## ORIGINAL PAPER

# Gerbu adjuvant modulates the immune response and thus the course of infection in C56BL/6 mice immunised with *Echinococcus multilocularis* rec14-3-3 protein

**Maxi Margos · Bruno Gottstein**

**Abstract** Vaccination with *Echinococcus multilocularis* 14-3-3 protein can protect mice against primary *E. multilocularis* infection. The present study investigated the efficacy and efficiency of the adjuvant muramyl dipeptide Gerbu, alone or together with recombinant 14-3-3 protein, to modulate the course of secondary *E. multilocularis* infection in C56BL/6 mice. The application of Gerbu alone already resulted in a parasite weight reduction when compared with infected control mice, while rec14-3-3 did not add to this effect. Immunological parameters were concurrently assessed with a mixed cell reaction including bone marrow-derived dendritic cells (BMDCs) together with lymph node cells from mice with or without immunisation and/or infection. While mice having received Gerbu adjuvant were found to highly proliferate in response to co-cultivation with 14-3-3-stimulated bone marrow dendritic cells, a sensitisation of BMDCs with vesicle fluid (VF) antigen lead to a striking decrease of the lymphoproliferative response in comparison to that of control mice, raising the hypothesis that immunosuppressive components may be part of this VF-antigen. Anti-14-3-3 antibody production was only found in those mice that had been previously 14-3-3-immunised, whereas all other only-infected mice failed to produce such antibodies. Conclusively, Gerbu adjuvant appears to directly generate a non-specific immune response that contributes to the control of the metacestode growth, putatively in association with a BMDC activity suppressed by components of the VF-antigen.

M. Margos · B. Gottstein (✉)
Institute of Parasitology, University of Berne,
Länggass-Strasse 122,
CH-3012 Bern, Switzerland
e-mail: bruno.gottstein@ipa.unibe.ch

## Introduction

Secondary experimental infection with *Echinococcus multilocularis* in mice goes along with an intense host–parasite interplay orchestrated predominantly by the parasite in view to provide survival (Bresson-Hadni et al. 1990; Emery et al. 1996; Vuitton et al. 1989). The proliferating metacestode of *E. multilocularis* consists of an inner, germinal layer, representing the living parasite, sheathed by an outer acellular laminated layer providing protection against effector mechanisms of the host (Gottstein and Hemphill 1997). Protection may also depend on immune-modulatory effects of parasite metabolites (Gottstein and Hemphill 1997; Meijri et al. 2010). How this modulation or evasion is achieved is not yet fully elucidated, either for *Echinococcus granulosus* (Diaz et al. 1997) or for *E. multilocularis* (Vuitton and Gottstein 2010). Known parasite molecules of respective relevance include, e.g. a carbohydrate antigen, Em2(G11), which acts as an T cell-independent antigen and therefore inhibits maturation of antibody avidity (Dai et al. 2001). Walker et al. (2004) found the Em492-antigen, a carbohydrate molecule with biological activity, supposed to exhibit immuno-supressive properties at the host–parasite interface. Meta-cestodic antigens [such as vesicle fluid (VF) from *E. multilocularis*] contain substances responsible for the inactivation of eotaxin by proteolytic activity in vitro. This inactivation may be relevant for the parasites survival, as eotaxin works as a main activator and chemo-attractant for eosinophils, and the impairment of eosinophils to infiltrate the site of parasite residence seems to be an important parameter in avoiding the host defense (Mejri and Gottstein 2009). Similar other studies revealed that other secreted metacestode products influence the outcome of an infection in favour of the parasite as well

(Allen and MacDonald 1998; Lightowlers and Rickard 1988; Pastrana et al. 1998).

In the case of primary (peroral egg) *E. multilocularis* infection, first trials had revealed that rec14-3-3 is able to induce protection in mice against the oncosphere, but not against a mature larval metacestode (Siles-Lucas et al. 2003). The protective immune response, yielded upon immunisation with Saponin as adjuvant, was not polarised, e.g. specific antibodies as well as cytokines accounting for both Th1 and Th2 orientation were found (Siles-Lucas et al. 2003). In the present study, we addressed the question, how a more potent adjuvant (Gerbu adjuvant) interacts with the host immune system, in relation to different types of *E. multilocularis* antigens.

## Materials and methods

### Mice

Female C56/BL6 mice were purchased from Charles River, Germany. Average age of animals was 8–10 weeks when mice received the first immunisation. All mice were handled under standard animal laboratory conditions according to Swiss regulations for animal experimentation.

### Parasite antigens

*E. multilocularis* VF-antigen was obtained by gentle disruption of in vitro maintained vesicles, following centrifugation and recovery of supernatant (Ingold et al. 1999). The protein concentration of the VF batch used in the present study was 0.94 mg/ml. *E. multilocularis* rec14-3-3 antigen was obtained and purified as described before (Dai and Gottstein 1999; Siles-Lucas et al. 1998), the batch concentration was 0.17 mg protein per millilitre.

### Immunisation of C57BL/6 mice and infection with *E. multilocularis* metacestodes

For immunisation 10 µg rec14-3-3 were mixed 1:1 with Gerbu adjuvant (GMDP; Biotechnik GmbH, Gaiberg, Germany) to yield a final inoculation volume of 100 µl per injection. Each group of mice ($n$=5) were subcutaneously injected at the shoulder site. A control group received a mixture of ovalbumin (10 µg per injection, Dr. Grogg AG) and Gerbu, and another group Gerbu mixed with an equal volume of phosphate-buffered saline (PBS). Non-treated control mice received PBS only. Booster injection ensued 2 weeks later. Another 14 days later, appropriate groups of mice were infected intraperitoneally with *E. multilocularis* clone KF5as described before (Dai and Gottstein 1999); the other groups

remained non-infected. Three months post-infection mice were killed by CO2-euthanasia.

### Parasite weighing

The parasite tissue (metacestode mass) was removed carefully from the peritoneal cavity, freed of any attached hosts' tissue and weighted.

### Cultivation of BMDCs

Naive C56BL/6 animals were killed and bone marrow progenitors were isolated and cultured as described elsewhere (Lutz et al. 1999). Culturing took place in Petri dishes (Greiner, 145/20) with feeding of the cells every 3 days with 5 ml RPMI 1640 medium (GIBCO Invitrogen) and 200 U rh GM-CSF (Immunotools, Friesoythe, Germany). On day 9, non-adherent cells were harvested and, after counting, adjusted to a concentration of $1\times10^6$ cells per millilitre. Cells were plated in 12-well plates and supplemented with medium without GM-CSF for further experiments. Cell preparation contained 75% to 80% CD11c$^+$ bone marrow-derived dendritic cells (BMDCs) as determined with fluorometric analysis.

Stimulation of immature DCs was carried out as described below using the following antigens:

- VF (2 µg/ml)
- Rec14-3-3 antigen (1 µg/ml)
- 100 ng/ml lipopolysaccharide (LPS) as a positive control
- Appropriate volume of medium alone as a negative control

Immature dendritic cells (DCs) were exposed to stimulation with the antigens as described above and maintained for 16–18 h. Cells were subsequently collected and washed with warm supplemented medium.

### Cell proliferation assay

Lymph node (LN) cells (renal, inguinal, lumbar, and brachial) from control and treated (immunised with rec14-3-3, Gerbu, ovabulmin, and PBS and later with or without *E. multilocularis* metacestode infection) C56/BL6 mice were isolated. LN cells ($1\times10^6$/ml) were co-cultivated with BMDCs ($1\times10^5$/ml) from naive or *E. multilocularis*-infected mice. These BMDCs had been cultured before in medium alone or in medium plus stimulation with LPS or the three parasite antigens for 18 h and washed thereafter. After 5 days of co-cultivation, cells were pulsed with 1 µCi/well $^3$H thymidine. After 18 h, the cells were harvested onto glass fibre filters and activity measured in a beta counter (PerkinElmer) upon thymidine incorporation.

## IgG ELISA

Sera of mice were obtained on the day before infection and on the day of being killed. Sera of day 0 (before vaccination) served as negative controls, and positive control sera were used from animals of a previous experiment (Siles-Lucas et al. 1998). The presence of anti-*E. multilocularis* 14-3-3 and anti-Em2(G11) [outgroup control antigen] IgG antibodies was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (Dai and Gottstein 1999; Siles-Lucas et al. 1998). Alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, Duebendorf, Switzerland), anti-IgG1, or anti-IgG2a (both from Southern Biotechnology Associates, Birmingham, USA) was used as second antibody (conjugate). Absorbance was measured at 405 nm ($A_{405}$, reference filter 630 nm) using a Dynatech MR 7000 ELISA reader and the corresponding Dynatech Biocalc software (Dynatech, Embrach, Switzerland). The threshold value arbitrarily discriminating between "positive" and "negative" (cutoff) was defined by adding three standard deviations to the mean $A_{405}$ value of the sera from non-infected and non-treated control mice.

## Results

### Parasite weight

The average parasitic mass recovered from PBS-control and subsequently, from *E. multilocularis*-infected mice was 1,930 mg (Fig. 1). Rec14-3-3-immunised-and-infected mice disclosed a significantly lower parasite weight with an average of 368 mg, as well as mice "control-immunised" with Gerbu adjuvant only and subsequently infected with *E. multilocularis* (average 442 mg; Fig. 1).
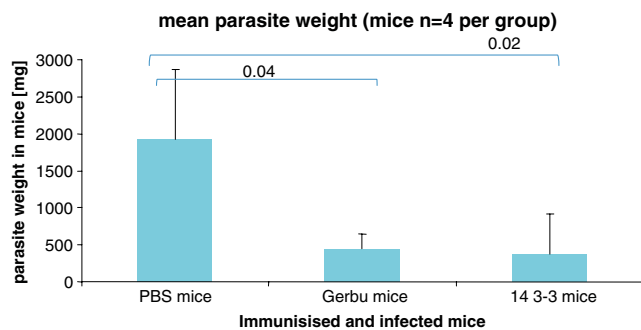


Fig. 1 Parasite weight recovered from C56BL/6 mice experimentally infected with *E. multilocularis*. Mice were either mock-immunised with PBS only or received Gerbu adjuvant supported immunisation with or without rec14-3-3-antigen. *Horizontally labelled bars* indicate *p* values for statistical significances

## Lymphoproliferative responses

The background proliferation activity of LN cells from 14-3-3 immunised mice was slightly elevated after the contact with immature BMDCs (Fig. 2a), indicating a potential contamination of the recombinant antigen with bacterial lipopolysaccharides. Otherwise, all control reactions with immature BMDCs yielded expected low background reactivities. LPS-matured BMDCs originating from all mouse groups resulted in an elevated LN cell proliferation when compared with the corresponding activities of
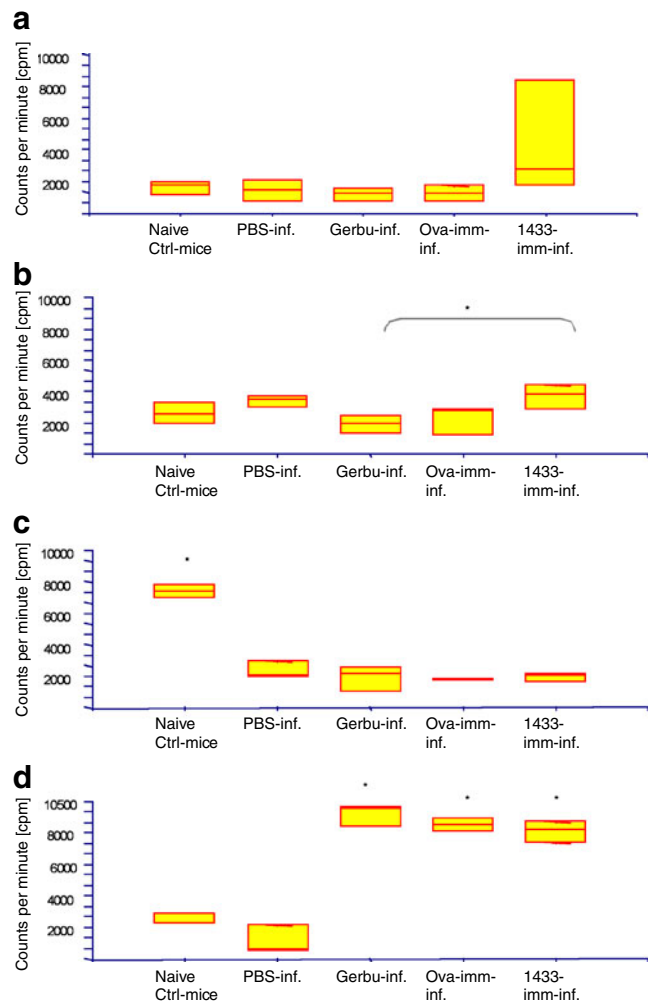


Fig. 2 Mixed BMDCs–lymphoproliferative assays. BMDCs were initially either kept with medium alone (immature DCs, iDCs) (a), or challenged for 16–18 h with LPS (b), VF (c), or rec14-3-3 (d), all iDCs were subsequently co-cultured with LN cells isolated from the following mouse groups: non-immunised/non-infected mice (*naive Ctrl mice*); *E. multilocularis*-infected mice: initially treated with PBS as a control (*PBS-inf.*), Gerbu-alone (*Gerbu-inf.*), ovabulmin plus Gerbu- (*Ova-imm-inf.*), or 14-3-3-plus Gerbu (*14-3-3-imm-inf*). Mice were killed 3 months after infection, control mice also on the same day. Incorporation of tritium-labelled thymidine was expressed as counts per minute (cpm). Statistical significances are labelled with an * and were at $p < 0.05$

immature DCs (Fig. 2b). The highest reaction to LPS-maturated DCs was again seen in the 14-3-3 LN cells. VF-presentation by BMDCs resulted in high proliferative activity of only LN cells from naive mice (significantly higher to all other groups), whereas all other mouse groups exhibited only moderate to low proliferative responses (Fig. 2c). BMDCs stimulated with 14-3-3 resulted in a high proliferative response of LN cells from all three immunised-and-infected mice (all co-exposed to the Gerbu adjuvant). Conversely, LN cells from non-infected control animals and PBS-control-and-infected mice demonstrated an only very moderate response.

IgG ELISA

Serological analyses were carried out after 14-3-3-immunisation and later after immunisation plus 3 months of infection. ELISA was used to determine anti-14-3-3 IgG antibodies and antibodies to an outgroup control antigen [Em2(G11)]. Mice immunised with rec14-3-3 without or not yet infected showed no antibody production to control Em2(G11)-antigen, while 14-3-3-immunised mice, as expected, presented a marked anti-14-3-3-specific antibody reactivity (Fig. 3b). All infected mice revealed a marked anti-Em2(G11) antibody response, while anti-rec14-3-3 antibodies were only detectable in mice previously immunised with 14-3-3 (Fig. 3a), thus demonstrating that

intraperitoneal *E. multilocularis* infection alone does not yield an anti-14-3-3-antibody production.

## Discussion

Former studies (Reuben and Tanner 1983; Siles-Lucas et al. 2003) provided evidence of distinct differences between the initial anti-oncospheral immune response and the subsequent immune response directed against an established, maturated and fully active metacestode of *E. multilocularis*. Siles-Lucas et al. (2003) also showed that vaccination with rec14-3-3 yielded a good protection against primary *E. multilocularis* infection (peroral egg infection), while this immunity was ineffective against secondary infection (intraperitoneal inoculation of mature metacestodes). The interplay between a mature metacestode (chronic stage of infection/disease) and its murine host was intensively studied by multiple research groups (reviewed, e.g. by Gottstein and Hemphill 2008; Mejri and Gottstein 2009; Vuitton 2003) and had provided insight into the mode the parasite is involved in the modulation of the host immunity. The question we now presently addressed was if 14-3-3, though not inducing protection in mice secondarily infected with the metacestode, influences nevertheless the immune response thus as to yield a different immune recognition pattern when compared with non-immunised animals. During our first experiments, we
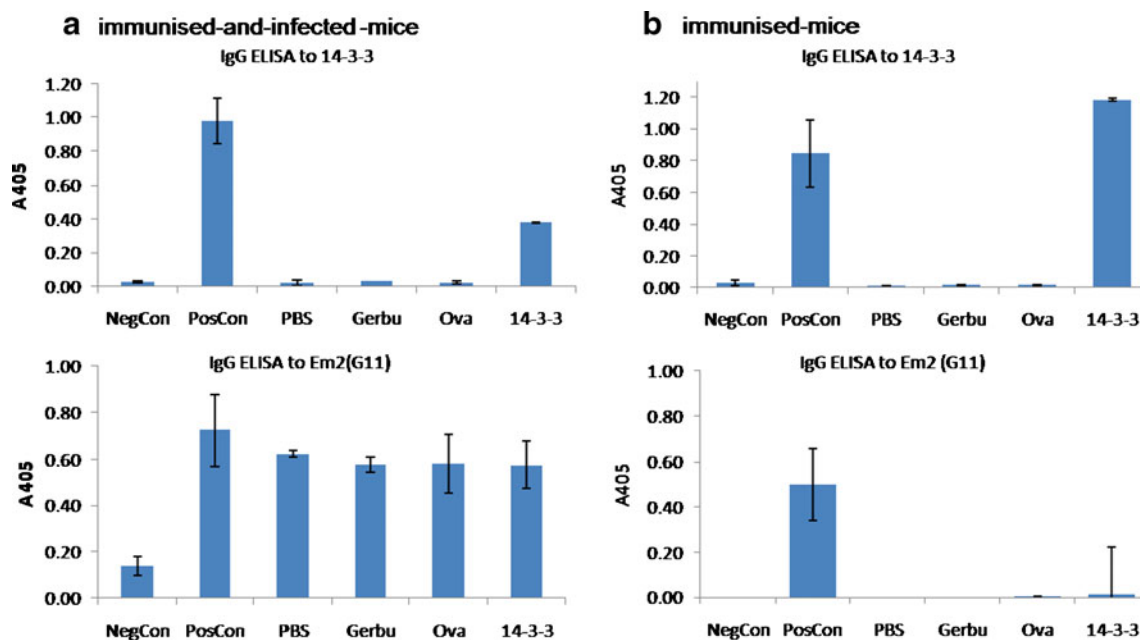


**Fig. 3** ELISA-based analyses of serological antibody reactivities against rec14-3-3-antigen or against an outgroup control antigen Em2 (G11). **a** immunised-and-infected mice, **b** mice only immunised. Positive and negative control sera are labelled as *NegCon* and *PosCon*, respectively. *PBS*-group: mice receiving a mock-

immunisation with PBS alone; *Gerbu*-group: mice receiving Gerbu adjuvant without antigen; *Ova*-group: mice immunised with Gerbu and ovalbumin; *14-3-3*-group: mice immunised with Gerbu and rec14-3-3-antigen

replaced the formerly used adjuvant saponin (Siles-Lucas et al. 2003) with the synthetic immunoadjuvant Gerbu. Gerbu is a muramyl dipeptide (MDP) representing the smallest subunit of bacterial cell walls still active as an adjuvant (Adam and Lederer 1984). Gerbu originates from Gram-positive cell walls and displays a good alternative to common adjuvants, as it can be injected directly and safely in vivo. Conversely to saponin, immunisation with Gerbu and subsequent secondary infection yielded in a reduction of metacestode growth when being compared with non-immunised alveolar echinococcosis (AE)-infected mice. This effect was independent of an additional use of antigen such as rec14-3-3 that we employed in the present study. At the early beginning of immunological *E. multilocularis* research, Reuben et al. (1978) had already revealed that the use of Bacillus Calmette-Guérin (BCG) (originating from an attenuated *Mycobacterium bovis* strain and inducing variable protection against tuberculosis and cancer) and also its cell wall skeleton (CWS) were inducing protection in cotton rats against the metacestode stage of infection. The use of lipoprotein from *Mycobacterium tuberculosis* is known to induce an effective Th1 cell-mediated immune response by TLR2 activation (Thoma-Uszynski et al. 2001), and so protection may be associated with the induction of a proper Th1 response. The former findings upon use of BCG or BCG-CWS had revealed a dose-dependent degree of protection; a low dose was not effective while a too-high dose formed tubercular granulomas in the host. So far, we have not addressed a dose-dependence of the Gerbu-effect in experimental murine AE. So far, most groups agreed that secondary murine AE includes a mixed Th1/Th2 response, so the working hypothesis discussed by several authors was that partial protection (=reduced metacestode proliferation) may be associated with a rather Th1-orientation of immune response. A Th2-orientation may be in favour of metacestode proliferation (Amiot et al. 1999; Emery et al. 1996; Godot et al. 2003; Jenne et al. 1998; Liance et al. 1998).

The results showing reduced parasite masses in both Gerbu and Gerbu-rec14-3-3 immunised mice are in congruence with those of the lymphocyte proliferation assays. We found that stimulation of BMDCs cultivated with rec14-3-3 protein of *E. multilocularis*, and the following combination of these cells with LN cells from Gerbu-treated mice resulted in antigen recognition and so to a significant LN cell proliferation. Conversely, non-Gerbu-treated groups showed no reactivity to 14-3-3 stimulation. This raises the question whether this adjuvant does somehow interfere with the parasite or the mode the parasite exerts immune modulation of the host. Investigation of the humoral immune response also provided data as expected. First, sera obtained after rec14-3-3-immunisation showed the presence and thus proved synthesis of anti-rec14-3-3 IgGs, whereas no antibodies against the outgroup

control antigen Em2(G11) were yet detectable. Anti-Em2 (G11) IgG levels were positive in the sera of all immunised-and-infected or only-infected mice, while naive (non-infected control) mice produced no anti-Em2(G11) antibodies, as expected. Immuno-stimulating activity of vaccine adjuvants, such as muramyl dipeptides, may not only orchestrate the mode of antigen delivery to immunological sites, but may also influence the induction of secondary signals like B-7 family receptors and CD40 on APCs, directly or indirectly (Schijns 2000). This will be the focus of forthcoming studies we will carry out on the expression of co-stimulatory molecules compared between BMDCs from naive versus infected mice after different stimulations. MDP and derivates are strong inducers of a variety of cytokines such as IL-1, IL-6, TNFα, and IFN-γ in mice and humans and are therefore activating different cell types including Th1, Th2, or Th17 (de Jong et al. 2010). Another point of investigation we plan to undertake is to analyse the cytokine production pattern of cultured BMDCs stimulated in vitro with Gerbu adjuvant. The recognition of rec14-3-3 by LN cells from Gerbu-treated mice may be induced by unblocking of certain receptors. An interesting presumption by Rock et al. (1998) is that mycobacterial adjuvants do serve as ligands for receptors on immune competent cells, inducing marked cellular responses. At an early beginning of AE infection, the parasite is assumed to already start its modulation of the hosts' immune response such as to allow its subsequent survival. The accompanying innate immune system, largely non-specific and constitutively expressing pathogen recognition receptors for the recognition of pathogen-associated microbial patterns (PAMPs), may also be involved in the subsequent modulation of the specific immune response (Gerard et al. 1998). In vivo, *E. multilocularis* is expressing the carboxylated protein 14-3-3 in its germinal layer. In our present study, however, this antigen was presented by respectively in vitro-stimulated BMDCs such as to induce a subsequent lymphocyte proliferation. BCG is known to activate multiple Toll-like receptors (TLR; Azuma and Seya 2001); Gerbu also may function as a ligand for TLRs, interfering in the parasites reaction by uncovering different TLRs, so putatively miscasting the parasites modulation efficacy and thus proliferation (growth) efficiency. In a similar context, several authors (Azuma and Seya 2001) favoured a hypothesis in which TLR receptors may induce cellular responses supported by similar signaling events. The lack of TLR ligation/activation was supposed to initiate a Th2-oriented humoral immune response, which in *E. multilocularis*-infected man and mice may—at least partially— account for the AE-specific (mixed Th1/Th2) immune orientation. Several MDP derivates also revealed the ability of re-stimulating immuno-suppressed mice, rats, and guinea pigs (Osada et al. 1982). A variety of TLRs were found to be expressed on DCs (Rock et al. 1998), allowing DCs to

recognise PAMPs, like LPS. Furthermore, a large panel of TLRs is expressed on DC surfaces, like TLR-2, which is generally thought to be the receptor for Gram-positive bacteria (Knapp et al. 2002). In view of future experiments, we may address the different steps that account for Gerbu or other adjuvants to influence the activation status of DCs from naïve or AE-infected mice. Schijns (2000) had already proposed that, beside several signals which are important for DC activation, along with antigen presentation and the delivery of co-stimulatory molecules and cytokines, adjuvants may act as an induction factor, too. Another aspect to study as a result from our findings is that of putative therapeutic effects of amphotericin B and other antibiotics, which showed synergistic effects in combination with MDP derivates (Rock et al. 1998). As treatment with amphotericin B already showed a reduction in vesicle proliferation in vitro (Reuter et al. 2003), a promising approach could arise from enhancing the treatment efficacy by co-stimulation with an appropriate adjuvant.

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