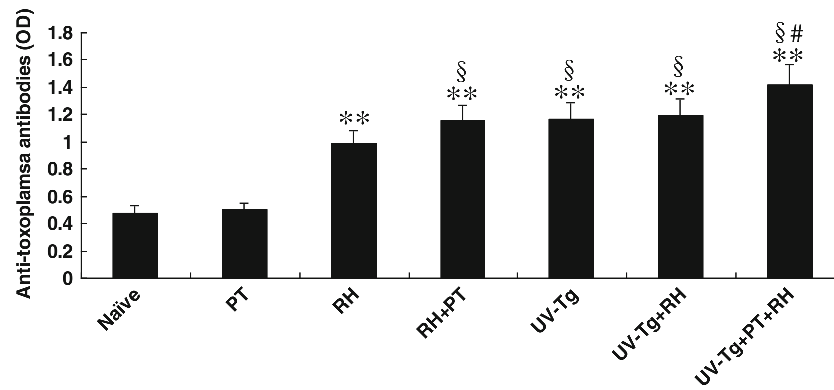

Fig. 6 Levels of anti-toxoplasma IgG in sera. Mice were infected or challenged by i.p. injection with 10^2 RH tachyzoites, and sera were taken from mice at various times when becoming moribund. Symbols indicate statistically significant differences ($P<0.01$) for comparison with the uninfected control mice (*double asterisk*), the infected untreated mice (*section symbol*), and the UV-*T.g* vaccinated and then challenged mice (*number sign*). Four mice were used in each group, and these data are representative of two separate experiments

gondii used did not multiply in vivo and induced no obvious liver pathology. It has been reported that pidotimod has shown biological and immunological activity on both the adaptive and innate immune responses (Riboldi et al. 2009). Pidotimod is able to positively affect certain pathologic disturbances associated with significant changes in immune response (Zervoudis et al. 2010). In addition, pidotimod stimulates the release of cytokines and drives T cell proliferation, and displays adjuvant properties at the nasal mucosa level in vivo (Giagulli et al. 2009). In this study, we investigated the feasibility of using pidotimod as an adjuvant combined with UV-attenuated *T. gondii* as a vaccine against acute murine toxoplasmosis. Protection against challenge by the virulent RH strain of *T. gondii* was mainly assessed by monitoring two parameters, survival rate and parasite burden in peritoneal lavage fluids. Compared with infected controls, we found that the severity of the infection was markedly reduced by pidotimod treatment, and *T. gondii*-infected mice with pidotimod treatment displayed significant higher survival rate and longer survival time, associated with significant decreased parasite burden in their peritoneal lavage fluids, and improved liver histopathological scores ($P<0.01$). Although all the pidotimod-treated mice died of virulent RH strain infection eventually, the protection was markedly enhanced and the severity of the infection was markedly reduced by pidotimod co-administration. The efficacy of pidotimod has been previously shown in vulvar papillomatosis with a good safety profile (Guerra et al. 1998). Meanwhile, our data also showed that, mice immunized with UV-attenuated *T. gondii* and subsequently challenged with viable parasites, had significantly higher survival rate and extended survival time, minimal liver pathology, associated with significantly decreased parasite burden in peritoneal lavage fluids. Consequently, mice co-administered with UV-*T. gondii*+pidotimod and then challenged showed even lower parasite count in peritoneal lavage fluids and milder liver pathology. Our results demonstrated that the immune efficacy induced by UV-*T. gondii*+pidotimod was better than that induced by UV-*T. gondii* alone.

T. gondii parasites multiply within the cells of their host species and a Thl response is believed to play a key role in early protective immunity mainly by an IFN-γ driven CTL response (Filisetti and Candolfi 2004). Th1-type response plays a major role in controlling both acute and chronic *T. gondii* infections (Gazzinelli et al. 1991), and $CD8^+$ T cells and IFN-γ protect against chronic *Toxoplasma* infection and challenge infections in previously immunized mice (Casciotti et al. 2002). In this study, we observed that pidotimod could stimulate a Th1-oriented immune response in *T. gondii*-infected mice, and mice vaccinated with UV-*T. gondii*+ pidotimod and then challenged with *T. gondii*, characterized by enhanced spleen IL-2, IFN-γ, and TNF-α mRNA expressions, and enhanced serum production of toxoplasma-specific IgG antibody in vivo. Th1 responses remained dominant after challenge in the mice till the end of the observation. Our previous study demonstrated that B cells are also necessary for vaccine-mediated protection against ocular challenge with virulent RH *T. gondii*, and the potential role for their antibody production is in limiting for parasite burden (Lu et al. 2009). It has been reported that when mice were administered with pidotimod, B cells are able to induce antibodies in humoral immunity (Coppi and Manzardo 1994). Various adjuvants and delivery systems have been evaluated for increasing protective immune responses of vaccines (Tafaghodi et al. 2011). Our results indicate that pidotimod can promote both Th1 and humoral immune responses, providing an effective Th1 adjuvant for use in vaccination against acute toxoplasmosis and significantly enhanced the protection and markedly reduced parasite burdens after both infection and challenge, which is desirable for vaccination against intracellular pathogens. The capability of pidotimod to improve antigen presentation in vivo to cells of adaptive immunity after intranasal delivery

may make this molecule an adjuvant for the development of mucosal vaccine formulations (Giagulli et al. 2009). Therefore, our data in this study further demonstrated that pidotimod has vaccine adjuvant activity.

In conclusion, our study demonstrated that combination of pidotimod and UV-*T. gondii* vaccine are synergistic for improving immune activity, which may provide an additional benefit of better efficacy against infection of *T. gondii*. Pidotimod is safe and easy for human use, an advantage over many of the vaccine trials to be a toxoplasma vaccine only when inoculated with Th1 cytokines. Thus, pidotimod, as an adjuvant, may be a valuable component of a future vaccine against toxoplasmosis.

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