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New Evidences of the Anti-SARS-CoV-2 Vaccine Candidate FINLAY-FR-02 Effects on Immune System Using Morphometric Techniques

Anti-SARS-CoV-2 Aşı Adayı FINLAY-FR-02'nin Morfometrik Teknikler Kullanılarak Bağışıklık Sistemi Üzerindeki Etkilerine Dair Yeni Kanıtlar

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Abstract

Introduction: As a part of a repeated dose toxicity and local tolerance study of the anti-Severe acute respiratory syndrome-Coronavirus-2 vaccine candidate FINLAY-FR-02, a morphometric histopathological analysis was carried out to obtain a more detailed understanding of its effects on organs within the immune system.
Materials and Methods: Sprague Dawley rats were divided into three groups: those vaccinated with three doses of the vaccine candidate (37.5 µg receptor binding domain), the placebo group, and controls. Tissue samples of the spleen, deep inguinal lymph node, and popliteal lymph node from five animals in each group were obtained at 3, 7, and 21 days after completing the vaccination schedule. Paraffin tissue sections for histological examination were prepared using standard procedures and stained with hematoxylin and eosin.
Results: In the spleen, there was an increase in the density of the Malpighian corpuscles. In the deep inguinal and popliteal lymph nodes, there was an increase in the density of subcapsular secondary lymphoid follicles and paracortical secondary lymphoid follicles, as well as the presence of apoptosis, plasmocytes, macrophages, and mast cells in vaccinated animals.
Conclusion: The histopathological and morphometric observations in the spleen and lymph nodes confirmed the development of an intense immune response against the vaccine candidate FINLAY-FR-02. The morphometric methods developed in this study could be valuable for future research studies related to the evaluation of new vaccine candidates.
Keywords: COVID-19, FINLAY-FR-02, morphometry, immunotoxicology, vaccines

Öz

Giriş: Anti-Şiddetli akut solunum sendromu-Koronavirüs-2 aşı adayı FINLAY-FR-02'nin tekrarlanan doz toksisitesi ve lokal tolerans çalışmasının bir parçası olarak, bağışıklık sistemine ait organlar üzerindeki etkilerinin daha ayrıntılı yorumunu elde etmek amacıyla morfometrik bir histopatolojik çalışma gerçekleştirilmiştir.

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Gereç ve Yöntem: Sprague Dawley sıçanları 3 doz aşı adayı (37,5 µg reseptör bağlama domaini) ile aşılananlar, plasebo ile aşılananlar ve kontroller şeklinde üç gruba ayrıldı. Aşılama programının tamamlanmasından sonraki 3., 7. ve 21. günlerde grup başına beş hayvandan dalak, derin kasık lenf düğümü ve popliteal lenf düğümü örnekleri alındı. Histolojik çalışmalar için parafin doku kesitleri rutin yöntemlerle elde edildi, hematoksilen-eozin ile boyandı.
Bulgular: Dalakta Malpighian cisimciklerinin yoğunluğu, derin inguinal ve popliteal lenf düğümlerinde ise subkapsüler ve parakortikal sekonder lenfoid foliküllerin yoğunluğu, apoptoz, plazmosit, makrofaj ve mast hücrelerinin varlığı aşılanmış hayvanlarda artmıştır.
Sonuç: Dalak ve lenf düğümlerindeki histopatolojik ve morfometrik gözlemler, aşı adayı FINLAY-FR-02'ye karşı yoğun bir immün yanıtın geliştiğini doğruladı. Bu çalışmada geliştirilen morfometrik yöntemler, yeni aşı adaylarının değerlendirilmesi ile ilgili gelecekteki çalışmalarda uygulanabilir.
Anahtar Kelimeler: COVID-19, FINLAY-FR-02, morfometri, immünotoksikoloji, aşılar

Introduction

Coronavirus disease-2019, caused by Severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2), is a rapidly spreading global disease that was declared a pandemic by the World Health Organization in 2020[1]. This fact generated the need to develop vaccines, diagnostic tools, and therapeutic substances aimed at addressing the pressing challenges posed by the disease[2-4].

In this context, the Finlay Vaccine Institute (IFV) of Havana, Cuba, developed anti-SARS-CoV-2 vaccine candidates (FINLAY-FR-01 and FINLAY-FR-02), using known vaccine platforms. These candidates have demonstrated efficacy in prior preclinical and clinical trials[5-7].

As a part of the preclinical requirements prior to its approval and use in humans, a study was conducted to evaluate the repeated dose toxicity and local tolerance of the anti-SARS-CoV-2 vaccine candidate FINLAY-FR-02, using Sprague Dawley (SD) rats as the model. The study found that FINLAY-FR-02, based on the monomeric recombinant receptor binding domain (RBDm) conjugated with tetanus toxoid (TT) and absorbed onto aluminum hydroxide, exhibited both efficacy and nontoxicity[8].

Additionally, morphometric analyses have gained significant importance in anatomic pathological studies, as they constitute a tool that allows objectively evaluating tissue-level changes, thus, providing a better interpretation. Since morphometric evaluations have demonstrated the effects of different substances on immune system organs in several species[9-11], it is of crucial interest to perform such analyses in the context of the aforementioned preclinical toxicological study.

In this sense, the present study aimed to conduct a morphometric histopathological study of immune system organs to obtain a more detailed interpretation of the effects of the vaccine candidate FINLAY-FR-02.

Materials and Methods

Product Under Evaluations

FINLAY-FR-02 consists of a suspension containing the active pharmaceutical ingredient, the SARS-CoV-2 RBD protein monomer (sequence: 319-541 residues with a polyhistidine fusion tag at its C-terminus), expressed in CHO cells. The purified RBD was chemically conjugated with the TT binding protein and adsorbed on aluminum hydroxide[8]. The vaccine was manufactured in accordance with Good Manufacturing Practice by IFV and the Center for Molecular Immunology in Havana, Cuba.

Animals and Accommodation

Male SD rats, aged 8-9 weeks, were obtained from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). They were housed at the IFV Animal Care Center in Tecniplast® rat boxes (2-3 rats per cage), at 21 °C±2 °C and a relative humidity of 55%±5%, with 12-h/12-h' light and dark cycles. The rats had access to rodent food and water *ad libitum.* The rats were allowed to acclimatize for one week before starting the experimental protocol. All protocols were approved by the FINLAY Vaccine Institute Institutional Committee for the Care and Use of Animals (code: P-05/20, date: 19.11.2020).

Experimental Design

A total of 45 rats were randomly assigned to one of the three groups: FINLAY-FR-02 (12.5 µg SARS-CoV-2 RBD protein monomer conjugated to 11.5 µg TT and adsorbed in 250 µg/0.25 mL aluminum hydroxide), the placebo group (excipients of the vaccine candidate, including aluminum hydroxide), and a Control group, which received 0.9% NaCl. Three doses were administered intramuscularly to each group with 24-h intervals between each one. The injection volume was 0.25 mL (representing 50% of the human dose), divided into two sites (on both hind limbs). The injection volume corresponds to the maximum allowed for this administration route and host species[12,13].

Five rats per group were euthanized by an overdose of sodium thiopental (80 mg/kg, AICA Laboratories, Havana, Cuba) at 3, 7, and 21 days. Samples from three organs of the immune system (spleen, deep inguinal lymph node, and popliteus lymph node) were removed[8].

All the procedures were carried out taking into account the standards, guidelines, and publications following the Canadian Council on Animal Care, the American Veterinary Medical

Association guidelines, the Center for the State Control of Medicines and Medical Devices of Cuba (regulation no. 39/04 CECMED, MINSAP, Cuba), and Morton's recommendations on the humanitarian endpoint (Morton, 1999)[14-17].

Histological Processing

The samples were fixed in 4% buffered formalin, longitudinally sectioned, following previously described methodology, and processed using routine histological methods. These processed samples were embedded in paraffin, and 4 µm-thick sections were obtained using a Sakura microtome. Also, sections were stained with hematoxylin and eosin (HE)[18].

Histopathological Analysis

The evaluation of the three organs (spleen, deep inguinal lymph node, and popliteal lymph node) was performed independently by two pathologists using a Zeiss Primo Star Light microscope. Microphotographs were obtained using a Canon EOS 1000 D#2 digital camera attached to the microscope.

Histopathological Variables

Density of Malpighian Corpuscles in the Spleen

The total number of Malpighian corpuscles (MC) in each histological section of the spleen was determined by direct observation under the microscope using a 100 X magnification. These MCs were identified within the white pulp, which consists of a mass of lymphocytes arranged around a central artery[19].

Besides, the area of each spleen section was measured using macroscopic images obtained with a 13 MP Xiaomi Redmi 6 camera, using the morphometry software Image J[20]. Then, the MC density was calculated by dividing the number of MCs by the corresponding spleen's section area. The results were expressed as the number of MC per square millimeter.

Diameter of the Malpighian Corpuscles in the Spleen

Microphotographs were taken at three zones (upper, middle, and lower) within each spleen histological section at 16 X magnification. The largest diameter of three MC in each image was measured using Image J[20].

Number of Secondary Lymphoid Follicles

The lymphoid follicles were identified based on their location in the different anatomical areas of the lymph node, cortex below the capsule (subcapsular), and the intermediate paracortex (paracortical). These follicles consist of pale-staining lymphocytes, with B lymphocytes in the subcapsular region, and T lymphocytes in the paracortical region. Secondary lymphoid follicles were recognized by their enlarged clear region[19].

The number of subcapsular secondary lymphoid follicles (SCSLF) and paracortical secondary lymphoid follicles (PCSLF) was counted in popliteal and deep inguinal lymph nodes through direct observation under the microscope using a 100 X magnification.

Presence of Apoptosis in SCSLF and PCSLF

The presence or absence of apoptotic cells in SCSLF and PCSLF in both popliteal and deep inguinal lymph nodes was quantified through direct microscopic observation at 400 X magnification. These cells were recognized by the retraction of cytoplasmic components and apoptotic bodies resulting from chromatin condensation[19].

Cell Types in the Sinuses and Hilum of the Lymph Nodes

The predominant cell types in the sinuses and the hilum of the popliteal and deep inguinal lymph nodes (plasmocytes, macrophages, and mast cells) were identified by direct microscopic observation. Three histological fields randomly selected from these aforementioned areas were observed at 400 X magnification. The presence of the mentioned cells was classified as "Scarce" if the average count from the three observations was less than 15 or "Abundant" if the average count exceeded 15.

Data Management and Statistical Analysis

The comparisons between groups were made using the non-parametric Mann-Whitney U test, except for the presence of apoptosis of the lymphoid follicles, where the Fisher's test was used. For all variables, the group of vaccinated rats was compared with the placebo and control groups. *A priori*, a significance level of $p \leq 0.05$ was set. Statistical analyses were performed using the commercial package GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

Density of MC in the Spleen

An increase in the number of MC was observed in the vaccinated group compared to the placebo and control groups at the 7- and 21-day sampling points (Table 1, Figure 1).

Diameter of MC in the Spleen

No significant differences in the diameter of the MCs were found among the study groups at any of the sampling points (Table 2).

Table 1. Density of Malpighian corpuscles in the spleen

Group	Day 3	Day 7	Day 21
FINLAY-FR-02	1.0±0.12	1.5±0.16*	1.7±0.11***
Placebo	0.96±0.05	0.75±0.12	0.71±0.05
Control	1.0±0.02	0.90±0.04	0.80±0.6

Density of MC (number of MC/mm2). Data as mean±mean standard error. Comparison with respect to placebo and control groups. Mann-Whitney U test (*p<0.05; ***p<0.001).

MC: Malpighian corpuscles

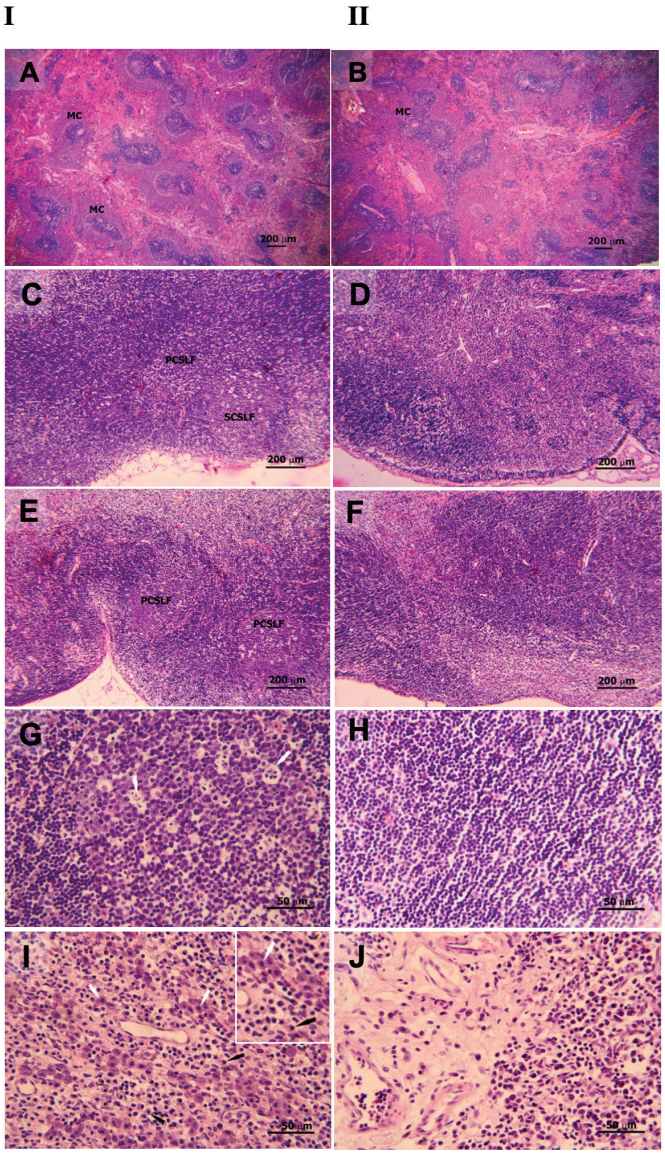

Figure 1. Cross sections. HE. I) Vaccine; II) Control. (A, B) Spleen. Observe a greater amount of Malpighian corpuscles in A. (C, D) popliteal lymph node. Note an increase in subcapsular secondary lymphoid follicles and paracortical secondary lymphoid follicles (PCSLF) in C. (E-H) Deep inguinal lymph node. Note an increase in PCSLF in E, as well as apoptosis in secondary lymphoid follicles in G (white arrows). (I, J) Popliteal lymph node. Observe the presence of abundant plasmocytes (black arrows) and mast cells (white arrows) IN sinuses in I. Bars: (A-F) 200 µm, (G-J) 50 µm

Table 2. Diameter of the Malpighian corpuscles in the spleen

Group	Day 3	Day 7	Day 21
FINLAY-FR-02	806.0±57.55	778.7±20.20	841.8±26.80
Placebo	752.1±27.99	799.2±24.46	833.4±45.58
Control	738.4±43.51	804.9±24.41	821.2±44.23

Data as mean±mean standard error. Comparison with respect to placebo and control groups. Mann-Whitney U test

Lymph Nodes

In the popliteal and deep inguinal lymph nodes, the presence of SCSLF and PCSLF with apoptosis, as well as plasmocytes, macrophages, and mast cells in the sinuses and hilum, was evident in all the three groups.

Numbers of SCSLF and PCSLF

In the popliteal lymph node, an increase in the number of SCSLF was observed, which was significant in the vaccinated group compared to the placebo and control groups in the samples at the 3- and 7-day samples, while the number of PCSLF increased only at day 3 (Figure 1, Table 3).

In the deep inguinal lymph node, an increase in the number of SCSLF and PCSLF was observed, with significance in vaccinated rats compared to placebo and control groups at days 3 and 7. However, no differences between groups were noted regarding SCSLF at day 21 (Figure 1, Table 4).

Presence of Apoptosis of SCSLFs and PCSLFs in Deep Inguinal and Popliteal Lymph Nodes

The presence of apoptosis in the follicles of both lymph nodes significantly increased in vaccinated rats compared to controls and placebo rats. In the popliteal lymph node, this was evident at all three sampling times (Figure 1, Table 5). However, in the deep inguinal lymph node, it was observed at days 7 and 21 (Table 5).

Cell Types in the Sinuses and Hilum of the Popliteal and Deep Inguinal Lymph Nodes

In the vaccinated animals, abundant plasmocytes, and macrophages, as well as mast cells, were present in the sinuses and the hilum of both lymph nodes, unlike the control and placebo groups, where these cells were scarce, at all sampling times (Figure 1).

Discussion

The animal model used in the present study to demonstrate the nontoxicity of the vaccine candidate FINLAY-FR-02 in SD rats is a well-characterized model for vaccine toxicological studies and has been used previously in preclinical studies on SARS-CoV-2 vaccines[21-24].

In this work, the importance of morphometric studies in interpreting the effects of the vaccine candidate FINLAY-FR-02 effects on immune system organs was demonstrated.

The spleen, with its various roles in blood filtering and controlling, as well as influencing the systemic immune response, serves as the main indicator to evaluate the condition of the organism and its adaptation to the external environment. MCs are part

Table 3. Numbers of SCSLF and PCSLF in the popliteal lymph node

Group	Day 3		Day 7		Day 21	
	SCSLF	PCSLF	SCSLF	PCSLF	SCSLF	PCSLF
FINLAY-FR-02	10.00±1.58*	4.60±0.74*	5.00±0.83*	2.40±0.67	5.80±1.06	2.40±0.74
Placebo	1.50±0.86	1.25±0.75	2.75±0.85	2.00±0.00	2.80±0.86	1.80±0.86
Control	1.00±0.44	0.60±0.60	1.40±0.97	0.80±0.58	2.40±1.12	0.40±0.40

Data as mean±mean standard error. Comparison with respect to placebo and control groups. Mann-Whitney U test (*p<0.05).

SCLF: Subcapsular secondary lymphoid follicles, PCSLF: Paracortical secondary lymphoid follicles

Table 4. Numbers of subcapsular and paracortical secondary lymphoid follicles in the deep inguinal node

Group	Day 3		Day 7		Day 21	
	SCSLF	PCSLF	SCSLF	PCSLF	SCSLF	PCSLF
FINLAY-FR-02	7.60±1.44*	4.20±0.66*	6.80±0.73*	3.20±0.58*	4.00±0.89	1.80±0.58*
Placebo	1.50±0.64	1.00±0.57	1.80±0.37	1.00±0.44	4.40±1.03	2.20±0.37
Control	1.25±0.94	0.75±0.25	0.60±0.40	0.20±0.20	4.00±1.22	0.00±0.00

Data as mean±mean standard error. Comparison with respect to placebo and control groups. Mann-Whitney U test (*p<0.05).

SCLF: Subcapsular secondary lymphoid follicles, PCSLF: Paracortical secondary lymphoid follicles

Table 5. Presence of apoptosis in secondary lymphoid follicles of popliteal and deep inguinal lymph nodes

Sampling day	Lymph nodes	Groups		
		FINLAY-FR-02	Placebo	Control
3	Popliteal lymph node	80*	0	0
	Deep inguinal lymph node	60	50	0
7	Popliteal lymph node	60*	25	0
	Deep inguinal lymph node	80*	20	0
21	Popliteal lymph node	100**	40	0
	Deep inguinal lymph node	80*	0	0

Data as number of rats with apoptosis/total of rats (%).

Comparison with respect to placebo and control groups. Fisher's test (*p≤0.05, **p≤0.01)

of the white pulp in which the germinal centers (GC) develop, containing abundant B lymphocytes that could be identified in sections stained with HE[25].

Upon exposure to systemic antigens, GCs play a pivotal role in generating and refining the humoral response. In GCs, B lymphocytes undergo proliferation and differentiation into antibody-producing plasmocytes that exit the GC. This process also involves somatic hypermutation and class switching of the GC, enabling B lymphocytes to produce antibodies with higher affinity and different subtypes (IgM, IgG, etc.), each with different functions. Changes in these GC structures provide information about the cell populations affected by xenobiotics[19,26].

In this study, vaccinated rats showed an increase in the number of MCs at days 7 and 21 compared to placebo and control groups. It is probably due to the effect of chemical substances on the hematopoietic system and, particularly, on the spleen, where early stages of the immunological responses involve the proliferation and differentiation of T and B lymphocytes activated by antigens[25].

However, no significant differences were observed between the study groups concerning the diameter of the MCs at any of the sampling points. This observation confirms the discrete MCs hyperplasia reported in the previous study based on the qualitative histological study[8].

Thus, these morphometric findings explain that, despite the increase in the number of MCs without altering their diameter in vaccinated animals, there were no changes in the area of the analyzed spleen histological sections. This contrasts with the outcomes obtained in the previous study after measuring area using macroscopic images[8].

Regarding the lymph nodes, the qualitative evaluation (presence or absence of secondary follicles) carried out in the previous study based on the presence of SCSLF and PCSLF was verified in the three groups, at the three sampling times, without observing significant differences between them[8].

Conversely, in the current study, an increase in the number of SCSLF and PCSLF was demonstrated in both lymph nodes of the vaccinated animals upon performing a quantitative analysis.

The increase in the number of SCSLF in the popliteal lymph node may be linked to the inoculated substances present in the vaccine candidate. This is possibly due to an increase in the production of antibodies from B lymphocytes in the subcortical area of the lymph nodes, especially if we take into account that the popliteal lymph node filters lymph originating from the muscle area where the vaccine candidate was inoculated[27,28].

Regarding the deep inguinal lymph node, the increase in the number of SCSLF and PCSLF in the vaccinated group may be attributed to these lymph nodes draining lymph from various areas of the animal's body, including the sites of inoculations[28].

These results correspond to the previously reported increase in antibody levels in rats vaccinated with the FINLAY-FR-02 vaccine candidate[8].

In addition, the increase in cell apoptosis at secondary follicles of lymph nodes among vaccinated animals compared to control and placebo rats can be attributed to its crucial role in sustaining the immune response of B and T lymphocytes. This mechanism come into play as soon as adequate antibody levels are reached, ensuring the maintenance of their quantitative and qualitative normality and contributing to adequate homeostasis[8,29,30].

Likewise, in the lymph nodes of the vaccinated animals, the presence of abundant plasmocytes, macrophages, as well as mast cells in the sinuses and hilum was observed, in contrast to the control and placebo groups, where these cells were scarce.

The participation of plasmocytes and mast cells in the immune response is widely recognized. The increased number of plasmocytes is associated with increased antibody generation, as a key goal of any vaccination is to stimulate the production of highly effective, specific antibodies[29]. Thus, the increase in mast cells observed in the vaccinated animals of study could indicate their intervention as activators of the immune response[31].

Furthermore, in the sinuses of lymph nodes, macrophages hold a strategic position at the interface of this tissue, where they entrap and present antigens to B lymphocytes. Macrophages have also been shown to prevent the systemic spread of lymph-borne pathogens[32].

Also, macrophages possess the capability of activating a wide range of innate and adaptive memory effector cells, including follicular memory T cells and memory B cells, which are rapidly prepositioned or recruited into the subcapsular sinus following infection. In addition, memory B lymphocytes are rapidly reactivated to differentiate into plasmocytes within subcapsular proliferative foci[31].

The vaccine candidate used in this study could also have influenced both lymph nodes, given it composition containing subunits from the SARS-CoV-2 virus. It is known that the entry of microorganisms into a host cell involves the binding of certain surface molecules to cell proteins[29].

The alterations described in both deep inguinal and popliteal lymph nodes of vaccinated rats, including the increased presence of SCSLF, PCSLF, cell apoptosis of the follicles, number of plasmocytes, macrophages, and mast cells, are consistent with the increased total area of both nodes as assessed through macroscopic evaluation in a previous study[8].

The increase in number of MCs in the spleen, as well as SCSLF and PCSLF in the lymph nodes close to the inoculation site, together with previously reported granulomatous formations[8], provides additional support for the immune response triggered by the vaccine candidate FINLAY-FR-02.

An added contribution of this study is the application of morphometric methods to illustrate the changes induced in the immune system organs following antigenic stimulation. These methods could be applied to future studies related to the evaluation of new vaccine candidates.

Study Limitations

Considering the observed changes in representative immune system organs, from histological preparations using routine techniques (HE staining), excluding immunohistochemical studies to better understand the effects of the vaccine candidate on specific cell types involved in the immune response may have limited our findings.

Conclusion

The histopathological and morphometric observations conducted on the spleen and lymph nodes confirm the development of an immunological response against the vaccine candidate FINLAY-FR-02.

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Ethics

Ethics Committee Approval: The study was approved by the Animal Care and Use of FINLAY Vaccine Institute of Institutional Committee (code: P-05/20, date: 19.11.2020).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.F-M., T.H-S., Concept: J.F.I-B., S.R-S., D.G.R., Design: J.F.I-B., S.R-S., D.G.R., Data Collection or Processing: J.F.I-B., S.R-S., L.O-N., M.F-M., T.H-S., Analysis or Interpretation: J.F.I-B., S.R-S., L.O-N., V.C-D-P., Literature Search: J.F.I-B., S.R-S., V.C-D-P., Writing: J.F.I-B., S.R-S., V.M-C., L.M.R-N., D.G.R.

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