**Article**

# Immunotherapeutical Potential of *Mycobacterium Vaccae* on *M. Tuberculosis* Infection in Mice

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**Tuberculosis remains the worldwide infectious disease. To identify the therapeutic potential of *M. vaccae* in treating tuberculosis, *M. vaccae* was injected into *Mycobacterium tuberculosis* (*M. tuberculosis*) infected mice. The optimal dose of *M. vaccae* (22.5 μg/mouse) treated mice showed lower pathological change index, spleen weight index, lung weight index and vital *M. tuberculosis* count than those of the untreated group. Treatment with *M. vaccae* enhanced the percentages of $CD3^+$ and $CD4^+$ T cells, IFN-$\gamma^+CD4^+$ T cells, innate immune cells including NK cells, $NK1.1^+$ T cells and γδ T cells, and reduced the percentage of IL-$4^+CD4^+$ T cells. Therefore, *M. vaccae* could protect the mice from *M. tuberculosis* infection and improved mouse innate and adaptive cell-mediated immunity, suggesting that *M. vaccae* is a potential immunotherapeutic agent in pulmonary tuberculosis. *Cellular & Molecular Immunology*. 2009;6(1):67-72.**

**Key Words:** tuberculosis, *Mycobacterium vaccae*, immunological intervention, cellular immunity

## Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), is a major public health problem in China, with about 130,000 deaths occurring annually and 550 million new cases of tuberculosis developing per year, in which approximately 50 million tuberculosis high-risk persons with strong positive tuberculin reaction have high tuberculosis incidence rate of 5%-10% (1). Therefore, preventive therapy in the high risk persons is the key point for tuberculosis control.

*Mycobacterium vaccae for injection* (*M. vaccae,* with the items of Wei-Ka) is an immunoregulatory agent as a heat-killed preparation of *M. tuberculosis*. In recent years, *M. vaccae* has been used as an adjuvant immunotherapy to chemotherapy for the treatment of tuberculosis and its efficacy has been observed (2-4). In addition, *M. vaccae* is very safe and has fewer side effects. Studies performed in mice and humans showed that *M. vaccae* could enhance *M. tuberculosis* antigen-specific cellular immunity and anti-virus/bacteria ability obviously (5-9). However, the mechanism by which *M. vaccae* influences the immune response to *M. tuberculosis* infection is unclear yet.

In this study, we developed mouse model of *M. tuberculosis* infection and treated the infected mice with different doses of *M. vaccae* to observe its effect on tuberculosis preventive treatment and immune system. Our results suggested that *M. vaccae* is an effective immunotherapeutic reagent for tuberculosis treatment.

## Materials and Methods

### *Reagents*

*M. vaccae* (batch number: 20041201) was provided by Anhui Longcome Biopharmaceutical Co. Ltd. Isoniazid (INH, batch number: 040304) was purchased from Shanghai Xinpasi Pharmaceutical Co., Ltd. The mAbs used in this study including FITC-conjugated anti-NK1.1, CD4, PE-conjugated anti-γδ TCR, IFN-γ, IL-4 and PE-Cy5-conjugated anti-CD3e, were purchased form BD PharMingen.

### *Mouse model of M. tuberculosis infection*

C57BL/6 mice weighing 18-20 g were purchased from the Shanghai SIPPR/BK Experimental Animal Co., Ltd.

H37Rv *M. tuberculosis* (Beijing Institute of Tuberculosis and Thoracic Tumor) were grown in modified Lowenstein-

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**Table 1.** *M. vaccae* treatment ameliorated tuberculosis pathological change of *M. tuberculosis* infected mice (n = 20)

| | Normal | Infection | *M. vaccae* | | | INH |
|---|---|---|---|---|---|---|
| | | | 11.25 μg | 22.5 μg | 45.0 μg | |
| Pathological change index | - | 65.00 ± 14.9 | 52.25 ± 9.52** | 48.50 ± 11.4** | 51.00 ± 11.5** | 16.75 ± 2.45** |
| Spleen weight index | 0.346 ± 0.084** | 0.973 ± 0.246 | 0.951 ± 0.255 | 0.783 ± 0.251* | 0.832 ± 0.263 | 0.337 ± 0.053** |
| Lung weight index | 0.812 ± 0.094** | 2.853 ± 0.930 | 2.578 ± 0.629 | 2.361 ± 0.338* | 2.323 ± 0.623* | 0.713 ± 0.080** |
| Liver weight index | 3.985 ± 0.364** | 5.448 ± 0.736 | 5.431 ± 0.693 | 5.171 ± 0.501 | 5.306 ± 0.741 | 4.325 ± 0.597** |

*$p < 0.05$, **$p < 0.01$, compared with infection model group.

Jenson egg medium for 3-4 weeks. The culture was harvested and vigorously agitated with agate mortar. The viability of the bacteria in suspension was evaluated using 0.05% Tween 80/PBS at the concentration of 2 mg/ml. Mice were injected *i.v.* with 200 μl of $5 \times 10^6$ CFU (colony forming units)/ml bacilli (about $1 \times 10^6$ CFU/mouse).

***Experimental design***

*M. tuberculosis* infected mice were randomly divided into 6 groups (30 mice/group, male:female = 1:1): (1) infection model group: *i.m.* injection at the inner thigh of 0.5 ml PBS each mouse on days 3, 10 and 17 after *M. tuberculosis* injection; (2) positive INH control group: intragastric administration of 5 mg/kg/d INH from day 1 of *M. tuberculosis* injection until 3 days before sacrifice; (3-5) high dose, medium dose or low dose of *M. vaccae* group: *i.m.* injection at the inner thigh of 45.0 μg, 22.5 μg, or 11.25 μg *M. vaccae* in 0.5 ml PBS each mouse on days 3, 10 and 17 after *M. tuberculosis* injection, respectively; and (6) normal control group: *i.m.* injection at the inner thigh of 0.5 ml of PBS each mouse on days 3, 10 and 17 after *M. tuberculosis* injection. After 8 weeks post-injection, the mice were sacrificed for further study.

***Assessment of organ pathological change***

At the end of week 8 after injection, 20 mice of each group were sacrificed to observe tuberculosis pathological changes of lungs, spleens and livers and calculate the weight index of each organ.

***Quantitative lung viable bacterial counts***

Randomly selected 20 mice in each group were sacrificed. The lungs were removed and homogenized with 4× vol. physiological saline. The homogenates were mixed with 5× vol. 4% $H_2SO_4$ for 15 min and diluted serially in physiological saline. Then 0.1 ml aliquots of 10-fold, 100-fold, and 1,000-fold serial dilutions of homogenates were plated on modified Lowenstein-Jenson egg medium at 37°C for 4 weeks to determine the CFU per gram of lung tissue.

***Histopathological assessments***

Ten mice of each group were randomly selected for histopathological analyses. The lung, spleen and liver tissues were fixed with 10% formalin in PBS, and embedded in paraffin for sectioning. The tissue sections were stained with hematoxylin & eosin and Ziehl-Neelsen acid-fast, and then evaluated by light microscope. Tuberculosis tubercles were divided into proliferative granuloma (mostly epithelial tubercle or tuberculosis granulomas), lymphoid granuloma (mostly lymphocytes) and necrotic granuloma (necrotic period of tuberculosis pathological changes).

***Isolation of splenocytes***

The splenocytes of 6 mice in each group were obtained by forcing tissues through stainless steel mesh. Before use, the erythrocytes were removed from the cell suspension by lysing solution (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 1 mM EDTA, and 170 mM Tris, pH 7.3).

***Flow cytometric analysis***

The cells were blocked and incubated with saturating amount of indicated fluorescence labeled mAbs in the darkness at 4°C for 30 min. After washed twice, the stained cells were analyzed by FACSCalibur (Becton Dickinson) and the data were analyzed by WinMDI 2.8 software.

***Intracellular IFN-γ and IL-4 detection***

For intracellular cytokine staining, cells were stimulated with PMA (30 ng/ml, Sigma), ionomycin (1 μg/ml, Sigma) and monensin (1.7 μg/ml, Sigma) at 37°C and 5% $CO_2$ for 4 h. Then the cells were stained with FITC-conjugated anti-NK1.1 mAb and PE-Cy5-conjugated anti-CD3e mAb. After fixation and permeabilization, intracellular cytokine staining was performed using PE-conjugated anti-IFN-γ or anti-IL-4 mAbs. After washed twice with permeabilization buffer, samples were analyzed by flow cytometry.

***Statistical analysis***

Data were expressed as mean ± SD. By using SPSS 10.0, the statistical analysis was performed by ANOVA. And q test was adopted to compare the differences among every group and the difference between the groups was considered statistically significant when *p* value was less than 0.05.

## Results

***M. vaccae treatment ameliorated tuberculosis pathological change of M. tuberculosis infected mice***

Mice were randomly divided into 6 groups, injected with *M.*

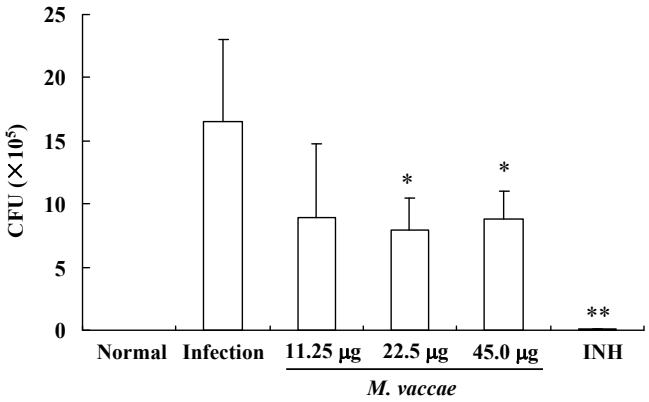


**Figure 1. *M. vaccae* treatment decreased the number of lung viable bacteria of *M. tuberculosis* infected mice.** Mice were injected *i.v.* with about $1 \times 10^6$ CFU *M. tuberculosis* per mouse and received different treatments as described in Materials and Methods. After 8 weeks, randomly selected 20 mice of each group were sacrificed. The lungs were removed and the numbers of lung viable bacteria were analyzed. Data were expressed as mean ± SD. Statistical analysis was performed using q test. $*p < 0.05$, $**p < 0.01$, compared with infection model group.

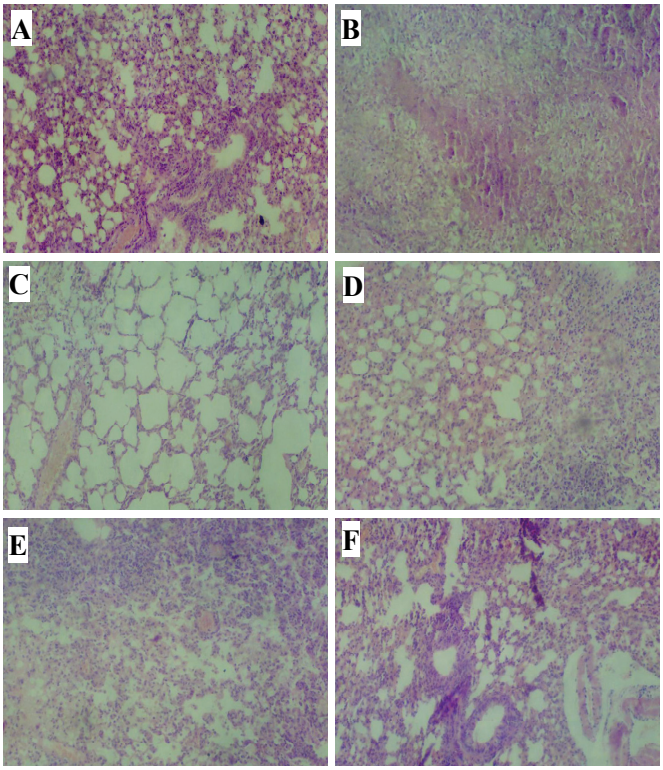


**Figure 2. *M. vaccae* treatment improved the histopathology of *M. tuberculosis* infected mice.** Mice were injected *i.v.* with about $1 \times 10^6$ CFU *M. tuberculosis* per mouse and received different treatments as described in Materials and Methods. After 8 weeks, 10 mice of each group were sacrificed. The lung tissues of normal control (A), infection model group (B), low dose of *M. vaccae* group (C), medium dose of *M. vaccae* group (D), high dose of *M. vaccae* group (E), and INH control (F) mice were fixed and stained for histopathological analyses.

*tuberculosis* and received different treatments. The organ pathological change index and weight index were observed after 8 weeks. The results showed that the organ pathological change indexes of three doses of *M. vaccae*- and INH-treated mice were significantly lower than that of infection model group. The weight indexes of mouse spleen, lung and liver were increased after *M. tuberculosis* infection and significantly decreased after INH treatment. The medium doses of *M. vaccae* treatment significantly improved the spleen weight index, and treatment with high and medium dose of *M. vaccae* obviously decreased the lung weight index, compared with infection model group mice. But the liver weight index of *M. vaccae* group was not different from that of infection model group (Table 1).

***Treatment with M. vaccae decreased the number of lung viable bacteria in M. tuberculosis infected mice***

Quantitative viable bacterial counts showed that the numbers of viable bacteria in the lungs of high dose, medium dose and low dose of *M. vaccae*-treated mice were lower than that of infection model group. The CFU of INH treatment group, high dose and medium dose of *M. vaccae* groups were $0.07 \pm 0.03 \times 10^5$, $8.85 \pm 2.14 \times 10^5$ and $7.90 \pm 2.54 \times 10^5$, respectively, significantly lower than that of infection model group ($16.55 \pm 6.44 \times 10^5$, $p < 0.05$, Figure 1).

***M. vaccae treatment improved the histopathology of M. tuberculosis infected mice***

The histopathological analyses showed that the histological structure of the spleens and livers of all the mice in this experiment was similar to those of normal mice (data not shown). As to the lung tissue, as shown in Figure 2, necrotic granuloma and proliferative granuloma were found in the infection model group mice, while proliferative granuloma was the mainly pathological change in *M. vaccae*- and INH-treated mice. And no lymphoid granuloma and necrotic granuloma was found in medium dose of *M. vaccae* group (Figure 2).

***Treatment with M. vaccae enhanced the percentages of $CD3^+$ and $CD4^+$ T cells***

Cellular immune response, especially T cell-mediated immunity, was important to tuberculosis pathology and $CD4^+$ T cells had an essential role in the host immune response to *M. tuberculosis* (10, 11). To further study the effect of *M. vaccae* on T cells and their subpopulations, we examined the percentages of total T cells and $CD4^+$ T cells in each group of mice. The results showed that the percentages of $CD3^+$ and $CD4^+$ T cells of infected mice were significantly lower than those of normal control mice ($p < 0.01$, Figure 3), but increased obviously when treated with INH ($p < 0.01$) and *M. vaccae* ($p < 0.05$). And we got better treatment effect in low dose and medium dose of *M. vaccae* groups than high dose of *M. vaccae* group (Figure 3).

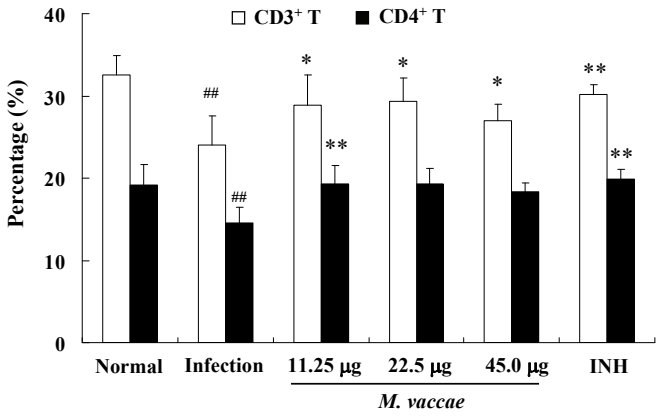


**Figure 3. *M. vaccae* treatment upregulated the percentages of $CD3^+$ and $CD4^+$ T cells.** Mice were injected *i.v.* with about $1 \times 10^6$ CFU *M. tuberculosis* per mouse and received different treatments as described in Materials and Methods. After 8 weeks, 6 mice from each group were sacrificed to examine the percentages of $CD3^+$ and $CD4^+$ T cells. Data were expressed as mean ± SD. Statistical analysis was performed using q test. $*p < 0.05$, $**p < 0.01$, compared with infection model group; $^{\#\#}p < 0.01$, compared with normal control group.

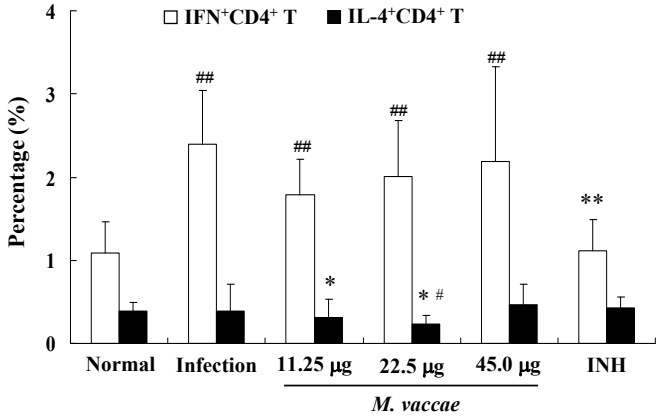


**Figure 4. *M. vaccae* treatment regulated the ratio of Th1/Th2.** Mice were injected *i.v.* with about $1 \times 10^6$ CFU *M. tuberculosis* per mouse and received different treatments as described in Materials and Methods. After 8 weeks, randomly selected 6 mice from each group were sacrificed and the expression of IFN-γ and IL-4 in $CD4^+$ T cells were examined. Data were expressed as mean ± SD. Statistical analysis was performed using q test. $*p < 0.05$, $**p < 0.01$, compared with infection model group; $^{\#}p < 0.01$, $^{\#\#}p < 0.01$, compared with normal control group.

***M. vaccae* treatment regulated Th1/Th2 ratio**

It was reported that *M. vaccae* could induce type 1 response to *M. tuberculosis* antigens (12). So we examined and compared the expressions of IFN-γ and IL-4 in $CD4^+$ T cells of the infected and treated mice. Compared with normal control mice, the percentage of IFN-$\gamma^+CD4^+$ T cells was significantly increased in infection model mice ($p < 0.001$, Figure 4). Treatment with INH significantly decreased the expression of IFN-$\gamma^+$ by $CD4^+$ T cells in *M. tuberculosis*-infected mice ($p < 0.01$) to the similar level of normal control mice. But when the infected mice were treated with *M. vaccae*, the expression of IFN-γ was not downregulated and still parallel to that of infection model mice (Figure 4).

There was no difference in the secretion of IL-4 by $CD4^+$ T cells between *M. tuberculosis* infected and normal control mice. And treatment with INH did not influence the expression of IL-4 by $CD4^+$ T cells either. But low dose and medium dose of *M. vaccae* treatment decreased the percentages of IL-$4^+CD4^+$ T cells greatly in comparison with infection model mice ($p < 0.05$, Figure 4). And the percentage of IL-$4^+CD4^+$ T cells in medium dose of *M. vaccae*-treated mice was even lower than that of normal control mice ($p < 0.05$, Figure 4).

***M. vaccae* effectively enhanced innate immunity in comparison with INH**

Innate immunity also played important roles in tuberculosis due to the participation of NK cells, NKT cells and γδ T cells (13-18). The results demonstrated that the percentage of γδ T cells was significantly increased in low dose and medium dose of *M. vaccae*-treated mice ($p < 0.01$, Table 2) and unchanged in INH group. *M. tuberculosis* infection did not affect the percentage of $NK1.1^+$ T cells. After INH treatment, it was decreased significantly ($p < 0.01$, Table 2). However, the percentage of $NK1.1^+$ T cells was not altered when treated with *M. vaccae*. The percentages of NK cells was downregulated after mice were infected with *M. tuberculosis* ($p < 0.05$). Treatments with high dose of *M. vaccae* and INH could not reverse the reduction of NK cells. But low dose, especially medium dose of *M. vaccae* treatment upregulated the percentage of NK cells significantly compared with infection model mice ($p < 0.05$, Table 2). So, compared with

**Table 2.** *M. vaccae* effectively enhanced innate immunity in comparison with INH (n = 6)

| | Normal | Infection | *M. vaccae* | | | INH |
|---|---|---|---|---|---|---|
| | | | 11.25 μg | 22.5 μg | 45.0 μg | |
| γδ T | 0.13 ± 0.02 | 0.12 ± 0.04 | 0.27 ± 0.05** | 0.28 ± 0.03** | 0.05 ± 0.01 | 0.13 ± 0.03 |
| $NK1.1^+$ T | 0.77 ± 0.15 | 0.72 ± 0.24 | 0.90 ± 0.33 | 0.69 ± 0.22 | 0.78 ± 0.32 | 0.34 ± 0.05** $^{\#\#}$ |
| NK | 3.54 ± 1.04 | 2.46 ± 0.31$^{\#}$ | 2.82 ± 0.74 | 3.84 ± 1.17* | 2.54 ± 0.80$^{\#}$ | 2.04 ± 0.39$^{\#}$ |

$*p < 0.05$, $**p < 0.01$, compared with infection model group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with normal control group.

INH, treatment with *M. vaccae* could effectively enhance the innate immunity of *M. tuberculosis* infected mice.

## Discussion

Tuberculosis remains a heavy burden of worldwide healthcare system. The emergence of multidrug resistant tubercle bacilli makes tuberculosis treatment more troublesome. So it is a key point to explore effective prevention and treatment means. It has been shown that *M. vaccae* was a potential immunotherapeutic agent for the treatment of tuberculosis (3, 19-21). In this study, we applied three doses of *M. vaccae* to treat *M. tuberculosis* infected mice, and analyzed their effects on tuberculosis development and immune systems.

The treatment with high dose and medium dose of *M. vaccae* decreased the organ pathological change index, and improved splenomegalia was also found in medium dose of *M. vaccae*-treated mice. The enlargement of lung and viable bacteria in the lungs of high dose and medium dose of *M. vaccae*-treated mice were significantly alleviated than those of infection model group. All above results indicated that *M. vaccae* had a protective effect on *M. tuberculosis*-infected mice. Different from necrotic and proliferative granulomas in the infection model group mice, the pathological change in *M. vaccae*-treated mice was mainly proliferative granuloma, and no lymphoid granuloma and necrotic granuloma was found in medium dose of *M. vaccae* group. So it suggested that the high-risk persons could be prevented from tuberculosis development if they received preventive treatment with appropriate dose of *M. vaccae*.

*M. tuberculosis* is an intracellular pathogen. So the promotion of Th1 response and suppression of the production of Th2 type cytokines are the effective approaches for protection against *M. tuberculosis* infection (22). Our results showed that INH, the medicine mainly used in clinic for tuberculosis treatment, significantly decreased IFN-γ expression in $CD4^+$ T cells of *M. tuberculosis* infected mice. However, *M. vaccae* treatment did not downregulate IFN-γ secretion. Meanwhile, treatment with low dose and medium dose of *M. vaccae* could decrease the percentage of $IL\text{-}4^+CD4^+$ T cells obviously. Therefore, the maintaining of Th1 response and suppression of Th2 cytokine production made *M. vaccae* a potential agent in clinic for tuberculosis treatment.

The immune response against tuberculosis is mainly cell mediated. So we examined the status of adaptive and innate immune system of *M. vaccae* treated mice. It showed that the percentages of $CD3^+$ and $CD4^+$ T cells of infected mice were significantly decreased after 8 week-*M. tuberculosis* infection, indicating the lower cellular immunity in tuberculosis mice. *M. vaccae* treatment, especially low and medium doses of *M. vaccae*, could upregulate the percentages of $CD3^+$ and $CD4^+$ T cells significantly and enhance the adaptive immunity. This result was similar with previous report that *M. vaccae* could upregulate $CD3^+$ and $CD4^+$ T cells, the ratio of $CD4^+/CD8^+$ T cells and promoted the recovery of tuberculosis (6, 23).

NKT cells played a role in antimycobacterial immunity and activated NKT cells could enhance immunity to tuberculosis, even though their absence does not impair resistance to tuberculosis (14, 18, 24, 25). The percentage of $NK1.1^+$ T cells was significantly decreased with INH treatment, but not affected by *M. vaccae*, demonstrating the advantage of *M. vaccae* in clinic. It was reported the impared NK cell activity and reduced γδ T cells in patients with active pulmonary tuberculosis (26, 27). Zhang et al. also indicated that human NK cells positively regulated γδ T cells in the immune response to *M. tuberculosis* by cell-cell contact and by soluble factors TNF-α, GM-CSF, and IL-12 (17). Our results showed that the percentages of γδ T cells were significantly increased in low and medium doses of *M. vaccae*-treated mice and medium dose of *M. vaccae* could reverse the reduction of low NK cell percentage. Thus, the innate immunity could be better stabilized by *M. vaccae*.

INH is one of the standard antituberculosis therapy medicines. But long-term usage could result in severe side effects, including hepatotoxicity, exanthema, and arthralgia, etc. (28). *M. vaccae* is an effective and safe preventive vaccine against tuberculosis (29). Its efficacy was observed in drug-sensitive, multidrug-resistant tuberculosis when used together with chemotherapy (30, 31). In this study, we demonstrated that although the treatment effect of *M. vaccae* was inferior to INH, the pathological change of *M. tuberculosis*-infected mice was greatly improved when treated with *M. vaccae* and *M. vaccae* had a protective effect on tuberculosis. Furthermore, in comparison with INH, *M. vaccae* could promote the immune response against *M. tuberculosis* infection much more through upregulating the ratio of Th1/Th2 and enhancing the innate immunity. Thus, this study provided some clues for the usage of *M. vaccae* in tuberculosis treatment and preventive treatment.

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