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## Original article

# Humoral and cellular immune response of mice challenged with *Yersinia pestis* antigenic preparations



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## ABSTRACT

**Objectives:** The plague, which is an infectious disease caused by *Yersinia pestis*, still threatens many populations in several countries. The worldwide increase in human plague cases and the potential use of the bacteria as a biological weapon reinforce the need to study the immunity that is induced by potential vaccine candidates. To determine the immunogenicity of antigenic preparations based on the F1 protein and the total extract from *Y. pestis*, we assessed the role of these antigens in inducing an immune response.

**Methods:** The immunogenicity of antigenic preparations based on the *Y. pestis* (YP) total extract and the *Y. pestis* fraction 1 capsular antigen protein (F1) was determined in Swiss-Webster mice immunized with 40 µg or 20 µg for each preparation. Immunophenotyping was performed by flow cytometry.

**Results:** Animals immunized with the YP total extract did not elicit detectable anti-F1 antibodies (Ab) in the hemagglutination/inhibition (HA/HI) test. Animals immunized with 40 µg or 20 µg of the F1 protein produced anti-F1 Abs, with titres ranging from 1/16 to 1/8132. The average of CD3<sup>+</sup>-CD4<sup>+</sup> and CD3<sup>+</sup>-CD8<sup>+</sup> T cells did not differ significantly between the groups. Neither YP total extract nor F1 protein induced a significant expression of IFN-γ and IL-10 in CD4<sup>+</sup> T lymphocytes. In addition, F1 failed to induce IFN-γ expression in CD8<sup>+</sup> T cells, unlike the YP total extract.

**Conclusion:** The results showed that F1 protein is not an immunogenic T cell antigen, although the YP total extract (40 µg dose) favoured CD8<sup>+</sup> T cell-mediated cellular immunity.

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## Introduction

The plague, which is caused by *Yersinia pestis*, is essentially a rodent-flea-transmitted disease that affects man and other mammalian species.<sup>1</sup> Due to the possibility of using *Y. pestis* for bioterrorism purposes and the high mortality rate of the disease,<sup>2</sup> there is a crucial need to study the immunity induced by potential vaccine candidates for future use as immunoprophylaxis. In recent years, effort has focused on developing a subunit vaccine based on virulence factors of this bacterium.<sup>3</sup>

*Y. pestis* F1 capsular antigen protein (Fraction 1 or F1) has been evaluated in several immunization studies that used experimental animals.<sup>4–6</sup> Furthermore, vaccines that are formulated with whole cells may contribute to the induction of an effective immune response.<sup>7</sup> The mechanism of protection conferred by these preparations has not been fully elucidated, but it has already been shown that an effective vaccine against the plague must induce both humoral immunity and Th1 type cellular response.<sup>8</sup>

Aiming to determine the immunogenicity of the antigenic preparations based on the total extract from *Y. pestis* and the F1 protein, we assessed the role of these antigens in inducing the production of antibodies, determining the phenotype of splenic T-lymphocytes and stimulating the production of IFN- $\gamma$  and IL-10 by subpopulations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## Materials and methods

### Animals

Female, 6–8-week-old, Swiss-Webster mice (20–24 g) were obtained from the Universidade Federal de Minas Gerais (UFMG) facilities. Four animals per cage were maintained with temperatures at 21–24 °C, a 12 h light/dark cycle, and fed with pelletized food and water *ad libitum*. Due to the policy of reducing the number of animals used in research protocols, only the minimum number of animals per group was utilized.

### Preparation of whole cell extract and *Y. pestis* F1 antigen

*Y. pestis* strain CYP 0579 from the culture collection Fiocruz-CYP was reactivated by inoculation in brain heart infusion broth media (Difco, USA) and incubated overnight at 37 °C. The presence of the genes *caf1*, *lcr*, *pla*, and *irp2*, which are prominent pathogenicity markers,<sup>9</sup> was determined by M-PCR according to Leal and Almeida.<sup>10</sup> The culture was inactivated by the addition of 2% formaldehyde (Sigma–Aldrich, USA), incubated overnight at room temperature, 23–25 °C, and plated on blood agar base (Difco, USA) to confirm bacterial death. The F1 antigen was extracted from the *Y. pestis* strain A1122 as previously described.<sup>11</sup>

The *Y. pestis* formaldehyde-killed suspension (YP) was fragmented by sonication for 90 s (2 cycles of 30 Hz and 2 cycles of 60 Hz intercalated with an ice bath). The YP total extract and F1 protein were subjected to gamma radiation. The protein concentration of the preparations was determined by the Lowry method<sup>12</sup> and the products were suspended in PBS at pH 7.2–7.4.

### Immunization

Four groups of four female Swiss-Webster mice were immunized with 40  $\mu$ g or 20  $\mu$ g of the YP total extract and the *Y. pestis* F1 protein suspensions in PBS plus 25% (v/v) aluminium hydroxide adjuvant, administered in two doses with a 21-day interval. Each animal was injected intramuscularly in the posterior thigh with a total volume of 0.1 mL. The control group received the same volume of aluminium hydroxide adjuvant in PBS. After primary immunization, on day 42, the mice received a booster intravenous injection at the base of the tail with 4  $\mu$ g or 2  $\mu$ g doses of the antigens without adjuvant.<sup>13</sup> On day 45 after primary immunization, mice were euthanized by anaesthetic overdose. Blood samples and spleens of the immunized and control mice were collected.

### Hemagglutination/inhibition test (HA/HI)

Sera were collected from immunized animals throughout the study and assayed for the presence of anti-F1 Abs by the HA/HI test.<sup>11</sup> The test was considered positive when the HA endpoint titre was depressed by three or more dilutions in the HI test. A titre of 1/16 was considered positive.

### Preparation of spleen cell suspensions

Single cell suspensions were prepared from each 2/3 spleen in RPMI 1640 (Gibco, Germany). Each spleen was aseptically collected, and the mononuclear cells were filtered through sterile nylon. The cells were washed in RPMI 1640 and spun down at 440  $\times$  g for 10 min at 18 °C. The erythrocytes were lysed with an ammonium chloride solution, and the cells were washed three times in RPMI 1640 and resuspended into RPMI 1640 supplemented with 5% heat-inactivated foetal calf serum (Cultilab, SP, Brazil), penicillin (100 UI/mL) and streptomycin (50  $\mu$ g/mL; Sigma–Aldrich, USA).

### Spleen cell culture and flow cytometry analysis

Mononuclear cells were isolated from the spleens of immunized and control mice by Ficoll-Hypaque (Sigma–Aldrich, USA) density gradient centrifugation. After counting the viable lymphocytes, suspensions were prepared containing 1  $\times$  10<sup>6</sup> splenic cells/mL in RPMI supplemented with 5% foetal bovine serum, 2 mM glutamine, 50 UI penicillin, and 0.05 mg streptomycin (Sigma–Aldrich, USA). Cell suspensions were used for the phenotypic analysis of lymphocytes and for intracellular cytokine analysis.

Cell surface marker staining was performed by adding the previously prepared cell suspension (0.1 mL) to flow cytometry tubes containing combinations of the following fluorochrome-labelled Abs: CD3-FITC (145-2C11), CD4-PerCPy55 (RM4-5) and CD8-APC (53-6.7) (Becton Dickinson, USA). Samples were vortexed gently and incubated for 30 min at room temperature. The fixing solution containing 4% paraformaldehyde was added to the samples, which were refrigerated until flow cytometric analysis. Lymphocytes were specifically analyzed by selective gating based on size and granularity of the cells using a flow cytometer (Forteza, Becton Dickinson, USA). As negative controls, the respective isotype control (BD) labelled with the

same fluorochrome was used. Data analysis was performed using the FlowJo software.

For intracellular cytokine analysis,  $1 \times 10^6$  spleen cells from each animal were plated in a 24-well plate and stimulated with  $10 \mu\text{g/mL}$  of YP total extract or *Y. pestis* F1 antigen for 72 h. Cultures without added *Y. pestis* proteins served as controls. Golgi Plug ( $1 \mu\text{g/mL}$  – Becton Dickinson, USA) was added during the last 4 h of incubation. Cells were surface stained with anti-CD3-FITC (145-2C11), anti-CD4-PerCPy55 (RM4-5) and anti-CD8-APC (53-6.7) Abs (Becton Dickinson, USA) and then fixed with 4% paraformaldehyde for 10 min. After permeabilization, cells were stained with anti-IFN- $\gamma$ -PeCy7 (XMG1.2) and anti-IL-10-PE (JES5-16E3) or isotype controls (Becton Dickinson, USA) and analyzed using flow cytometry (Forteza, Becton Dickinson, USA).

### Statistical analyses

The difference of the data among groups was compared by analysis of variance (ANOVA) using the R software<sup>14</sup> and probability values of  $<0.05$  were considered significant. The number of animals per group (N) was determined using the standard deviation, difference to be detected, and significance level<sup>15,16</sup> and was confirmed by the website <http://www.lee.dante.br/esquisa/amostragem/amostra.html>.<sup>17</sup>

### Ethics

Mice were handled following the Guide for the Care and Use of Laboratory Animals guidelines.<sup>18</sup> The experimental protocols were approved by the Ethics Committee on Animal Experimentation (CETEA) of UFMG (Protocol No. n°148/2014).

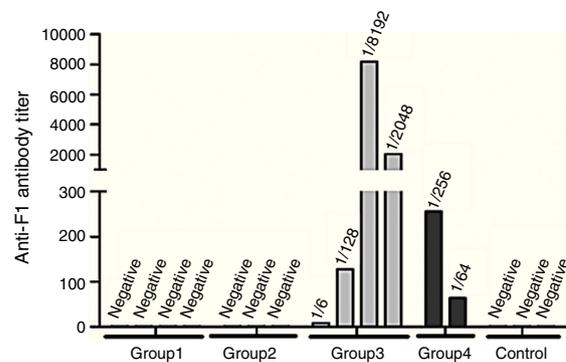
## Results

### *Y. pestis* anti-F1 antibody (Ab) in animals immunized with different immunization protocols

To characterize the humoral response induced by immunization with different doses of the YP total extract and the *Y. pestis* F1 protein, anti-F1 Ab titres were determined by the HA/HI test from serum samples of immunized mice that were collected 45 days post-immunization. Anti-F1 Abs were not detected in the two groups immunized with  $40 \mu\text{g}$  (Group 1) and  $20 \mu\text{g}$  (Group 2) of YP total extract or in the control group, which received aluminium hydroxide adjuvant in PBS. In the animals immunized with the *Y. pestis* F1 antigen, the anti-F1 Ab titres in HA/HI ranged from 1/16 to  $\geq 1/8132$  in the group receiving  $40 \mu\text{g}$  F1 (Group 3) and from 1/64 to 1/256 in the group receiving  $20 \mu\text{g}$  F1 (Group 4). It should be pointed out that the volume of sera from some mice of groups 2 (1), 4 (2) and control (1) was not enough for the HA/HI tests (Fig. 1).

### Phenotypic analysis of splenic T cells after immunization

Because the response to immunization involves activation at the cellular level, flow cytometric analysis was performed to investigate whether immunization resulted in changes in cellular activation markers or gross changes of cell counts of

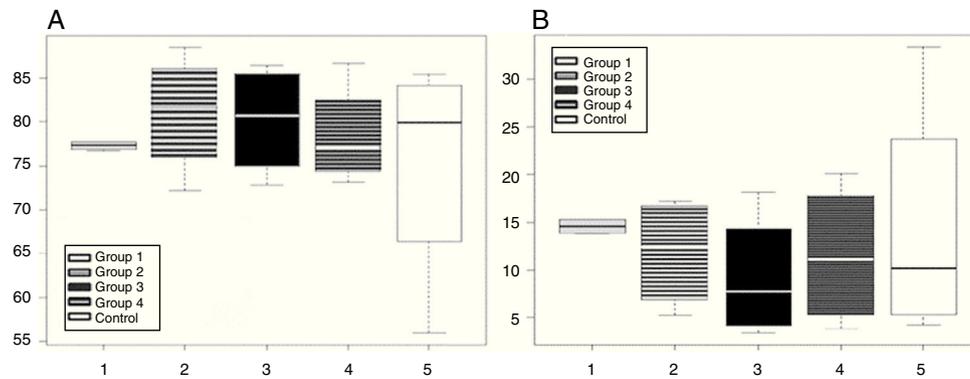


**Fig. 1 – *Yersinia pestis* anti-F1 antibody in mice immunized with different immunization protocols. Group 1:  $40 \mu\text{g}$  of YP total extract; Group 2:  $20 \mu\text{g}$  of YP total extract; Group 3:  $40 \mu\text{g}$  of *Y. pestis* F1 antigen; Group 4:  $20 \mu\text{g}$  of *Y. pestis* F1 antigen; Control: aluminium hydroxide adjuvant.  $n < 4$  (groups 2, 4 and control): the volume of sera was insufficient for the HA/HI tests.**

mice given different preparations of *Y. pestis*. Therefore, within the lymphocyte population, the changes in the percentage of splenic T cells were evaluated among immunized groups. No significant differences were observed in the percentages of  $\text{CD3}^+\text{CD4}^+$  and  $\text{CD3}^+\text{CD8}^+$  T cells among the four immunized groups ( $p > 0.05$ ). However, a clear predominance of  $\text{CD4}^+$  T cells was observed in all groups (Fig. 2A and B).

### Detection of intracellular cytokines IFN- $\gamma$ and IL-10 in T cells

To investigate the response of  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells after *in vitro* stimulation with YP total extract and *Y. pestis* F1 antigen, spleen mononuclear cells from immunized mice that were collected 45 days post-immunization were cultured and assessed for the production of IFN- $\gamma$  and IL-10 by flow cytometry. A higher percentage of  $\text{CD4}^+$  T cells that expressed IFN- $\gamma$  was observed among the group that received  $40 \mu\text{g}$  YP total extract compared to the other groups (Fig. 3A). The levels of  $\text{CD4}^+$  T cells that expressed IL-10 were higher in the groups that received  $40 \mu\text{g}$  and  $20 \mu\text{g}$  of YP total extract (Fig. 3B). However, no significant differences were observed in the percentages of  $\text{CD4}^+$  T cells that produced IFN- $\gamma$  and IL-10 cytokines after stimulation with YP total extract ( $p > 0.05$ ). The percentage of  $\text{CD4}^+$  T cells that produced IFN- $\gamma$  (Fig. 3C) and IL-10 cytokines (Fig. 3D) was lower after stimulation with the *Y. pestis* F1 antigen than after stimulation with YP total extract. No significant differences were observed in the percentages of  $\text{CD4}^+$  T cells that produced IFN- $\gamma$  and IL-10 cytokines among the groups after stimulation with the *Y. pestis* F1 antigen ( $p > 0.05$ ). The percentage of  $\text{CD8}^+$  T cells that produced IFN- $\gamma$  after stimulation with YP total extract was significantly higher in the group that received  $40 \mu\text{g}$  YP total extract (Fig. 3E) compared with the other groups ( $p < 0.05$ ). No significant differences were observed in the percentage of  $\text{CD8}^+$  T cells that expressed IFN- $\gamma$  (Fig. 3F) among the four groups after stimulation with *Y. pestis* F1 antigen ( $p > 0.05$ ).



**Fig. 2 – Phenotypic analysis of splenic T cells after immunization. A: CD3<sup>+</sup>–CD4<sup>+</sup> subpopulations. B: CD3<sup>+</sup>–CD8<sup>+</sup> subpopulations. Group 1: 40 µg of YP total extract; Group 2: 20 µg of YP total extract; Group 3: 40 µg of *Y. pestis* F1 antigen; Group 4: 20 µg of *Y. pestis* F1 antigen; Control: aluminium hydroxide adjuvant.**

## Discussion

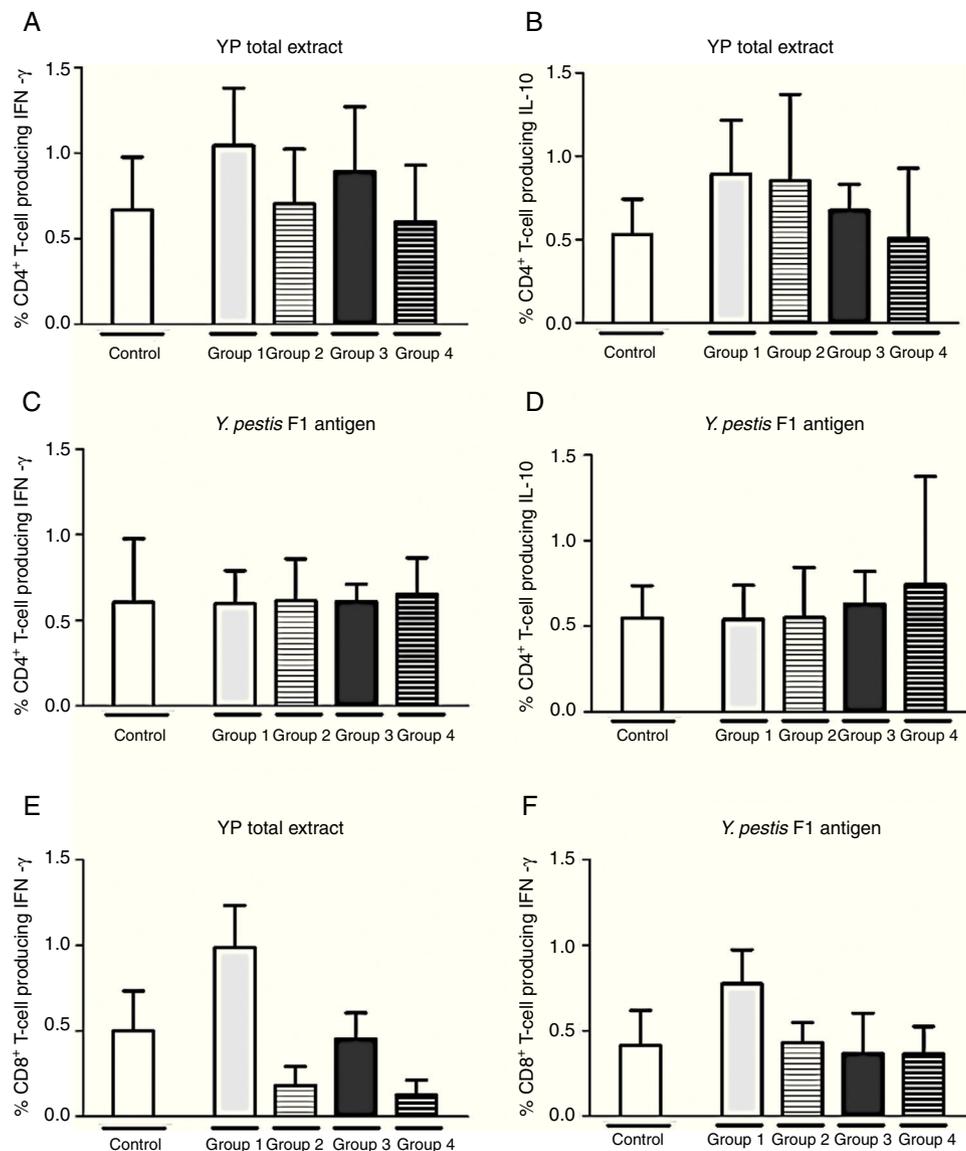
The present study aimed to investigate the immunogenicity of the antigenic preparations based on the *Y. pestis* F1 protein and the YP total extract in inducing the production of anti-F1 Abs and in determining the phenotype of splenic T lymphocytes and the stimulation of the production of IFN- $\gamma$  and IL-10 among the subpopulations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mice. The immunization protocol utilized in our animal experiments was based on a previously established model<sup>16</sup> that evaluated the protective efficacy of *Y. pestis* F1 protein and V subunit vaccines in mice against subcutaneous challenges of the virulent *Y. pestis* strain. Our results showed that animals immunized with the F1 antigen exhibited a strong humoral response with high titres of anti-F1 Ab in the HA/HI test. Furthermore, it was observed that the animals receiving the lowest (20 µg) F1 dose showed titres >1:16, which was the cut-off point in the HA/HI test. These findings were in accordance with another study<sup>16</sup> that demonstrated that a dose of 20 µg of the F1 antigen induced significantly high IgG titres in mice. The *Y. pestis* F1 and V subunit vaccines have been the focus of several studies. Although the value of the *Y. pestis* V antigen is recognized in its protection against non-encapsulated strains (deficient in F1 production), the significant contribution of the F1 protein in the induction of early immune responses is also important. In a study to characterize the immune response of mice after immunization with the F1 protein, quantitative ELISA analysis demonstrated that the levels of anti-F1 IgM antibodies were detectable in serum of animals as early as day 1 post-immunization and that anti-F1 IgG antibodies were detected after three days.<sup>19</sup> The ability of anti-F1 Abs to provide effective protection against the bubonic plague has been demonstrated in several studies.<sup>19,20</sup> Total protection (100%) against a challenge with a virulent *Y. pestis* strain was observed in mice immunized with the F1 antigen.<sup>19</sup> In another animal model, 80% survival rate was demonstrated with active immunization with the recombinant F1 and V antigen.<sup>20</sup> The immunogenicity of recombinant V and F1 proteins may be at maximum because their conformations are similar to those of native proteins.<sup>21</sup> Therefore, this finding can explain the high titres of antibodies observed in our study because the

antigens used in the immunization experiment were obtained from *Y. pestis* cultures and not by using recombinant DNA techniques. The use of the aluminium hydroxide adjuvant could also explain the results obtained for the humoral immune response. Furthermore, other studies have reported that subunit vaccines comprising F1 and V antigens in aluminium hydroxide induced a predominant IgG1 response in both mice and humans.<sup>16,22</sup> Our results showed that the anti-F1 titres were variable among individuals from the same group, which can be attributed to the mouse lineage used in our study. We chose the experimental model with Swiss-Webster mice, which is a genetically heterogeneous outbred lineage<sup>23</sup> that responded in different ways to the same stimulus. The purpose of using that mouse line was to adopt an experimental model that could demonstrate the real efficacy of our putative vaccine because the response to vaccinations may vary among individuals in a population.

Due to the policy of reducing the number of animals in research protocols, only experiments with the minimum number of animals per group were approved by the ethics committee. This would be the best alternative, although the possibility of loss throughout the experiments, e.g., insufficient volume of sera, loss of mononuclear cells when preparing the spleen for cell culture, or microbial contamination of cultivated spleen cell culture, was discussed. In fact, the loss when working with the exact number of animals per group determined an “N” lower than the minimum calculated.

It must be noted that the absence of the humoral immune response to both doses of the YP total extract could indicate that the immunization scheme did not induce detectable levels of anti-F1 antibodies. However, it could be that the YP total extract did not contain a sufficient concentration of F1 protein to induce the humoral immune response in mice.

The role of antibodies in protection against *Y. pestis* has been verified by several studies.<sup>24,25</sup> However, it seems that the humoral immune response induced by subunit vaccines alone may not be sufficient for complete protection against the plague. It was observed that there was 80% protection against a challenge with *Y. pestis* in mice immunized with the F1 and V antigens; however, 20% of them did not survive, even though high levels of antibodies were found. This finding suggests that



**Fig. 3 – Detection of intracellular cytokines IFN- $\gamma$  and IL-10 in T cells. (A) CD4<sup>+</sup>T cells producing IFN- $\gamma$  and (B) IL-10 cytokines after stimulation with YP total extract; (C) CD4<sup>+</sup>T cells producing IFN- $\gamma$  and (D) IL-10 cytokines after stimulation with *Y. pestis* F1 antigen; (E) CD8<sup>+</sup>T cells producing IFN- $\gamma$  after stimulation with YP total extract and (F) after stimulation with *Y. pestis* F1 antigen. Control: aluminium hydroxide adjuvant; Group 1: 40  $\mu$ g of YP total extract; Group 2: 20  $\mu$ g of YP total extract; Group 3: 40  $\mu$ g of *Y. pestis* F1 antigen; Group 4: 20  $\mu$ g of *Y. pestis* F1 antigen.**

protection against *Y. pestis* is not exclusively mediated by antibodies and depends on the involvement of T cells and their cytokine secretion.<sup>20</sup>

To determine the changes in the percentage of T cells that respond to antigenic stimulus, we performed immunophenotypic characterization and quantified the mononuclear splenic cells. Our findings indicated a clear predominance of CD4<sup>+</sup> T cells in all immunized groups studied. Another study also reported a higher percentage of CD4<sup>+</sup> T cells than CD8<sup>+</sup> T cells in peripheral blood from Swiss mice. However, our findings showed numbers of CD4<sup>+</sup> T cells that were higher than those previously reported,<sup>24</sup> which can be justified by the fact that our experiments were performed in spleen T cells. No significant differences were observed in the percentage of T

cells among mice from different groups, which suggested that there was no T cell proliferation in response to antigenic stimulation.

Intracellular cytokine analysis showed that the higher dose of YP total extract (40  $\mu$ g) induced higher expression of IFN- $\gamma$  in CD4<sup>+</sup> T cells (group 1). Although no significant differences were observed, this finding may indicate that T cell activation is higher in animals immunized with a higher dose of bacterial total extract. Therefore, increasing the dose of antigen candidates for the vaccine or administering additional doses may represent an alternative to improve the cellular immune response to *Y. pestis*.

Our study demonstrated that YP total extract induced a lower production of IL-10 in CD4<sup>+</sup> T cells; however, the

difference was not significant. A higher percentage of CD4<sup>+</sup> T cells that expressed IL-10 in groups immunized with the YP total extract (40 µg and 20 µg) was observed. These results may suggest a Th2 response, which could be confirmed by determining the presence of other cytokines, such as IL-4, IL-5 and IL-6. Given the importance of Th1 and Th2 in protecting against *Y. pestis* and the possible use of appropriate adjuvants to direct the adaptive immune response, it is highly necessary to find new co-stimulatory molecules that can induce a mixed response based on cytokines and antibody production, which would represent an important innovation in vaccine research field for the plague.

No significant differences were observed in the percentage of T cell activation in response to the F1 antigen. Additionally, our findings revealed that the T cell response induced by the F1 protein was lower than that of the groups immunized with the YP total extract. This finding is consistent with a previous study<sup>26</sup> that demonstrated that F1 antigen is unable to induce a strong cellular response in animals immunized with the live attenuated EV76 vaccine, suggesting that the F1 protein may not represent a dominant antigen in T cell activation. Our results also showed that the percentage of CD8<sup>+</sup> T cells that expressed IFN-γ was higher in the group that received 40 µg YP total extract than in the other groups, which may suggest the role of YP total extract in eliciting a Th1 response mediated by this cellular subpopulation. This finding is highly important because the production of IFN-γ and TNF-α by CD8<sup>+</sup> T cells plays a crucial role in activating macrophages and neutrophils, which, in turn, exhibit an increased production of reactive oxygen intermediates that act in the control of intracellular bacteria.<sup>27</sup> It was demonstrated that *Y. pestis* cannot replicate in macrophages of mice activated by IFN-γ.<sup>28</sup> Mice whose T cells could not produce IFN-γ and TNF-α did not survive a lethal pulmonary *Y. pestis* infection.<sup>29</sup> Anti-F1 and anti-V Abs protect mice against experimental pneumonic plague, but this protection is highly dependent on IFN-γ and TNF-α production. Therefore, animals that can produce and respond effectively to cytokines seem to protect against *Y. pestis* infections.<sup>30</sup> Our intracellular cytokines analysis exhibited a differentiated response of animals from group 1, which received 40 µg YP total extract. These findings may suggest the activation of CD8<sup>+</sup> T cells by *Y. pestis* immunodominant epitopes. To confirm this hypothesis, further studies are needed to identify the proteins in the YP total extract and their contributions to inducing the cellular immune response.

In brief, we showed that the immunization regimen based on the F1 antigen induced a strong humoral immune response, whereas 40 µg and 20 µg of YP total extract did not induce anti-F1 Ab production in mice. Furthermore, immunization with the YP total extract and the *Y. pestis* F1 antigen neither promoted CD4<sup>+</sup> or CD8<sup>+</sup> T cell proliferation nor induced the expression of IFN-γ and IL-10 by CD4<sup>+</sup> T cells; however, it did stimulate the production of IFN-γ by CD8<sup>+</sup> T cells. Therefore, we can suggest that *Y. pestis* F1 protein is not an immunogenic antigen to T cells.

### Conflicts of interest

The authors declare no conflicts of interest.

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