IMMUNOGENICITY OF FULL-LENGTH AND MULTI-EPITOPE MRNA VACCINES FOR *M. TUBERCULOSIS* AS DEMONSTRATED BY THE INTENSITY OF T-CELL RESPONSE: A COMPARATIVE STUDY IN MICE

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Development of the new tuberculosis vaccines that would be effective in adults is an urgent task: worldwide, the annual death toll of this disease exceeds 1.5 million. In the recent decades, the matter has been addressed in numerous studies, but none has yielded an effective vaccine so far. There are many factors to resistance against tuberculosis; this study focuses on the T-cell response, a mechanism that enables elimination of intracellular pathogens, such as *M. tuberculosis*. We aimed to develop an mRNA vaccine capable of triggering a pronounced T-cell response to the *M. tuberculosis* antigens. The *in silico* analysis allowed us to select epitopes of the *M. tuberculosis* secreted protein ESAT6 (Rv3875) and design a multi-epitope mRNA vaccine thereon. We assessed the intensity of T-cell response in mice immunized with mRNA vaccines that encode a full-length or multi-epitope antigen. The results of this study in mice show that immunization with a multi-epitope mRNA vaccine produces twice as many IFNγ-secreting splenocytes in response to specific stimulation than immunization with an mRNA vaccine encoding the full-length protein. Thus, the developed multi-epitope mRNA vaccine can be an effective *M. tuberculosis* prevention agent the mode of action of which involves formation of a pronounced T-cell response.

Keywords: mRNA vaccine, multi-epitope vaccine, T-cell response, ELISpot

Funding: the study was supported by the Ministry of Science and Higher Education of the Russian Federation (agreement № 075-10-2021-113, unique project identifier RF----193021X0001).

Acknowledgements: the authors express their gratitude to the staff of the Sirius University: Terenin IM for *in vitro* transcription, Zaborova OV for the formulation of mRNA into lipid nanoparticles, Gavrilyak MA and Golovin EV for purification and characterization of the Rv3875 protein; Shevyrev DV for setting up the intracellular staining experiment and Sitikova VA for assistance in the context of the animal experiment.

Author contribution: Vasileva OO, Tereschenko VP — work with the cells, data analysis, article authoring, figures authoring; Krapivin BN, Pateev II, Rybtsov SA — work with the cells, animal experiment, data analysis; Muslimov AR, Kukushkin IS — design of structures, cloning; Ivanov RA, Reshetnikov VV — text editing, data analysis, project coordination.

Compliance with the ethical standards: animal study was approved by the Ethics Committee of the Institute of Cytology and Genetics (2022); carried out in accordance with the laboratory animals care guidelines (2010), Directive 2010/63/EU of the European parliament and of the Council on the protection of animals used for scientific purposes (2010), Good Laboratory Practice guidelines (2016).

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Received: 24.04.2023 **Accepted:** 15.06.2023 **Published online:** 29.06.2023

DOI: 10.24075/brsmu.2023.021

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ИММУНОГЕННОСТИ ПОЛНОРАЗМЕРНОЙ И МУЛЬТИЭПИТОПНОЙ МРНК-ВАКЦИН ПРОТИВ *M. TUBERCULOSIS* ПО ВЫРАЖЕННОСТИ Т-КЛЕТОЧНОГО ОТВЕТА У МЫШЕЙ

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Разработка новых вакцин против туберкулеза, эффективных в том числе и у взрослых, является актуальной задачей, поскольку ежегодная смертность от этого заболевания во всем мире превышает 1,5 млн случаев. Несмотря на множество исследований в последние десятилетия, эффективной вакцины все еще не получено. Сопротивляемость туберкулезу состоит из многих факторов, в этом исследовании сделан акцент на Т-клеточном ответе — механизме, позволяющем элиминировать внутриклеточные патогены, такие как *M. tuberculosis*. Целью исследования было разработать мРНК-вакцину, способную формировать выраженный Т-клеточный ответ на антигены *M. tuberculosis*. С помощью анализа *in silico* были выбраны эпитопы секреторного белка ESAT6 (Rv3875) *M. tuberculosis* для дизайна мультиэпитопной мРНК-вакцины. Проведена оценка эффективности Т-клеточного ответа у мышей, иммунизированных мРНК-вакцинами, которые кодируют полноразмерный или мультиэпитопный антиген. Результаты показали, что при иммунизации мультиэпитопной мРНК-вакциной количество IFNγ-секретирующих спленоцитов в ответ на специфичную стимуляцию в два раза выше, в сравнении с количеством IFNγ-секретирующих клеток у мышей, иммунизированных мРНК-вакциной, кодирующей полноразмерный белок. Таким образом, разработанная мультиэпитопная мРНК-вакцина может стать эффективным препаратом для профилактики *M. tuberculosis* посредством формирования выраженного Т-клеточного ответа.

Ключевые слова: мРНК-вакцина, мультиэпитопная вакцина, Т-клеточный ответ, ELISpot

Финансирование: исследование выполнено при поддержке Министерства науки и высшего образования Российской Федерации (соглашение № 075-10-2021-113, уникальный идентификатор проекта РФ----193021X0001).

Благодарности: авторы выражают благодарность сотрудникам АНО ВО «Университет «Сириус»: И. М. Теренину за постановку транскрипции *in vitro,* О. В. Заборовой за формуляцию мРНК в липидные наночастицы, М. А. Гавриляк и Е. В. Головину за очистку и характеризацию белка Rv3875; Д. В. Шевыреву за постановку эксперимента по внутриклеточному окрашиванию и В. А. Ситиковой за помощь в проведении эксперимента с животными.

Вклад авторов: О. О. Васильева, В. П. Терещенко — клеточные работы, анализ данных, написание статьи, подготовка рисунков; Б. Н. Крапивин, И. И. Патеев, С. А. Рыбцов — клеточные работы, проведение эксперимента с животными, анализ данных; А. Р. Муслимов, И. С. Кукушкин — дизайн конструкций, клонирование; Р. А. Иванов, В. В. Решетников — редактирование текста, анализ данных, координация проекта.

Соблюдение этических стандартов: исследование на животных одобрено этическим комитетом ИЦиГ СО РАН (2022 г.); проведено в соответствии с руководством по уходу и использованию лабораторных животных (2010 г.), Directive 2010/63/EU of the European parliament and of the council on the protection of animals used for scientific purposes (2010 г.), «Правилами надлежащей лабораторной практики» (2016 г.).

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Статья получена: 24.04.2023 **Статья принята к печати:** 15.06.2023 **Опубликована онлайн:** 29.06.2023

DOI: 10.24075/vrgmu.2023.021

Every year, more than 10 million new cases of tuberculosis and about 1.5 million deaths from the disease are registered worldwide. The COVID-19 pandemic has boosted the spread of tuberculosis: it develops against the backdrop of the viral infection, and the global incidence of tuberculosis in 2020 and 2021 has grown by 3–6% compared to previous years [1]. Despite this, in the pandemic years of 2020 and 2021, the incidence of tuberculosis in the Russian Federation decreased, but at the same time, its course became more complicated, with more first-time patients suffering from lung tissue destruction, massive bacterial excretion, and fibrous-cavernous variety of tuberculosis [2].

Currently, the only approved tuberculosis prevention vaccine is BCG. It has been used for more than 100 years; in addition to tuberculosis, BCG can help develop non-specific protection against other respiratory and viral diseases, training the immunity [3]. In the Russian Federation, children (provided there are no contraindications) receive 3 injections of BCG after birth. In addition to its protective effect, the vaccine can cause a number of post-vaccination complications, inflammatory reactions manifesting as infiltrates, abscesses, fistulas, and ulcers [4]. Another disadvantage of BCG is that it does not shield adults from pulmonary tuberculosis [5]: if the bacteria is multidrug-resistant or extensively drug-resistant, it is necessary to use expensive and toxic chemotherapy drugs [6]. The foregoing adds urgency to the task of finding new effective vaccines against tuberculosis for adolescents and adults, including for the purposes of prevention and to enable the immunity to combat multidrug-resistant tuberculosis.

The main purpose of vaccination is to prevent development of the disease. In the case of tuberculosis, T-cell response drives elimination of the pathogen, and the vaccine protects by triggering production of $CD4^+$ memory cells. In turn, $CD8^+$ cells control the spread of mycobacteria in chronic tuberculosis cases. Secretion of the pro-inflammatory IFNγ cytokine by T-cells is associated with the vaccine's protective effect [7].

Searching for the new drugs to fight and prevent tuberculosis, researchers most often consider subunit protein vaccines or viral vectors [8], but the new trend in the scientific community is to suggest mRNA-based vaccine designs. One of the studies has demonstrated the ability of a self-replicating mRNA vaccine to induce a specific response of $CD4^+$ and $CD8^+$ cells [9]. mRNA vaccines have a number of advantages over vaccines of other types. Compared to the vaccines based on protein antigens, they guarantee an order of magnitude higher antibody titer, and the cost of production and scaling mRNA vaccines is lower, since the process is cell-free. Compared to DNA vaccines, mRNA vaccines are less genotoxic, since they cannot merge with the genome [10], and more effective, since, unlike DNA vaccines, they act in the cell's cytosol and not in the nucleus, which means their performance is independent from the cell cycle stage during transfection [11].

A transition from attenuated vaccines to protein or nucleic acid vaccines raises the problem of selecting the epitope for immunization. The most common choice are subunit vaccines that contain a single protein sequence [12], but there are also vaccine variants with epitopes from one or more pathogen antigens that may induce a synergistic immune response [13]. Such vaccines are called multi-epitope. Humoral and T-cell responses are triggered by different epitopes. B-cell epitopes can be non-linear (repeating the native protein conformation) and linear, and T-cell epitopes are short peptides. Multi-epitope vaccines can induce both types of immune responses simultaneously. Bioinformatic tools are actively used to predict the epitopes for mRNA tuberculosis vaccines [14, 15]. Short peptides in the composition of a multi-epitope vaccine mitigates the risk of allergic reactions peculiar to attenuated vaccines [16]. In addition, a multi-epitope vaccine can contain epitopes of antigens of various microorganisms, which translates into effective simultaneous immunization against several pathogens [13].

However, there are few experimental studies that compare the effectiveness of mRNA vaccines encoding full-length and multi-epitope variants of antigens. Therefore, this study in mice sought to assess the intensity of T-cell response following immunization with mRNA vaccines that encode the full-length or multi-epitope *M. tuberculosis* antigen.

METHODS

Experiment design

The experiment involved 15 male C57BL6/J mice, age 8–9 weeks, SPF status, weight 19–21 g. The animals were provided by the Center for Genetic Resources of Laboratory Animals of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. They were kept in a conventional vivarium with a fixed 12 light/12 dark cycle; there were no restrictions imposed on the standard feed (granules) and water the mice received. The two different variants of mRNA vaccines — mRNA Rv3875 (582 nucleotides) or mRNA mEpitope (699 nucleotides), 50 μg of RNA each, — were administered intramuscularly. In addition, mice were immunized with full-length Rv3875 protein (25 mg) adjuvanted with mRNA-free lipid nanoparticles in an amount equivalent to (± 10%) the number of particles in the RNA vaccine groups. Besides the test groups, there were two control groups in the experiment, first of which received lipid nanoparticles without mRNA, second — a phosphate buffer. Each group included three animals. Second immunization (same doses) took place 4 weeks after the first one. The mice were killed 4 weeks after second immunization and their spleen isolated (Fig. 1).

Selection of epitopes for the Rv3875 multi-epitope construct

The software that enabled prediction of the MHC I epitopes was the MHC Lurry 2.0 version 2.0.4 package [17], and for MHC II epitopes we used the NetMHCIIpan 4.1 package [18]. The "-length" values selected for the epitope calculations were 13,14,15,16,17, plus the -BA modifier to enable the epitope-allele affinity prediction mode. Quantitative characteristics of the allele frequencies were taken from the published data [19, 20]. Python 3.9 was used to filter the predictions output by MHCFlurry 2.0 and NetMHCIIpan 4.1. The criteria were that each epitope-allele pair should interact with an affinity of no more than 500 nM, and each epitope should bind to at least 5 different alleles. The algorithm allowed selecting two epitopes for MHC I (LLDEGKQSL and AAWGGSGSEAY) and three epitopes for MHC II (WNFAGIEAAASAIQG, KQSLTKLAAAWGGSG and LNNALQNLARTISEA). The final construct contained five epitopes (1 copy of each epitope) connected by linkers (KK for MHCII and AAY for MHCI). In addition, we added to the construct a sequence of the cytoplasmic and transmembrane domain of MHC class I (IVGIVAGLAVLAVVVIGAVVATVMCRRKSSGGKGGS YSQAASSDSAQGSDVSLTA), which enables colocalization of the target protein with histocompatibility complexes in various endocytic compartments [21].

Cloning

Seeking to produce the constructs for subsequent *in vitro* transcription of RNA, we extended the pSmart vector (Lucigen;

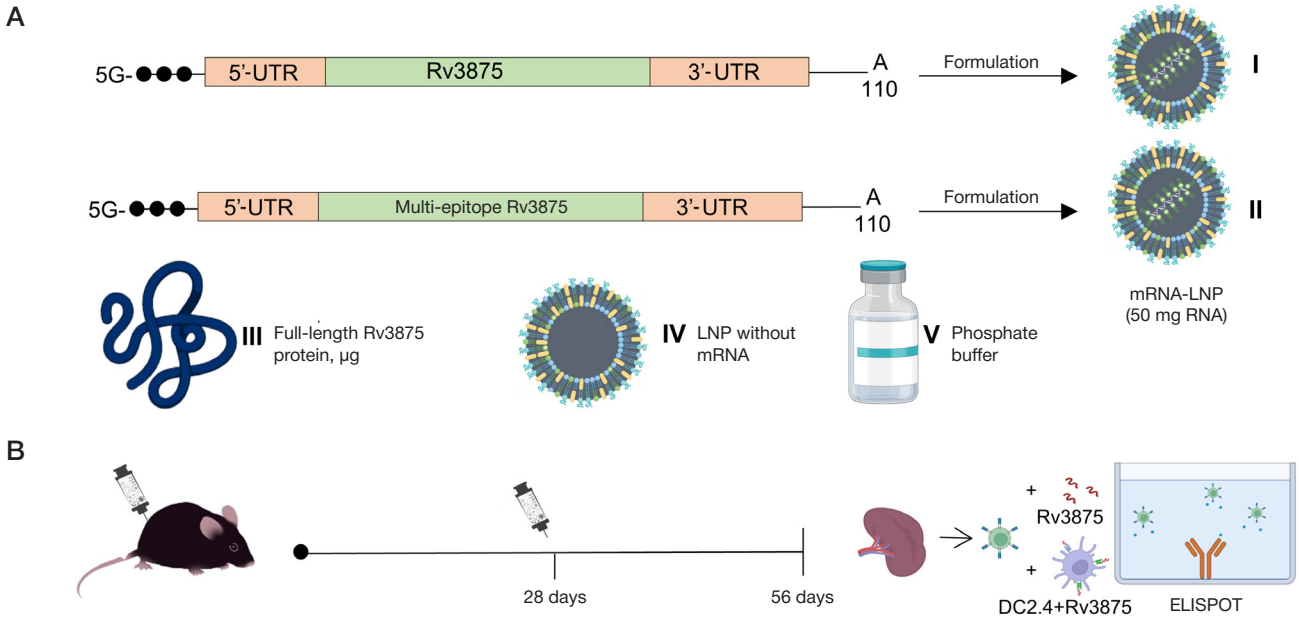

Fig. 1. Experiment design. **A**. Schematic representation of composition of the mRNA-LNP vaccine and other drugs. **B**. Design of the experiment in C57BL6/J mice. The two different variants of mRNA vaccines — mRNA Rv3875 (582 nucleotides) or mRNA mEpitope (699 nucleotides), 50 μg of RNA each, — were administered intramuscularly. In addition, mice were immunized with full-length Rv3875 protein (25 mg) adjuvanted with mRNA-free lipid nanoparticles in an amount equivalent to (±10%) the number of particles in the RNA vaccine groups. Besides the test groups, there were two control groups in the experiment, first of which received lipid nanoparticles without mRNA, second — a phosphate buffer. Each group included three animals. Second immunization (same doses) took place 4 weeks after the first one. The mice were killed 4 weeks after second immunization and their spleen isolated. We used the ELISpot assay to count splenocytes secreting IFNγ in response to the Rv3875 tuberculosis protein post vaccination, and thus measured the level of the resulting T-cell response

USA) with a cassette with 5'UTR (gggaaataagagagaaaagaag agtaagaagaaatataagaccccggcgccgccacc) sequence, Rv3875 protein sequence (full-length or multi-epitope), and 3'UTR sequence (gctggagcctcggtggcctagcttcttgccccttgggcctcccc ccagcccctcctcccctcctgcacccgtacccccgtgtctttgaataaagtctga gtgggcggca). Next, we applied the EcoRI and BglII restriction enzymes to the resulting cassette, purified it on the agarose gel and ligated with a similarly prepared pSmart commercial vector (Lucigen; USA). Chemically competent NEB-stable cells (New England Biolabs; UK) were transformed with a ligation mixture and plated on LB agar with 100 μg/mL of ampicillin. To check the colonies for the insert, we used PCR. The plasmids were verified by Sanger sequencing, and to analyze the sequencing chromatograms, we used the Ugene v38.1 software. *E. coli* NEB-stable with the vector were cultivated at 30°C and 180 rpm. QIAGEN Plasmid Maxi Kit (Qiagen; USA) was used to isolate and purify the plasmid DNA. To obtain a linearized plasmid, we applied SpeI to the preparation at the unique restriction site located after the poly(A) tail.

A pET30 vector (Agilent; United States) was used to build the construct and produce the full length Rv3875 protein. The Rv3875 sequence with an additional sequence for six histidines at the N-terminus was assembled from the oligonucleotides with the help of PCR. We applied the EcoRI and NdeI restriction enzymes to the resulting cassette, purified it on the agarose gel and ligated with a similarly prepared pET30 vector (Lucigen; USA). For transformation, we used the BL21DE3 cells (Agilent; USA). The plasmids were verified by Sanger sequencing.

In vitro mRNA transcription

The *in vitro* transcription was done as described previously [22]. We used 5 μg of linearized plasmid, buffer (20 mM DTT, 2 mM spermidine, 80 mM HEPES-KOH pH 7.4, 24 mM $MgCl_2$), 500 units of T7 RNA polymerase (Biolabmix; Russia), 200 units of ribonuclease inhibitor RiboCare (Evrogen; Russia) and 1 μl of a mixture of enzymes from the RiboMAX Large Scale RNA Production System (Promega; USA) as a source of inorganic pyrophosphatase. The reaction mixture also contained 12 mM of the ARCA cap analogue (Biolabmix; Russia) and 3 mM of each of the ribonucleoside triphosphates (Biosan, Russia). The reaction was supported for 2 hours at 37 °C; after that, we added another 3 mM of each of the ribonucleoside triphosphates and incubated for another 2 hours. For DNA hydrolysis, we used the RQ1 nuclease (Promega; USA); the RNA was precipitated by adding LiCl to the concentration of 0.32 M and EDTA pH 8.0 to the concentration of 20 mM, after which the solution was put on ice for 1 hour of incubation. Next, the solution was centrifuged for 15 min (25,000 g at 4 °C). The RNA precipitate was washed with 70% ethanol, dissolved in ultrapure water and then precipitated again with alcohol as per the standard procedure. Spectrophotometry enabled identification of the RNA concentration; we measured absorption at 260 nm. The target length and homogeneity of the synthesized RNA molecules were assessed with the help of capillary electrophoresis done in a TapeStation (Agilent; USA).

Formulation of mRNA into LNP

To formulate mRNA into lipid nanoparticles (LNP), we mixed an aqueous solution (10 mM citrate buffer, pH 3.0) of 0.2 mg/mL mRNA with alcoholic solution of a lipid mixture in a microfluidic cartridge using The NanoAssemblr™ Benchtop (Precision Nanosystems; USA). The components of the lipid mixture were ionizable lipidoid ALC-0315 (BroadPharm; USA), distearoylphosphatidylcholine (DSPC) (Avanti Polar Lipids; USA), cholesterol (Sigma-Aldrich; USA), DMG-PEG-2000 (BroadPharm; USA); the molar ratio (%) was 46.3 : 9.4 : 42.7 : 1.6. The mass fraction of mRNA in LNP was 0.04% wt. To form particles, we mixed aqueous and alcoholic phases 3 : 1 by volume, the overall mixing rate was 10 mL/min.

Next, the particles were filtered (in sterile conditions) through a 0.22 μm PES membrane filter (Merck; USA) and

stored at 4 °C. After filtration, we analyzed the quality of the resulting particles by two parameters, particle size (measured with Zetasizer Nano ZSP, Malvern Panalitycal; USA) and mRNA loading. Concentration of the mRNA loaded onto lipid nanoparticles was established by the difference in fluorescent signal levels upon staining with the RiboGreen reagent (Thermo Fischer Scientific; USA) before and after their destruction. To destroy the particles, we used the Triton X-100 detergent (Sigma-Aldrich; USA).

Rv3875 protein production and purification

To produce protein, we incubated *E. coli* BL21DE3 with the vector at 37 °C and 180 rpm until OD(600) equaled 0.65, then added IPTG to the final concentration of 1 mM, and cultured for 6 h at 30 °C. The cells were resuspended in buffer containing 50 mM Tris-HCl pH 8.0 and 300 mM of sodium chloride. Next, we induced lysis of the cells with ultrasound, 15-second pulses every 15 seconds. The amplitude of ultrasonic vibrations was 50% of the maximum. After lysis, the samples were centrifuged for 15 minutes (20,000 g at 4 °C). The pH of the resulting supernatant was adjusted to equal 7.5 by addition of a 1 M Tris-OH solution. Then, we filtered the sample through a polyethersulfone membrane with 0.22 µm pores.

For the first stage of purification of the Rv3875 protein we used a metal chelate affinity sorbent with an average particle size of 30 µm: cell lysate was applied to the sorbent with immobilized chelating ligand (nitrilotriacetic acid) and eluted in an imidazole gradient. Next, the purified protein fractions were dialyzed against a buffer containing 25 mM Tris-HCl. The dialyzed sample was applied onto a potent cation-exchange sorbent (average particle size 45 µm) with immobilized functional sulfo groups (–SO_3–). To verify the mass of the Rv3875, we used both the classical polyacrylamide gel electrophoresis method and a maXis 4G ETD mass spectrometer (Bruker; USA).

Assessment of the post vaccination T-cell response level

We used the ELISpot assay to count splenocytes secreting IFNγ in response to the Rv3875 tuberculosis protein post vaccination, and thus measured the level of the resulting T-cell response.

Splenocytes were obtained by rubbing spleens of the experimental and control group animals through a 70 µm filter. Once available, 300,000 splenocytes were seeded onto the Elispot PVDF membrane plates and simultaneously stimulated with Rv3875 protein (50 µg/mL) and DC2.4 dendritic cells, 30000 cells per well. We did two technical repetitions for each experimental well. With the seeded splenocytes and added stimulants, the total volume of each well was 200 µl of RPMI-1640 nutrient medium (PanEco; Russia) with 10% FCS. Next, we left the splenocytes to culture for 18 hours in a CO_2 incubator (5% CO_2, 37 °C).

After culturing, we identified the IFNγ-secreting splenocytes with the help of a Mouse IFNg ELISpot Set (BD; USA) and an AEC Substrate Set (BD; USA) as per the manufacturer's instructions. To count the dots of splenocytes secreting IFNγ, we used S6 Ultra (CTL; USA).

Cytometry of the IFNγ-producing cells

Cytometry of the IFNγ-producing cells started with seeding splenocytes of the vaccinated mice onto 200 µl of RPMI-1640 medium in a 96-well culture plate, 300,000 splenocytes per well; there, they were stimulated with the Rv3875 protein (50 µg/mL)

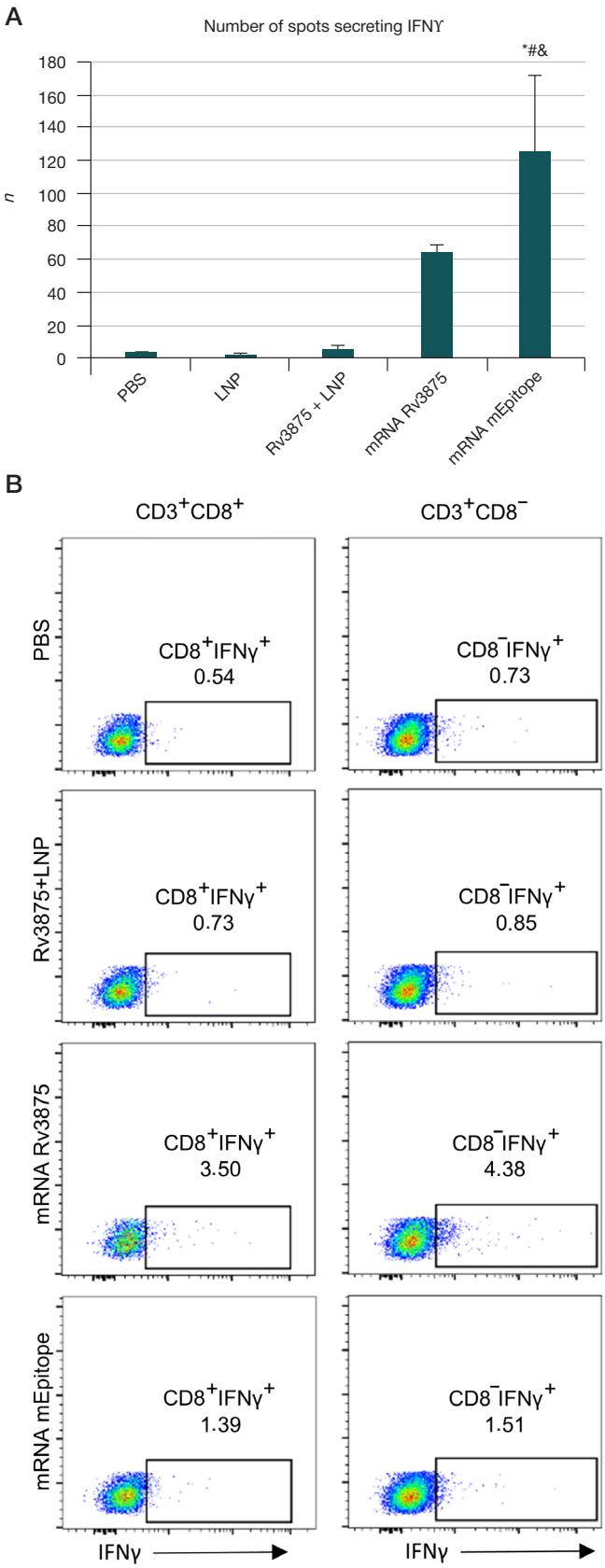

Fig. 2. Evaluation of the T-cell response of splenocytes of the immunized mice. **A**. ELISpot results. Number of cells secreting IFNγ in response to DC2.4 stimulation, activated by the Rv3875 protein. The data are presented as mean ± error of mean. Three animals per group. * — $p < 0.05$ compared with the PBS group; # — $p < 0.05$ compared with the LNP group; & — $p < 0.05$ compared with the Rv3875 + LNP group. **B**. Representative data on $CD3^+$ T lymphocytes ($CD8^+$ and $CD8^-$) producing IFNγ (intracellular staining). The figures show the percentage of cells from the total pool of $CD3^+$ $CD8^+$ and CD^{3+} $CD8^-$ T-lymphocytes that produce IFNγ

or left unstimulated. Two hours after beginning of the stimulation we added 10 μg/mL of Brefeldin A (Abcam; USA) to the wells. Next, the cells were cultured overnight in a CO_2 incubator. At the end of the incubation, they were stained with anti-CD3 (PE Anti-Mouse CD3, Elabscience) and anti-CD8 antibodies (FITC Anti-Mouse CD8a, Elabscience), the concentration thereof as per the manufacturer's instructions, then immobilized and permeabilized with a commercially available kit (BD; USA) as prescribed by its maker. To analyze the resulting samples, we used the LSRFortessa Cell Analyzer, a flow cytometer (BD; USA). Supplement 1 contains the example of gating. The obtained data were processed with the help of the FlowJo software (Treestar Inc.; USA).

Statistical analysis

We applied the one-way ANOVA and Turkey's test as a post-hoc analysis in the context of statistical processing of the data. Differences between the experimental groups were considered statistically significant at $p < 0.05$. Statistica 6.0 software package (StatSoft; USA) enabled statistical analysis of the data.

RESULTS

ELISpot assay used to analyze the T-cell response through identification of the IFNγ-secreting cells has proven the vaccination to be effective [$F(10.4) = 6.02$; $p = 0.012$]. The number of IFNγ-secreting cells after stimulation with DC2.4+RV3875 was higher in the splenocytes of mice vaccinated with the multi-epitope variant of the mRNA vaccine (mRNA mEpitope) than in the control groups (PBS, LNP) and splenocytes of mice immunized with Rv3875 protein with nanoparticles but without RNA (Rv3875+LNP) (Fig. 2A). As for the mRNA vaccine encoding the full-length Rv3875 protein, the respective experimental group had the number of IFNγ-secreting cells higher than the control group (64 ± 2.7 vs 2.5 ± 1.0), but these changes were not significant. A fact of note is the insignificant growth of the IFNγ-secreting cells count in mice immunized with Rv3875 with LNPs not loaded with mRNA compared to the animals that received mRNA vaccines (Rv3875: 5.7 ± 1.8; mRNA Rv3875: 64 ± 2.7; mRNA mEpitope: 126.0 ± 46.2). Supplement 2 presents the data on splenocytes from individual animals.

For cytometry, we used splenocytes from one animal of each group. This approach rendered statistical data processing impossible; the respective results are given for the purpose of clarity only. According to our data, the share of $CD3^+CD8^+$ and $CD3^+CD8^-$ T-lymphocytes producing IFNγ is higher in splenocytes of mice immunized with mRNA vaccines (Fig. 2B). Thus, cytometry confirms that the secretion of IFNγ in splenocytes of mice vaccinated with mRNA Rv3875 and mRNA mEpitope grows, inter alia, because of the T-lymphocytes, which is a clear sign of development of the T-cell response to Mycobacterium tuberculosis antigens in the vaccinated mice.

DISCUSSION

Our study has shown that immunization with an mRNA vaccine encoding the *M. tuberculosis* Rv3875 (ESAT6) multi-epitope protein yields a more pronounced T-cell response than both immunization with an mRNA vaccine encoding the full-length variant of the protein and immunization with a recombinant protein vaccine. The possible reasons behind these results are both a more efficient presentation of epitopes by type I and II histocompatibility complexes and the choice of the most immunogenic epitopes [11]. The enhanced presentation may be caused by the proteasome and lysosomal pathways of breaking of the protein molecule enabled by adding of the sequences of breaking linkers [23]. Another factor affecting immunogenicity is the leader sequence. In our case, we used the sequence of the cytoplasmic and transmembrane domain of MHC class I at the carboxyl end of the protein molecule. It has previously been shown that the leader sequence with the MHC class I transport signal ensures co-localization of the target protein and histocompatibility complexes in various endocytic compartments, which leads to efficient activation and expansion of antigen-specific $CD8^+$ and $CD4^+$ T-cells [21].

Another clear advantage of multi-epitope vaccines is the possibility to tune the T-cell response by selecting specific epitopes. In our study, we used the vaccine that encodes five Rv3875 epitopes, three presented by MHC class II and two by MHC class I, which guarantees specific activation of $CD8^+$ and $CD4^+$ T-cells. The epitopes were selected based on their affinity to human alleles rather than mouse alleles, and yet the multi-epitope mRNA vaccine showed high efficiency, probably due to high interspecies cross-reactivity. The T-cell response is one of the key components of the body's fight against M. tuberculosis. $CD8^+$-T-lymphocytes can destroy infected phagocytic cells with the help of perforin, granzyme and granulisin proteins; they are also capable of inducing apoptosis through the FasL receptor, while the secreted granulosin can kill M. tuberculosis directly. The most important function of $CD4^+$ cells in the fight against *M. tuberculosis* is the production of IFNγ, which activates macrophages and cytotoxic T-lymphocytes. In some people, insufficient induction of the $CD8^+$ T-cells may render BCG not as effective as expected [24].

There are two experimental studies that demonstrated efficacy of the mRNA vaccines against tuberculosis, but their results are inconsistent [9, 25]. One of these studies has shown immunogenic properties of the developed anti-tuberculosis mRNA vaccine based on the full-length MPT83 antigen [25]. Immunization with that vaccine triggered production of specific antibodies. T-cells isolated from the spleens of mice secreted IFNγ in response to the antigen challenge, and CTLs were able to lyse antigen-transfected cells; this is consistent with our results. In a similar 2022 study, the researchers used the ID91 mRNA containing four full-length tuberculosis antigens fused with a synthetic TLR4 agonist; the mRNA in this study was self-replicating [9]. Compared to a protein vaccine, such mRNA vaccine showed the capacity to raise the antibody titer to the same level, but the associated T-cell response was less pronounced. Our study is the first one to use a multi-epitope mRNA vaccine against tuberculosis; we have shown that, compared to the full length mRNA vaccine, the effects of multi-epitope mRNA vaccine are more intense. Use of multi-epitope vaccines for prevention of tuberculosis may be more promising than using protein and full-length mRNA vaccines for the purpose.

CONCLUSIONS

Administered intramuscularly, multi-epitope mRNA vaccine induces a pronounced T-cell response in mice. Enhancement of the T-cell response may be key to the development of an effective *M. tuberculosis* prevention vaccine. Vaccines against new epitopes of tuberculosis could enhance the T-cell response and accelerate the involvement of adaptive immune cells in the immune response within the first two weeks of infection, ensuring a more effective elimination of the pathogen. Thus, mRNA-based multi-epitope vaccines may be a promising direction of development of an effective vaccine against *M. tuberculosis*.

References

1. Dartois VA. Rubin EJ. Anti-tuberculosis treatment strategies and drug development: challenges and priorities. Nat Rev Microbiol, 2022. 20 (11): p. 685–701.
2. Vasileva IA, Testov VV, Sterlikov SA. Ehpidemicheskaya situaciya po tuberkulezu v gody pandemii COVID-19–2020-2021 gg. Tuberkulez i bolezni legkix. 2022. 100 3: 6–12. Russian.
3. Ahmed A, et al. A century of BCG: Impact on tuberculosis control and beyond. Immunological reviews. 2021; 301 (1): 98–121.
4. Shalofast EI, Ershova Yul. Postvakcinal'nye oslozhneniya pri «BCZh» immunizacii. Vestnik nauki. 2022; 4, 11 (56): 341–55. Russian.
5. Covian C., et al., BCG-Induced Cross-Protection and Development of Trained Immunity: Implication for Vaccine Design. Front Immunol. 2019; 10: 2806.
6. Nikolenko NYu, Kudlaj DA, Doktorova NP. Farmakoehpidemiologiya i farmakoehkonomika tuberkuleza s mnozhestvennoj i shirokoj lekarstvennoj ustojchivost'yu vozbuditelya.Farmakoehkonomika. Sovremennaya farmakoehkonomika i farmakoehpidemiologiya. 2021; 14 (2): 235–48. Russian.
7. Counoupas C, Triccas JA. The generation of T-cell memory to protect against tuberculosis. Immunol Cell Biol. 2019; 97 (7): 656–63.
8. Bouzeyen R, Javid B, Therapeutic Vaccines for Tuberculosis: An Overview. Front Immunol. 2022; 13: 878471.
9. Larsen SE, et al. An RNA-based vaccine platform for use against Mycobacterium tuberculosis. Vaccines. 2023; 11 (1): 130.
10. Tian Y, Deng Z, Yang P. mRNA vaccines: A novel weapon to control infectious diseases. Front Microbiol. 2022; 13: 1008684.
11. Melo A, et al. Third-generation vaccines: features of nucleic acid vaccines and strategies to improve their efficiency. Genes (Basel). 2022; 13 (12).
12. Heidary M, et al. A comprehensive review of the protein subunit vaccines against COVID-19. Front Microbiol. 2022; 13: 927306.
13. Teran-Navarro H, et al. A comparison between recombinant listeria GAPDH proteins and GAPDH encoding mRNA conjugated to lipids as cross-reactive vaccines for Listeria, Mycobacterium, and Streptococcus. Front Immunol. 2021; 12: 632304.
14. Al Tbeishat H. Novel in silico mRNA vaccine design exploiting proteins of M. tuberculosis that modulates host immune responses by inducing epigenetic modifications. Scientific Reports. 2022. 12 (1): 1–19.
15. Shahrear S, Islam ABMMK. Modeling of MT. P495, an mRNA-based vaccine against the phosphate-binding protein PstS1 of Mycobacterium tuberculosis. Molecular Diversity. 2022; 1–20.
16. Kar T, et al. A candidate multi-epitope vaccine against SARS-CoV-2. Sci Rep. 2020; 10 (1): 10895.
17. O'Donnell TJ, Rubinsteyn A, Laserson U. MHCflurry 2.0: improved pan-allele prediction of MHC class I-presented peptides by incorporating antigen processing. Cell Syst. 2020; 11 (1): 42–48.
18. Reynisson B, et al. Improved prediction of MHC II antigen presentation through integration and motif deconvolution of mass spectrometry MHC eluted ligand data. J Proteome Res. 2020; 19 (6): 2304–15.
19. Greenbaum J, et al. Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. Immunogenetics. 2011; 63 (6): 325–35.
20. Weiskopf D, et al. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for $CD8^+$ T cells. Proc Natl Acad Sci U S A. 2013; 110 (22): E2046–53.
21. Kreiter S, et al. Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. J Immunol. 2008; 180 (1): 309–18.
22. Kirshina A, Kolosova E, Imasheva E, Vasileva O, Zaborova O, Terenin I, et al. Effects of various mRNA-LNP vaccine doses on neuroinflammation in BALB/c mice. RSMU. 2022; 6.
23. Yano A, et al. An ingenious design for peptide vaccines. Vaccine. 2005; 23 (17–18): 2322–6.
24. Hess J, Kaufmann SH. Live antigen carriers as tools for improved anti-tuberculosis vaccines. FEMS Immunol Med Microbiol. 1999; 23 (2): 165–73.

Литература

1. Dartois, V.A. and E.J. Rubin, Anti-tuberculosis treatment strategies and drug development: challenges and priorities. Nat Rev Microbiol, 2022. 20 (11): p. 685–701.
2. Васильева И. А., Тестов В. В., Стерликов С. А. Эпидемическая ситуация по туберкулезу в годы пандемии COVID-19–2020-2021 гг. Туберкулез и болезни легких. 2022. 100 3: 6–12.
3. Ahmed A, et al. A century of BCG: Impact on tuberculosis control and beyond. Immunological reviews. 2021; 301 (1): 98–121.
4. Шалофаст Е. И., Ершова Ю. И. Поствакцинальные осложнения при «БЦЖ» иммунизации. Вестник науки. 2022; 4, 11 (56): 341–55.
5. Covian, C., et al., BCG-Induced Cross-Protection and Development of Trained Immunity: Implication for Vaccine Design. Front Immunol. 2019; 10: 2806.
6. Николенко Н. Ю., Кудлай Д. А., Докторова Н. П. Фармакоэпидемиология и фармакоэкономика туберкулеза с множественной и широкой лекарственной устойчивостью возбудителя. Фармакоэкономика. Современная фармакоэкономика и фармакоэпидемиология. 2021; 14 (2): 235–48.
7. Counoupas C, Triccas JA. The generation of T-cell memory to protect against tuberculosis. Immunol Cell Biol. 2019; 97 (7): 656–63.
8. Bouzeyen R, Javid B, Therapeutic Vaccines for Tuberculosis: An Overview. Front Immunol. 2022; 13: 878471.
9. Larsen SE, et al. An RNA-based vaccine platform for use against Mycobacterium tuberculosis. Vaccines. 2023; 11 (1): 130.
10. Tian Y, Deng Z, Yang P. mRNA vaccines: A novel weapon to control infectious diseases. Front Microbiol. 2022; 13: 1008684.
11. Melo A, et al. Third-generation vaccines: features of nucleic acid vaccines and strategies to improve their efficiency. Genes (Basel). 2022; 13 (12).
12. Heidary M, et al. A comprehensive review of the protein subunit vaccines against COVID-19. Front Microbiol. 2022; 13: 927306.
13. Teran-Navarro H, et al. A comparison between recombinant listeria GAPDH proteins and GAPDH encoding mRNA conjugated to lipids as cross-reactive vaccines for Listeria, Mycobacterium, and Streptococcus. Front Immunol. 2021; 12: 632304.
14. Al Tbeishat H. Novel in silico mRNA vaccine design exploiting proteins of M. tuberculosis that modulates host immune responses by inducing epigenetic modifications. Scientific Reports. 2022. 12 (1): 1–19.
15. Shahrear S, Islam ABMMK. Modeling of MT. P495, an mRNA-based vaccine against the phosphate-binding protein PstS1 of Mycobacterium tuberculosis. Molecular Diversity. 2022; 1–20.
16. Kar T, et al. A candidate multi-epitope vaccine against SARS-CoV-2. Sci Rep. 2020; 10 (1): 10895.
17. O'Donnell TJ, Rubinsteyn A, Laserson U. MHCflurry 2.0: improved pan-allele prediction of MHC class I-presented peptides by incorporating antigen processing. Cell Syst. 2020; 11 (1): 42–48.
18. Reynisson B, et al. Improved prediction of MHC II antigen presentation through integration and motif deconvolution of mass spectrometry MHC eluted ligand data. J Proteome Res. 2020; 19 (6): 2304–15.
19. Greenbaum J, et al. Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. Immunogenetics. 2011; 63 (6): 325–35.
20. Weiskopf D, et al. Comprehensive analysis of dengue virus-

specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci U S A. 2013; 110 (22): E2046–53.
21. Kreiter S, et al. Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. J Immunol. 2008; 180 (1): 309–18.
22. Kirshina A, Kolosova E, Imasheva E, Vasileva O, Zaborova O, Terenin I, et al. Effects of various mRNA-LNP vaccine doses on neuroinflammation in BALB/c mice. RSMU. 2022; 6.
23. Yano A, et al. An ingenious design for peptide vaccines. Vaccine. 2005; 23 (17–18): 2322–6.
24. Hess J, Kaufmann SH. Live antigen carriers as tools for improved anti-tuberculosis vaccines. FEMS Immunol Med Microbiol. 1999; 23 (2): 165–73.