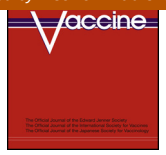
Vaccine 31 (2013) 4528–4535

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Nasal vaccination with attenuated *Salmonella* expressing VapA: TLR2 activation is not essential for protection against *R. equi* infection

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ARTICLE INFO

Article history:
Received 6 May 2013
Received in revised form 15 July 2013
Accepted 25 July 2013
Available online 7 August 2013

Keywords:
Rhodococcus equi
Vectored vaccine
VapA
Attenuated *Salmonella*
Toll-like receptors

ABSTRACT

Virulent strains of *Rhodococcus equi* have a large plasmid of 80–90 kb, which encodes several virulence-associated proteins (Vap), including VapA, a lipoprotein highly associated with disease. We have previously demonstrated that oral immunisation with attenuated *Salmonella enterica* Typhimurium strain expressing the antigen VapA (STM VapA+) induces specific and long-term humoral and cellular immunity against *R. equi*. It was shown that VapA activates Toll-like receptor 2 (TLR2) on macrophages by establishing an interaction that ultimately favours immunity against *R. equi* infection. The purpose of this study was to evaluate the immune response triggered by nasal immunisation with STM VapA+ and to determine whether TLR2 supports the vaccine effect. We developed an optimised protocol for a single nasal immunisation that conferred protection against *R. equi* infection in mice, which was manifested by efficient *R. equi* clearance in challenged animals. Nasal vaccination with STM VapA+ has also induced protection in *Tlr2*$^{-/-}$ mice and mice with non-functional TLR4. Moreover, spleen cells of vaccinated mice augmented T-bet expression, as well as the production of IL-12, IFN-γ, nitric oxide and hydrogen peroxide. Notably, the population of CD4^{+} T cells with memory phenotype significantly increased in the spleens of vaccinated mice challenged 1 or 5 months after immunisation. In these animals, the spleen bacterial burden was also reduced. When similar experimental procedures were performed in TLR2 knockout mice, an increase in CD4^{+} T cells with memory phenotype was not observed. Consequently, we conclude that nasal vaccination with attenuated *Salmonella* expressing the *R. equi* virulence factor VapA confers long-lasting protection against experimental rhodoccocosis and that TLR2 engagement was not crucial to induce this protection but may be required for a long-term immune response.

1. Introduction

Rhodococcus equi is a soil organism distributed worldwide that infects foals aged 1–6 months and causes severe granulomatous pneumonia [1]. As *R. equi* survives and reproduces inside host cells, pneumonia caused by this pathogen must be treated with lipophilic drugs over a prolonged period of time [2]. Currently, no vaccines are available, although several immunisation strategies have been tested to prevent rhodococcosis [3].

As *R. equi* harbours an 80–90-kb plasmid, proteins (Vaps) encoded by this plasmid are considered good candidates for vaccinal antigens. VapA is the most investigated immunogen encoded by this pathogenicity island and has been reported to account for *R. equi* virulence [4,5] and elicit specific immune responses [6,7]. In this context, our group has previously demonstrated that oral immunisation with live attenuated *Salmonella enterica* Typhimurium expressing VapA confers long-term protection against *R. equi*. Protection is due to the induction of strong mucosal, humoral and cell-mediated Th1-immunity, which is appropriately regulated, as verified by the profile of cytokines expressed in the organs of vaccinated mice after bacterial challenge [8,9]. These promising results have motivated us to advance studies on the vaccinal conception of VapA.

Studies on the innate immune response to *R. equi* have shown that infected macrophages with viable bacteria undergo *NFkB*

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http://dx.doi.org/10.1016/j.vaccine.2013.07.067

translocation and produce a variety of pro-inflammatory mediators, including TNF, IL-12, and nitric oxide (NO). This response was attributed to a pathway that is strongly dependent on the activation of Toll-like receptor 2 (TLR2) but not of TLR4. As purified VapA induces responses that are similar to those triggered by the whole bacteria, TLR2 activation was attributed to its interaction with VapA. These observations prompted us to investigate whether TLR2 accounts for protection conferred by vaccination with VapA.

In the present study, we standardised a new route and regimen of immunisation with the VapA antigen in the context of a *Salmonella*-vectored vaccine and investigated the role that TLR2 plays in the vaccine effect. We found that nasal vaccination of mice with a single dose of VapA-expressing *Salmonella* confers protection against *R. equi* infection via a mechanism that does not depend on TLR2 activation. Moreover, the vaccination procedure stimulates memory T $CD4^+$ cells and promotes specific immune response recall.

2. Materials and methods

2.1. Experimental animals

Groups of female mice, 6–8 weeks of age, including BALB/c, C3H/HeJ, C3H/HePAS, *Tlr2*$^{-/-}$ and wild type (WT) C57BL/6 mice (five mice per group), were housed under specific pathogen-free conditions in the Animal Research Facilities of the Medical School of Ribeirão Preto-USP. The studies were conducted as required by the Ethics Committee on Animal Research of the University of São Paulo.

2.2. Bacterial strains

The attenuated *S. enterica* Typhimurium χ3987 strains, carrying either the VapA antigen (STM VapA+) or the empty vector (control VapA−), were grown and prepared for the immunisation of animals as described by Oliveira et al. [9]. The virulent strain of *R. equi* (ATCC 33701) was grown and the titre of the challenge inoculum was calculated as described by Oliveira et al. [8].

2.3. Immunisation and challenge protocol

Groups of animals were immunised with a single dose or two doses of attenuated *Salmonella* harbouring the VapA antigen via intranasal route (1×10^9 CFU, 5 μL/animal). As a control, groups were inoculated with attenuated *Salmonella* carrying an empty vector according to the same schedule.

The challenges were conducted using an ATCC virulent, *R. equi* strain 33701 30 days after the first immunisation. In the Balb/c recall experiments, the animals were challenged 5 months after immunisation. In contrast, in the C57Bl/6 recall experiments, WT and *Tlr2*$^{-/-}$ animals were challenged 120 days after immunisation.

Organs were collected 5 days after challenge for *R. equi* clearance evaluation and other analyses as described below.

2.4. Estimation of bacterial clearance in the organs of infected mice

Quantification of viable *R. equi* recovered from the spleen and liver of challenged mice was performed on day 5 post-infection as reported elsewhere [8]. Briefly, 30 days after the first immunisation procedure with STM VapA+, mice were infected with 4×10^6 CFUs (Colony Forming Units) of virulent *R. equi* via intravenous route. Five days after the challenge, spleen and liver were collected and aseptically homogenised. To determine the number of CFUs, aliquots of 100 μL of the homogenates were serially diluted in PBS, plated onto BHI agar in duplicate, and incubated at 37 °C for 36 h.

2.5. Detection of cytokines in spleen homogenates

Spleen homogenate samples were analysed via ELISA for IL-12p40, INF-γ, and TNF-α using commercially available kits (OptEIA set; Pharmingen, San Diego, CA, USA). The assays were performed according to the manufacturer's recommendations.

2.6. Real time quantitative PCR analysis

Total cellular RNA was extracted from spleen cell samples using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. Real time PCR was performed as previously described by Oliveira et al. [9] using primers for GATA-3, T-bet, TLR2, and β-actin.

2.7. Flow cytometry analysis

Staining for flow cytometry was performed on spleen cells isolated from immunised and control mice 30 days post-infection. After harvesting the spleen cells, 1×10^6 cells were washed with ice-cold PBS and incubated for 30 min at 4 °C with 0.5 μg of anti-CD16/CD32 mAb (Fc block, clone 2.4G2, BD Pharmingen, San Diego, CA). Next, the cells were incubated for over 30 minutes with 5 μg of phycoerythrin-conjugated anti-CD62L, fluorescein isothiocyanate-conjugated anti-CD44, anti-TLR2, and PECy5-conjugated anti-CD3 (BD Pharmingen, San Diego, CA). Washing steps were performed with PBS 0.5% BSA and the cells were then submitted for analysis on a Guava flow cytometer (Millipore, Billerica, MA, USA) using Guava CytoSoft version 4.2.1 (Millipore, Billerica, MA, USA).

2.8. Statistical analysis

Statistical analysis (one-way analysis of variance) was performed in conjunction with the Tukey multiple comparison test using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). The results are presented as the mean and SE. Differences between groups were considered significant at the level of $p < 0.05$.

3. Results

3.1. Nasal immunisation with STM VapA+ protects mice against *R. equi* infection

Our previous studies have demonstrated that oral immunisation of Balb/c mice with live attenuated *S. enterica* Typhimurium (STM) expressing VapA antigen (STM VapA+) is an effective strategy to induce protection against *R. equi* infection [8,9]. In the present study, we performed nasal immunisation of Balb/c mice with a single dose or two doses of the STM VapA+. Both procedures resulted in a significant reduction in the *R. equi* burden in the spleen and liver of mice challenged 30 days after the first immunisation compared with the organs of animals injected with control $VapA^-$ (negative control) (Fig. 1A and B). Because both immunisation regimens promoted comparable *R. equi* clearance, the protocol using a single nasal inoculation of STM VapA+ was elected for subsequent experiments.

To optimise the quantity of the vaccine inoculum, groups of Balb/c mice were immunised with different doses of STM VapA+ (10^2–10^9) and challenged with *R. equi* 30 days after immunisation. A significant reduction in splenic *R. equi* burden was observed when the inoculum of the VapA+ vaccine strain was at least 10^7 CFU (Fig. 1C).

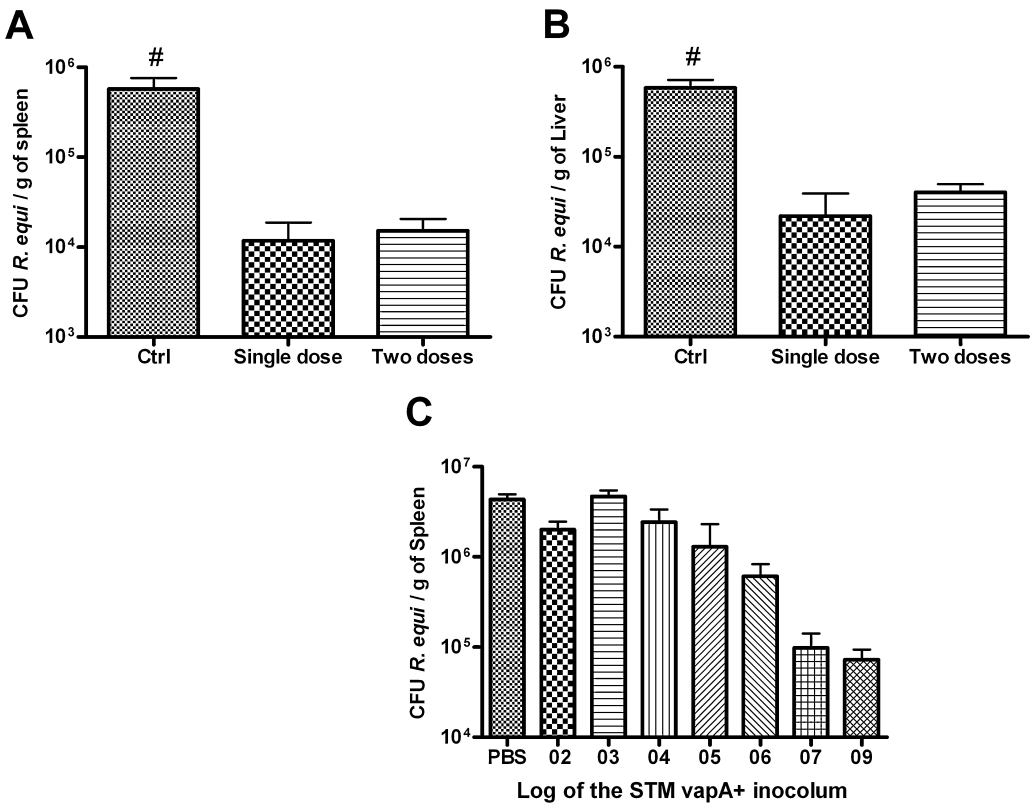

Fig. 1. Nasal immunisation with STM VapA+ reduces *R. equi* burden in infected mice. Balb/c mice were immunised with a single dose or two doses of attenuated *Salmonella enterica Typhimurium* carrying VapA antigen (STM VapA+). All animals were euthanised on day 5 after infection, and *R. equi* burden was evaluated in the spleen (A) and liver (B). Panel C shows the recovering of *R. equi* from the spleen of the animals that were immunised with a single dose of various vaccine inoculum. Attenuated *Salmonella* carrying empty vector was used as a negative control (Control). Data represent the mean ± SE, $n = 5$ animals. $^{\#}p < 0.05$.

3.2. Nasal immunisation with STM VapA+ induces Th1 immunity

To evaluate whether the protection conferred with the novel vaccination procedure was associated with the development of Th1 immunity as previously verified for oral vaccination, we assessed the level of some mediators in the spleens of mice immunised with the STM VapA+ strain and challenged the animals with *R. equi*. IL-12 (Fig. 2A), nitric oxide (Fig. 2B) and hydrogen peroxide levels (Fig. 2C) were significantly higher in the spleen tissue of VapA+-immunised mice, while the levels of TNF-α remained lower (Fig. 2D).

To better characterise the immune response induced by the vaccination procedure, we determined the mRNA levels for the transcription factors GATA-3 and T-bet 30 days after immunisation. Compared with the negative controls, i.e., mice injected with control VapA− or PBS, mice immunised with STM VapA+ displayed higher levels of T-bet mRNA (Fig. 2E) and lower levels of GATA-3 mRNA (Fig. 2F). Similar analyses were performed 5 days after challenging, and no difference in the levels of T-bet mRNA was observed among all groups (Fig. 2E). GATA-3 mRNA levels remained lower in vaccinated/challenged animals (Fig. 2F).

3.3. Nasal immunisation with STM VapA+ induces memory T cells and immune response recall

To evaluate the induction of T cells with memory phenotype via nasal vaccination with STM VapA+, we have assessed the frequency of cells co-expressing CD3, CD62L and CD44 (high) markers, which characterise T cells with memory phenotype, 30 days after immunisation. This population, according to the flow cytometric analysis, was larger in the spleens of vaccinated mice compared with the control groups (Fig. 3A). We also investigated whether the expansion of the population of cells was associated with immune response recall. Accordingly, Balb/c mice were immunised and challenged 5 months later. Next, spleen and liver were collected for analysis 5 days after challenge. Our results demonstrate a significant decrease in *R. equi* burden in the group immunised with STM VapA+ (Fig. 3B and C).

3.4. TLR-2 expression is upregulated after immunisation and downregulated after challenge

It is well established that VapA can activate Toll-like receptor 2; this interaction is considered a major mechanism involved in triggering specific immune responses to *R. equi*. Therefore, the expression levels of TLR2 in the spleen cells of mice vaccinated with STM VapA+ were evaluated via real time PCR (Fig. 4A) and flow cytometry (Fig. 4B), which was compared with the expression levels in the cells of mice injected with the controls VapA− or PBS. Thirty days after immunisation, mice spleen cells displayed an increase in TLR2 expression levels, as demonstrated by elevated mRNA (Fig. 4A) and protein levels (Fig. 4B). Five days after challenge with *R. equi*, murine spleen cells displayed reduced TLR2 expression levels similar to that detected in the negative control group.

3.5. Protection against R. equi conferred by STM VapA+ is not dependent on TLR2

We further investigated the role that TLR2 plays in the VapA+ vaccine-induced protection against *R. equi* infection. Accordingly, groups of TLR2 knockout mice (C57Bl/6 *Tlr2*$^{-/-}$) or wild type mice (WT) were treated with the standard vaccination and challenge protocols. As expected, among all WT vaccinated mice, *R. equi*

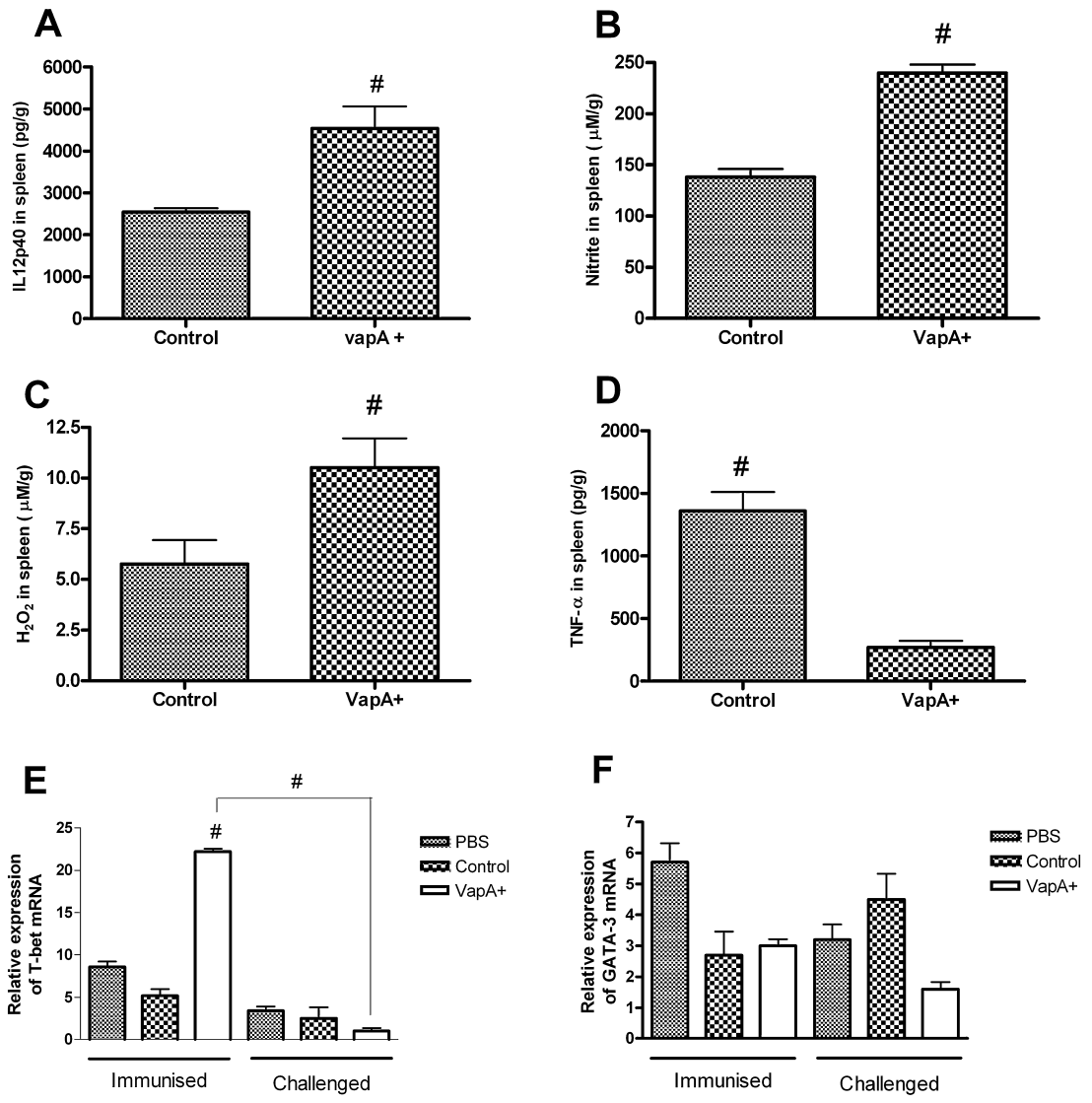

Fig. 2. Nasal immunisation with STM VapA+ induces Th1-immunity. Balb/c mice were immunised with a single dose of STM VapA+ and the levels of IL-12 (A), nitric oxide (B), hydrogen peroxide (C) and TNF-α (D) in the spleen were evaluated. The relative mRNA expression levels of T-bet (E) and GATA-3 (F) were also analysed. Attenuated *Salmonella* carrying empty vector was used as negative control. Data represent the mean ± SE, $n = 5$ animals. $^{\#}p < 0.05$.

burden in the spleen (Fig. 5A) and liver (Fig. 5B) was less than that detected in the organs of negative control mice injected with the controls VapA− or PBS. Nonetheless, similar results were obtained with the *Tlr2*$^{-/-}$ group, thereby indicating that TLR2 is not essential for the protection conferred by our VapA^{+} method of vaccination using attenuated *Salmonella*. This indication was reinforced by the fact that similar levels of IL-12 (Fig. 5C), IFN-γ (Fig. 5D) and TNFα (Fig. 5E) were detected in the spleen tissue of the *Tlr2*$^{-/-}$ and WT animals. Additionally, these results show that STM VapA+ vaccination of C57BL/6 and Balb/c mice is able to confer protection against *R. equi* infection and induces Th1 immune response as denoted by increased production of IL-12 and IFN-γ.

Similar immunisation and challenges were performed in mice with non-functional TLR4 (C3H/HeJ). In these animals, *R. equi* clearance was comparable to that observed in the spleen (Fig. 5F) and liver (Fig. 5G) of vaccinated mice expressing functional TLR4 (C3H/HePAS). This result indicates that the protection conferred by vaccination with the STM VapA+ strain is also independent of TLR4.

3.6. High frequency of memory phenotype CD4 T cells is maintained in the absence of TLR2

To understand the function of TLR2 in the nasal immunisation-mediated response, we investigated whether the emergence of memory T cells induced by vaccination could be affected by the absence of TLR2. The frequency of CD4^{+} or CD8^{+} T cells expressing CD44^{high}/CD62L was measured in the spleen of WT and *Tlr2*$^{-/-}$ mice 30 days after immunisation. As shown in Fig. 6A, vaccination led to an increased cell population with a CD4^{+}CD62L^{+}CD44^{+high} phenotype. Notably, this increase was similar for WT and *Tlr2*$^{-/-}$ mice. In contrast, the population of CD8^{+}CD62L^{+}CD44^{+high} cells was elevated only in the spleens of vaccinated WT mice, thereby indicating that vaccination had no effect on the frequency of these cells in *Tlr2*$^{-/-}$ animals (Fig. 6B).

Finally, we assessed the frequency of T cells with memory phenotype in challenged animals (120 days post-immunisation). In this particular case, the CD4^{+}CD62L^{+}CD44^{+high} population remained

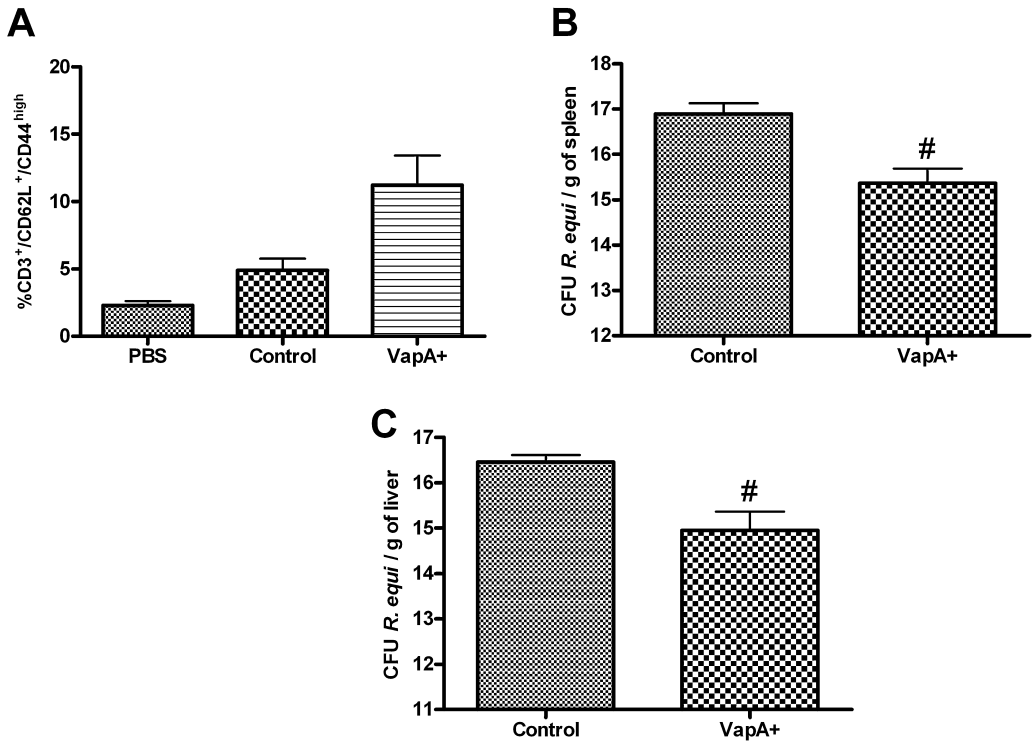

Fig. 3. Nasal immunisation with STM VapA+ increases the splenic population of T cells with memory phenotype and the recall of immune response. Vaccinated Balb/c mice with STM VapA+ were challenged with *R. equi* 30 days after immunisation, and the frequency of $CD3^+/CD62L^+/CD44^{high}$ cells (A) was analysed. A second group of Balb/c mice was challenged 5 months after immunisation and evaluated in terms of spleen (B) and liver (C) *R. equi* burden. Attenuated *Salmonella* carrying empty vector was used as a negative control. Data represent the mean ± SE, $n = 5$ animals. $^{\#}p < 0.05$.

elevated in WT, but not in *Tlr2*$^{-/-}$ mice (Fig. 6C). Regarding the $CD8^+CD62L^+CD44^{+high}$ cells, no noticeable difference was detected among all groups of animals (Fig. 6D).

4. Discussion

Various strategies have been proposed for the development of a safe and effective vaccine against rhodococcosis. For instance, the passive immunisation with hyperimmune plasma [10], vaccination procedures with inactivated *R. equi* strains [11], VapA DNA vaccine [12] and, more recently, attenuated *R. equi* vaccine [13] have been reported. However, none of these approaches have provided an efficient method to control the disease. Among the different strategies of vaccination for intracellular pathogens, a promising approach may be the use of live oral vaccines. In this context, our group has demonstrated that oral immunisation with an attenuated *S. enterica* Typhimurium strain expressing the VapA protein induces protection against experimental rhodococcosis in mice [8]. More recently, we have demonstrated that the oral immunisation protocol induces a strong and specific humoral and cell-mediated Th1-immunity against the heterologous antigen, with an appropriate regulatory response and a long-term protection against *R. equi* infection [9]. In the present work, we report that nasal delivery is an effective route for immunisation, which confers protection with a single dose of the vaccine. We have also verified that nasal vaccination induces a Th1 immune response, which is as protective and specific as the oral immunisation [9].

Nasal route has been proposed as an alternative route for vaccination due to ease of the process of immunisation and the presence of nasopharynx-associated lymphoid tissue (NALT). As with the Peyer's patches, NALT accomplishes the requirements to induce

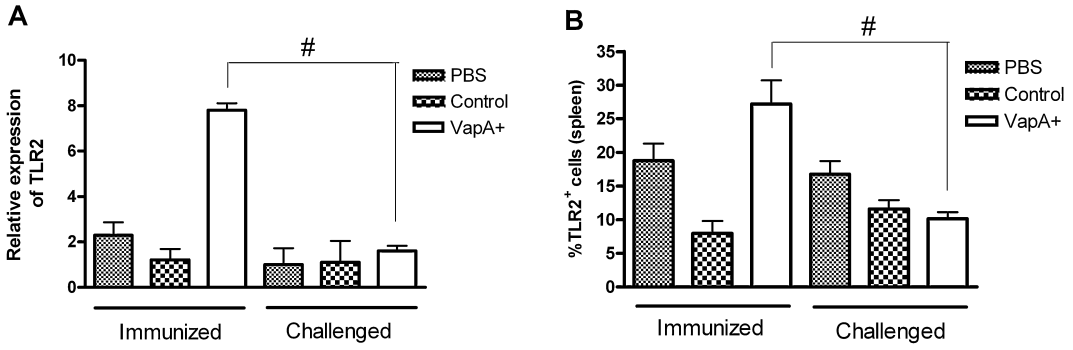

Fig. 4. Nasal vaccination with STM VapA+ enhances TLR2+ expression levels in spleen cells, which is reverted by *R. equi* challenge. The relative expression levels of *TLR2* mRNA in the spleen cells of vaccinated Balb/c mice either unchallenged or challenged or with *R. equi* is shown in panel A. Panel B shows the relative frequency of spleen cells positive for TLR2 staining as detected via flow cytometry. Attenuated *Salmonella* harbouring empty vector was used as a negative control. In (A), cDNA content was normalised on the basis of predetermined β-actin levels. Data represent the mean ± SEM ($n = 5$ animals). $^{\#}p < 0.01$. In (B), data represent the mean ± SE. $^{\#}p < 0.05$.

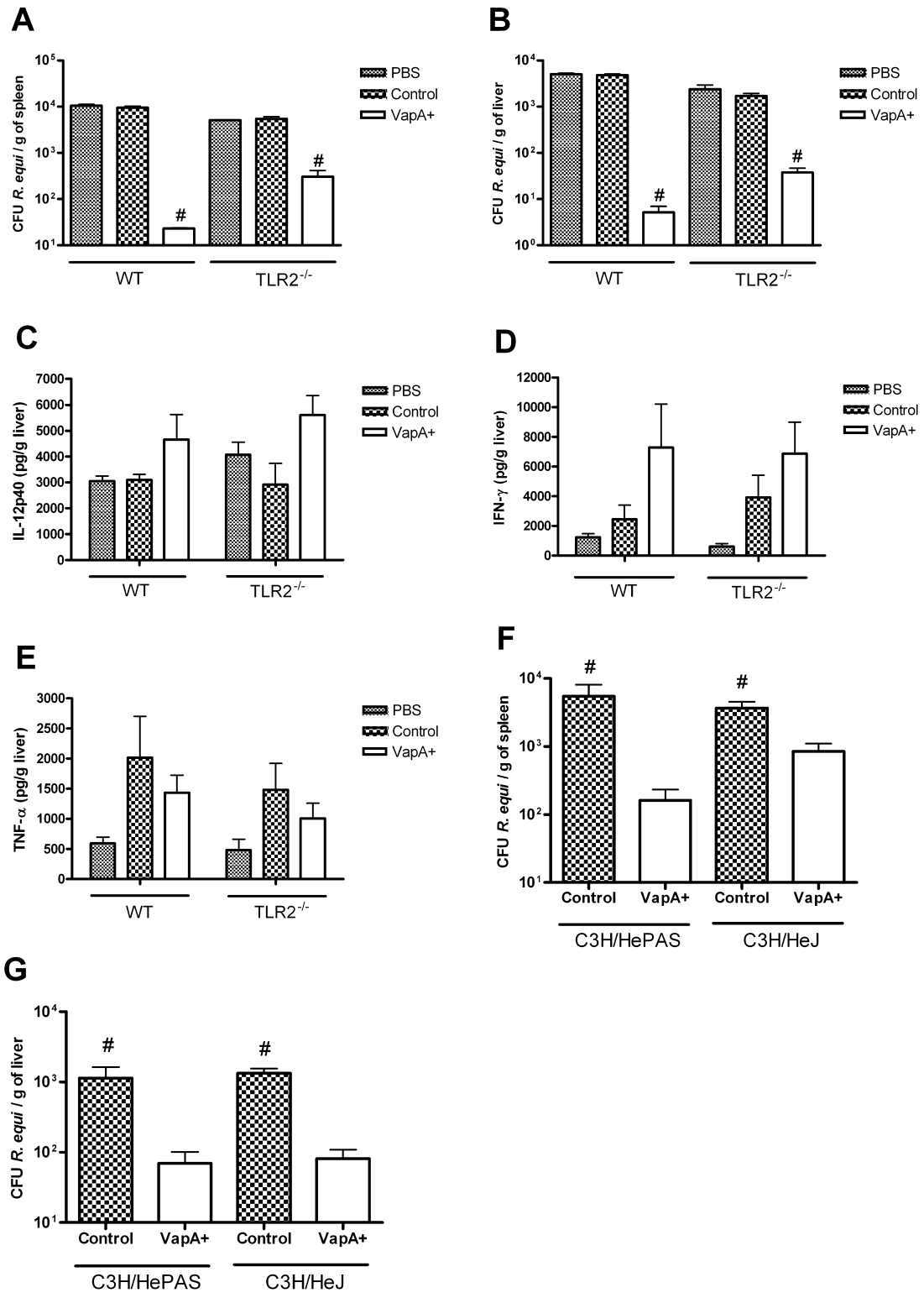

Fig. 5. Protection induced by nasal vaccination with STM VapA+ is independent of TLR2 activation. WT and *Tlr2*$^{-/-}$ C57BL/6 mice were challenged with *R. equi* 30 days after vaccination with STM VapA+. Five days after challenge, *R. equi* burden in the spleen (A) and liver (B) was evaluated. The spleen tissue was also analysed for the levels of IL12p40 (C), IFN-γ (D), and TNF-α (E). Similar experimental procedures were performed with C3H/HePAS and C3H/HeJ mice; *R. equi* burden was assessed in the spleen (F) and liver (G) samples. Attenuated *Salmonella* carrying empty vector was used as a negative control. The bars represent the mean ± SEM ($n = 5$ animals). $^{\#}p < 0.05$.

and regulate a protective immune response, which involves M cells, APCs, T cells and B cells [14]. Nonetheless, NALT is characterised as a Th0 environment, which can be reverted to either Th1 or Th2 prone ambiance [15]. Although the oral administration is a natural route for *Salmonella*-based vaccines, the intranasal delivery allows for the immunogen to reach the same organs, including the lungs and Peyer's patches and, as a consequence, can induce serologic and proliferative responses [16]. Currently, Kim et al. [17] have demonstrated that attenuated *Salmonella* Typhimurium is an efficient carrier of heterologous antigens, which is able to involve

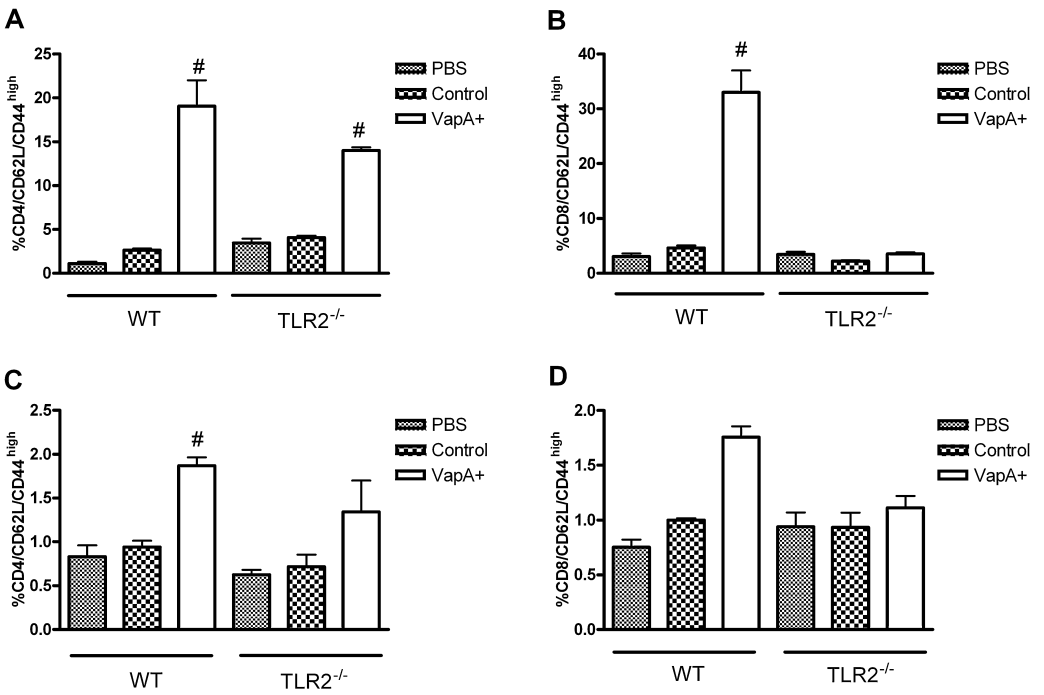

Fig. 6. Nasal immunisation with STM VapA+ induces increased frequency and recall of $CD4^+$ T cells with memory phenotype via a TLR2-dependent mechanism. Immunised WT and *Tlr2*$^{-/-}$ C57BL/6 mice were challenged with *R. equi* 30 days (panels A and B) or 120 days (panels C and D) after vaccination. The frequencies of $CD4^+/CD62L^+/CD44^{high}$ (A and C) and $CD8^+/CD62L^+/CD44^{high}$ cells (B and D) were assessed via flow cytometry in spleen samples. Attenuated *Salmonella* carrying empty vector was used as a negative control. The bars represent the mean ± SEM ($n = 5$ animals). $^{\#}p < 0.05$.

M cells in nasopharynx and tracheobronchial lymphoid tissue and induce local and systemic-specific immune responses. Such findings have motivated several groups to develop *Salmonella*-vectored vaccines for administered via nasal route in mice [18,19] and mares [20].

Darrah and co-workers [21] have demonstrated that the lipoprotein VapA activates macrophage TLR2, a receptor that is a major mediator of the innate response to *R. equi*. Therefore, along with the knowledge that TLR2 activation favours the differentiation of Th1 cells, it is likely that this receptor could play a role in the protection conferred by *Salmonella*-expressing VapA used herein. This idea was reinforced with the fact that vaccination elevated TLR2 expression levels on murine macrophages. However, the high TLR2 expression levels were not maintained when the immunised mice were challenged with *R. equi*. With regards to this findings, they might be related to (i) a supposed TLR2 internalisation, which accompanies the internalisation of *R. equi* or (ii) triggering a signal via TLR2 activation, thereby inducing a negative feedback mechanism able to control inflammation after challenge. The latter assumption is supported by the observation that TNF-α levels also decreased after *R. equi* challenge.

Activation of TLRs directly modulates T cell biology [22–24]. In this context, alteration of TLR2 expression levels by these cells could affect vaccine efficiency and the frequency of T cell populations. Surprisingly, the results of protection with the STM VapA+ vaccine were somewhat similar for both the WT and the *Tlr2*$^{-/-}$ animals, as shown by a similar reduction in *R. equi* burden in the spleen and liver. Furthermore, the population of $CD4^+$ T cells with memory phenotype was similarly expanded in *Tlr2*$^{-/-}$ and WT vaccinated mice. The frequency of $CD8^+$ T cells with memory phenotype; however, was only greater in WT mice after vaccination. These results are supported by the study of Cottalorda and co-workers [25], which demonstrated that TLR2 plays a critical role in the generation of memory $CD8^+$ T cells. Our results indicate that $CD4^+$ T cells play a major role in *R. equi* immunity. This is in agreement with the fact that activated $CD4^+$ T cells express TLR3 and TLR9 but not TLR2 and TLR4 [24]. Following vaccination, activation of $CD4^+$ T cells occurs independent of their direct interaction with VapA or *Salmonella* components.

Our results do not exclude the possibility that TLR2 might participate in the protective response against *R. equi* induced by STM VapA+. A closer examination of our results reveals that vaccinated WT mice displayed a reduced bacterial burden of almost 3 logs, while the vaccinated *Tlr2*$^{-/-}$ mice displayed a reduced burden of approximately 1.5 logs. Thus, the VapA antigen, in the context of the *Salmonella* cell, is able to induce a protective immune response via a pathway that is independent of TLR2 activation. Nevertheless, TLR2 seems to act synergistically to mount a strong protective response against *R. equi*.

The exact mechanism by which the VapA+ antigen is presented by the attenuated *Salmonella* remains unclear. *S. enterica* serovars survive inside the host cells. Although attenuated strains were shown to be unable to cause disease, they retain the ability to colonise the host tissue and trigger certain signals in the context of the host cells. Likewise, *R. equi* is an intracellular pathogen and natural infection should be mimicked via vaccination with STM VapA+, which has the advantage of providing better conditions for presentation of the VapA antigen. Such presentation seems to be crucial, as VapA is highly associated with *R. equi* virulence and thus is extensively described as a good antigen for vaccination. Currently, many strategies have been proposed based on the immunity developed against VapA, but only few of strategies were considered to have potential for vaccine application. The results presented herein suggest that the χ3987 attenuated strain of *Salmonella* is an efficient carrier for oral or nasal immunisation with VapA. The vaccine stimulates antigen-specific $CD4^+$ T cells without the participation of TLR2 and leads to a profile of immune response that confers resistance to the *R. equi* infection. Most importantly, the efficient

clearance of *R. equi* from the organs of vaccinated mice and the long-term recall of immunity indicate that the strategy used herein may be applicable for vaccination against rhodococcosis.

Acknowledgments

We would like to thank Sandra O. Thomaz and Patricia Vendruscolo for technical support. This study was in part supported by grants from FAPESP (05/50460-1) and CNPq (154963/2006-2). S.A.C. was a PhD fellow of CAPES, and MCRB is senior fellow of CNPq.

Contributors: S.A.C. and S.G.S. designed experiments, performed assays, interpreted data and wrote the manuscript. A.F.O. designed experiments and performed assays with TLR2KO mice. M.M.T. performed assays with TLR4-deficient animals. L.P.R. performed PCR and flow cytometry analysis. L.L.O., M.C.R.B. and E.S.H. interpreted data and contributed to the manuscript. *Competing interests*: The technology described in this paper is subject to pending patents. The data and materials described herein adhere to the Vaccine journal policies for academic, non-commercial research.

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