COMPARISON OF THE EFFICACY OF MRNA VACCINES AGAINST *M. TUBERCULOSIS* BASED ON LINEAR AND CIRCULAR RNAS

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The success of mRNA-based vaccine formulations against viral infections motivated many researchers to develop mRNA vaccines against bacterial infections. The development of new anti-tuberculosis vaccine is an urgent task since the only approved BCG vaccine is not effective enough in terms of infection prevention, despite the fact that it reduces the risk of severe disease. The study aimed to compare two anti-tuberculosis mRNA vaccines based on the classic linear mRNA (mRNA-MTB-mEp-5-1) and circular RNA (circRNA-MTB-mEp-5-1) by immunogenicity and the capability of protecting I/St mice against *M. tuberculosis* infection. The efficacy of mRNA vaccines in the formulations with lipid nanoparticles was compared with the BCG efficacy. The findings suggest that immunization with the mRNA vaccine based on the linear mRNA resulted in the cell-based and humoral immune response (OD IgG = 0.36 ± 0.12) that was less pronounced than after BCG vaccination (OD IgG = 0.54 ± 0.14). At the same time, immunization with the mRNA vaccine and BCG ensured comparable reduction of bacterial load in the lung and spleen of experimental mice (CFU in lung tissue for BCG: $4.00 \times 10^5 \pm 2.13 \times 10^5$, $p = 0.0068$; mRNA: $4.72 \times 10^5 \pm 3.44 \times 10^5$, $p = 0.0059$; LNP: $4.91 \times 10^6 \pm 3.89 \times 10^6$, ns; PBS: $4.01 \times 10^6 \pm 1.69 \times 10^6$) and increased survival of mice after getting infected with *M. tuberculosis*. Immunization with the vaccine based on the circular RNA resulted in developing humoral mmunity only (OD IgG = 0.52 ± 0.13) and did not ensure protection after getting infected with *M. tuberculosis* (CFU in the lung for circRNA: $2.12 \times 10^6 \pm 5.30 \times 10^5$, $p = 0.85$). Thus, in our studies, anti-tuberculosis vaccines based on circular RNAs are inferior in effectiveness to formulations based on linear RNAs.

Keywords: mRNA vaccine, circular RNA, tuberculosis, immunogenicity, protective immunity

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СРАВНЕНИЕ ЭФФЕКТИВНОСТИ МРНК ВАКЦИН ПРОТИВ *M. TUBERCULOSIS* НА ОСНОВЕ ЛИНЕЙНЫХ И КОЛЬЦЕВЫХ РНК

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Успехи вакцинных препаратов на основе мРНК против вирусных инфекций побудили многих исследователей к разработке мРНК-вакцин против бактериальных инфекций. Разработка новой вакцины против туберкулеза является актуальной задачей, поскольку единственная одобренная к использованию вакцина BCG, хотя и снижает риск развития тяжелых форм заболевания, не является достаточно эффективной для предотвращения инфицирования. Целью исследования было сравнить две мРНК-вакцины против туберкулеза на основе классической линейной мРНК (mRNA-MTB-mEp-5-1) и на основе кольцевой мРНК (circRNA-MTB-mEp-5-1) по иммуногенности и способности защищать мышей I/St от заражения *M. tuberculosis*. Эффективность мРНК-вакцин в составе с липидными наночастицами сравнивали с эффективностью BCG. Результаты свидетельствуют о том, что иммунизация мРНК-вакциной на основе линейной мРНК привела к формированию клеточного и гуморального иммунного ответа (OD IgG = 0,36 ± 0,12), который был менее выражен, чем после вакцинации BCG (OD IgG = 0,54 ± 0,14). В то же время иммунизация мРНК-вакциной и BCG обеспечила сопоставимое снижение бактериальной нагрузки в легких и селезенке экспериментальных мышей (КОЕ в легочной ткани BCG: $4,00 \times 10^5 \pm 2,13 \times 10^5$, $p = 0,0068$; линРНК: $4,72 \times 10^5 \pm 3,44 \times 10^5$, $p = 0,0059$; LNP: $4,91 \times 10^6 \pm 3,89 \times 10^6$, ns; PBS: $4,01 \times 10^6 \pm 1,69 \times 10^6$) и повысила их выживаемость после заражения *M. tuberculosis*. Иммунизация вакциной на основе кольцевой мРНК привела к формированию только гуморального иммунитета (OD IgG = 0,52 ± 0,13) и не обеспечила защиту после заражения *M. tuberculosis* (КОЕ в легких колРНК: $2,12 \times 10^6 \pm 5,30 \times 10^5$, $p = 0,85$). Таким образом, в наших исследованиях противотуберкулезные вакцины на основе кольцевых мРНК уступают в эффективности препаратам на основе линейных РНК.

Ключевые слова: мРНК-вакцина, кольцевые мРНК, туберкулез, иммуногенность, протективный иммунитет

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Tuberculosis (TB) is a highly contagious disease caused by the bacterium *Mycobacterium tuberculosis*, which is still the leading cause of death from infectious diseases. In 2023, a total of 10.8 million of incident TB cases and 1.25 million of deaths from this infection were reported [1]. Tuberculosis prevention is hampered by often asymptomatic course of the early-stage disease and delayed manifestation, which make it difficult to monitor the epidemiological situation. The spread of antimicrobial-resistant mycobacteria is associated with further challenges. TB is still a deadly disease, and the search for effective prevention and treatment methods is an urgent problem.

The development and introduction of a new tuberculosis vaccine represents the most important step on the way to elimination of this dangerous infection. BCG vaccine is not recommended by the World Health Organization for tuberculosis prevention in adults due to the risk of adverse effects. Variable efficacy in different geographic regions, strain heterogeneity, and low efficacy against pulmonary tuberculosis being the most prevalent tuberculosis form can be also considered the BCG disadvantages [2]. Thus, there is an urgent need to create new drugs capable of preventing TB in all age groups. Currently, a total of 15 vaccines are through various phases of clinical development: between phase I and phase III clinical trials [3]. All these represent different formulation types: viral vector-based, inactivated mycobacteria, live-attenuated mycobacteria, protein subunit and mRNA vaccines.

mRNA vaccines have become widely used due to the success of the RNA-1273 (Moderna) and BNT162b2 (Pfizer) vaccines against SARS-CoV2 [4]. This approach to vaccination has a number of benefits compared to other immunization variants: low cost of production, rapid development, high efficacy, lack of infectivity, and no integration into the genome. At the same time, the experience of using mRNA vaccines against infectious diseases caused by bacteria is limited [5]. Moreover, not all mRNA-based formulations show high efficacy: low RNA stability in the cell results in premature RNA degradation, reduced target protein translation [6]. The use of the alternative mRNA platforms, such as self-amplifying and circular RNAs, are considered as a possible way to solve these problems.

Circular RNAs were discovered in the 1980s. Since then these attracted attention due to their features. In contrast to linear mRNAs, the circular RNA-based formulations are less prone to degradation due to the lack of free 5' or 3' end, through which cleavage by exonucleases occurs [7]. Furthermore, circular RNAs are not subject to the degradation mechanisms, such as nonsense-mediated mRNA decay (NMD) or non-stop decay (NSD) [8]. Circular RNAs are translated through IRES (internal ribosome entry site), the indirect mechanism activated primarily under conditions of cellular stress, when the cap-dependent translation is inhibited; it is ensured by recruiting of the so-called IRES-transacting factors (ITAF) together with the eIF and eEF [9]. Currently, no clinical trials of the circular RNA-based tuberculosis vaccines are conducted [3]. At the same time, the circular RNA-based formulations have shown high efficacy in experimental models of viral infections [10, 11]; these are currently tested in clinical trials of the drugs for radiation-induced xerostomia [NCT06714253] and SARS-CoV-2 [NCT06205524]. We assume that these features of circular RNAs can make it possible to consider the circular RNA-based formulations as a promising platform for future tuberculosis vaccines. Therefore, the aim of our study was to evaluate the effectiveness of an anti-TB vaccine based on circular RNA.

METHODS

Animals

The experiments involved female inbred C57BL/6Cit (B6) and I/StSnEgYCit (I/St) mice obtained from the breeding nursery of the Central Tuberculosis Research Institute. The animals aged 2–4 months with the body weight of 20–25 g were included in the study. Lineages were maintained through brother-sister inbreeding; the animals had *ad libitum* access to food and water. A total of 25 B6 and 75 I/St females were used.

Experimental design

A total of 5 experimental groups for each mouse lineage were formed for the study: for B6 (5 animals per group) and I/St (15 animals per group) mice.

1. Group with the double intramuscular administration of the mRNA-MTB-mEp5-1 vaccine in a dose of 5 μg/mouse with the 4-week interval between injections.
2. Group with the double intramuscular administration of the circRNA-MTB-mEp5-1 vaccine in a dose of 5 μg/animal with the 4-week interval between injections.
3. Group with a single BCG administration in a dose of 1×10^5 CFU/mouse (5 weeks before tissue collection).
4. Control group with the double intramuscular administration of the lipid nanoparticles (LNP) with no mRNA in a dose equivalent to that administered to groups I–II with the 4-week interval between injections.
5. Control group with the double intramuscular administration of phosphate buffered saline (PBS) with the 4-week interval between injections.

The B6 mice were used to assess T cell-mediated response (ELISpot), as well as to assess the titer of IgG against antigens of *M. tuberculosis*.

The I/St mice were used to assess protective immune response, since mice of this lineage show increased sensitivity to tuberculosis infection [12]. The I/St mice were intravenously infected with the virulent *M. tuberculosis* strain 4 weeks after the second vaccination with mRNA vaccines / 5 weeks after BCG vaccination. Mycobacterial load in the spleen and lung (5 mice in each group) and the dynamics of deaths of animals after getting infected (10 mice in each group) were assessed 50 days after infection in these mice.

Obtaining mRNA vaccines

The MTB-mEp5-1 multi-epitope mRNA vaccine was described in detail earlier [13]. The coding sequence for the circMTB-mEp5-1 circular RNA-based vaccine was the same as that for MTB-mEp5-1. The cassette for circRNA comprised type I introns from Anabaena (on the 5' and 3' ends), homology arms, spacers, type I IRES (CVB3). The construct was also built by PCR and cloned into pSmart (Lucigen, USA) at the *EcoRI* and *AhlI* restriction endonuclease sites. The MTB-mEp5-1 circular RNA sequence is provided in Appendix.

RNA synthesis was accomplished using the mRNA-20 kit (Biolabmix, Russia) supplemented with pyrophosphatase (NEB, USA) and RNAse inhibitor (Biolabmix, Russia). The $m_2^{7,3'}$-OGpppAmG synthetic cap analogue (Biolabmix, Russia) was used for *in vitro* linear RNA transcription. To obtain circular RNA, guanosine triphosphate (GTP) was added to the reaction mixture after the transcription termination to a final concentration of 2 mM (Biolabmix, Russia) in order to induce circularization. The mixture was incubated at 55 °C for 15 min,

cooled on ice for 5 min and purified on the magnetic particles (VAHTS RNA Clean Beads, China). To eliminate linear RNA forms from the circular RNA-based formulation, the sample was treated with RNAse R (Abcam, USA). For that RNA was heated at 70 °C for 3 min, then cooled rapidly on ice (5 min). After that we added 0.5 µL of RNAse R (Abcam, USA) per 20 µg of RNA and 10× RNAse R buffer and incubated at 37 °C for 7 min, after the end of which another 0.5 µL of RNAse R were added, further incubated for 7 min at 37 °C and purified on the magnetic particles (VAHTS RNA Clean Beads, China).

After the *in vitro* transcription termination, the DNA matrix was eliminated by treating with 2 U of DNAse I (NEB, USA) per 1 µg of DNA at the temperature of 37 °C for 15 min. RNA was purified using magnetic particles (VAHTS RNA Clean Beads, China) in accordance with the manufacturer's instructions. The RNA quality was assessed by capillary electrophoresis (Qsep1-Plus, BiOptic, Taiwan).

mRNA was formulated into lipid nanoparticles using the microfluid cartridge in the NanoAssemblr™ Benchtop system, as earlier reported [14]. RNA was mixed with the lipid mixture ethanol solution in the microfluid cartridge; the aqueous and ethanol phases were mixed in a ratio of 3 : 1 v/v. The five-component lipid mixture consisted of the ionized lipidoid ALC-0315 (Sinopeg, Xiamen, China), SM-102 (Sinopeg, Xiamen, China), DSPC (Avanti Polar Lipids, USA), cholesterol (Merck Millipore, USA), and DMG PEG-2000 (Merck Millipor, USA) in a molar ratio (%) of 23.15 : 23.15 : 9.4 : 42.7 : 1.6. Particles were concentrated and sterilized using the PES membrane with the pore diameter of 0.22 µm. The quality of the LNPs obtained was assessed based on three parameters: particle size, polydispersity index (PDI) (Zetasizer Ultra ZSP; Malvern PanalityCal, USA), and mRNA payload of the particles. The particle size for the MTB-mEp-5-1 sample was 90.5 nm, polydispersity index was 0.22. The hydrodynamic diameter for the circMTB-mEp-5-1 formulation was 85.7 nm, polydispersity index was 0.19. The RNA payload was above 90% in both formulations. Lipid nanoparticles were stored at +4 °C for no more than 2 weeks prior to formulation administration.

T-cell response assessment: quantification of IFNγ-producting cells

The level of protective T-cell immune response in B6 mice was assessed based on the counts of cells secreting IFNγ in response to stimulation with mycobacterial antigens isolated from the spleen by the ELISpot method using the Mouse IFNγ ELISpot Set (BD; USA) and AEC Substrate Set (BD; USA) kits in accordance with the manufacturers' instructions [15]. The M. tuberculosis sonicate in a dose of 10 µg/mL was used as a source of mycobacterial antigens. Sonicate for the study was kindly provided by V.G. Avdienko. To produce it, mycobacteria were grown in the Sauton medium for 28 days at 37 °C. Sonicate was obtained from the washed clean bacterial mass using the MSE ultrasonic disintegrator by the method earlier reported by V.G. Avdienko et al. [16].

Humoral immunity assessment: determination of the titer of IgG immunoglobulins against *M. tuberculosis* sonicate

The titer of the IgG immunoglobulin against the *M. tuberculosis* sonicate was determined in the serum samples of experimental animals. Blood serum of the B6 experimental mice was subjected to titration in the phosphate buffered saline (PBS) from 1 : 50 to 1 : 400. The humoral immune response intensity was assessed based on optical density (OD) by the earlier reported method [17].

Vaccination protective effect assessment

To induce experimental tuberculosis infection 4 weeks after the second mRNA vaccine dose, the I/St mice were intravenously infected by the virulent *M. tuberculosis* H37Rv strain (Pasteur) from the collection of the Central Tuberculosis Research Institute in a dose of 5×10^5 CFU per mouse. BCG vaccination was performed 5 weeks before subcutaneous infection in a dose of 1×10^5 CFU per mouse. The experiments involving assessment of survival after getting infected with *M. tuberculosis* were terminated on day 111 after infection.

Determination of mycobacterial counts in organs of infected animals

Mycobacterial load in the lung tissue and the spleen was determined on day 50 after infection (5 animals per group). For that the organs were homogenized in 2 mL of saline. The 10-fold dilutions of homogenates were sown on the Middlebrook 7H10 agar (HiMedia Laboratories LLC, USA) (50 µL/Petri dish). After 18–21 days, colonies were enumerated, and CFU/organ were calculated using the following formula: $N = 2N_1 \times D / 0.05$, where N was CFU per organ; N_1 was the number of colonies in the dish; D was dilution.

Statistical analysis

Statistical data processing was performed by two-factor analysis of variance and Tukey's test for multiple comparisons to assess the IgG titer. The Dunn's test as a post-hoc test used after the Kruskal-Wallis test was applied to the ELISpot analysis data and the data on the bacterial load in the lung and spleen of the immunized mice infection with M. tuberculosis. Overall survival was assessed using the Kaplan–Meier estimator. Significance of differences in overall survival was calculated the Mantel–Cox log-rank test. The differences between experimental groups were considered significant at $p < 0.05$. Data analysis and visualization were performed using the GraphPad Prism 10.4.1 software tool (GraphPad Software, USA).

RESULTS

Tuberculosis mRNA vaccine immunogenicity assessment

We assessed the efficacy of two different tuberculosis mRNA vaccines with the antigen sequence MTB-mEp-5-1 based on the linear and circular RNAs. The MTB-mEp-5-1 and circMTB-mEp-5-1 vaccines (Fig. 1A) formulated into lipid nanoparticles (LNPs) were tested for the ability to activate humoral (Fig. 1C) and cell-mediated immune responses (Fig. 1D) compared to the BCG vaccine and control groups (PBS and LNP). When the animals were immunized with the LNPs containing no mRNA, the testing results showed no differences from that of the group of mice receiving PBS.

The highest anti-mycobacterial IgG titers were found in the groups of mice vaccinated with BCG and circMTB-mEp-5-1 ($p < 0.001$ for most dilutions compared to the control; see Table), and IgG levels for BCG and circRNA-MTB-mEp were comparable (Fig. 1C; Table). The increase in IgG titer was less prominent after immunization with MTB-mEp-5-1; the increase in the IgG immunoglobulin titer relative to non-vaccinated animals was reported for the serum dilution 1 : 50 only ($p = 0.001$). The

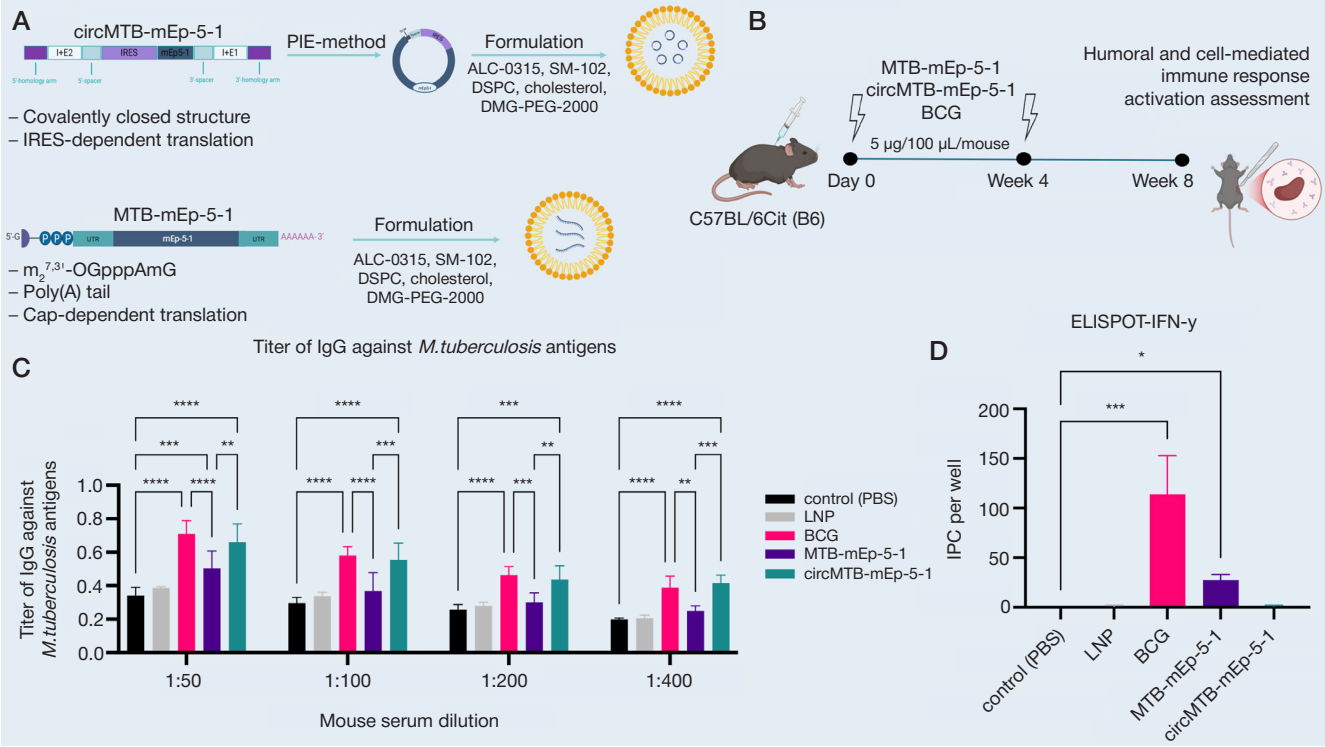

Fig. 1. mRNA vaccine immunogenicity assessment. **A**. Structure of the tuberculosis RNA vaccines with the MTB-mEp-5-1 antigen sequence based on the linear and circular RNAs. **B**. Experimental design scheme. **C**. Titer of IgG immunoglobulins to *M. tuberculosis* antigens in blood serum of mice after immunization with the tuberculosis vaccines. **D**. Differences in IPC counts in the spleen of mice vaccinated with various mRNA vaccine variants. The data are presented as the mean ± standard deviation. Mice per group $n = 5$. * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$; **** — $p < 0.0001$.

anti-mycobacterial IgG levels reported after immunization with MTB-mEp-5-1 were inferior to both circMTB-mEp-5-1 and BCG for almost all serum dilution variants (Fig. 1C; Table). Thus, both mRNA vaccines yielded the humoral immune response to *M. tuberculosis*, but when using the circular RNA-based vaccine, the humoral immune response was stronger and comparable with the immune response observed after BCG vaccination.

To determine the ability of the tuberculosis vaccines based on various RNA platforms to activate specific cell-mediated immune response, we assessed the counts of IFNγ-producing cells (IPC) of the spleen after the mycobacterial sonicate stimulation (Fig. 1D; Table). The highest IPC counts were reported for the group of BCG vaccinated mice (more than 100 spots, $p < 0.001$ compared to the control; Table). Vaccination with MTB-mEp-5-1 also led to the increase in IPC counts relative to the control (more than 20 spots, $p = 0.026$; Table). The mice immunized with circMTB-mEp-5-1 showed no significant differences in IPC counts from the control groups.

Thus, among RNA-based vaccines, only circMTB-mEp-5-1 could yield the humoral immune response comparable to that observed after BCG immunization. However, vaccination with this vaccine did not result in the T-cell immunity development, while immunization with the MTB-mEp-5-1 linear mRNA-based vaccine yielded moderate humoral and cell-mediated immune responses to mycobacterial antigens.

Tuberculosis mRNA vaccine protective effect assessment

For a vaccine, one of the most important criteria is its ability to generate the protective immune response after infection. We conducted comparative assessment of the efficacy of two tuberculosis vaccines based on different RNA platforms by the ability to protect mice after getting infected with *M. tuberculosis* (Fig. 2A). Fig. 2B and the Table show that vaccination with MTB-mEp-5-1 and BCG can ensure the decrease in mycobacterial counts in the lung and spleen on day 50 after infection compared to the control ($p = 0.007$ and $p = 0.006$ for the lung, $p = 0.009$ and $p = 0.022$ for the spleen, respectively). As for mice vaccinated with circMTB-mEp-5-1, no significant decrease in bacterial load in the lung and spleen relative to the control was reported after infection.

When assessing the dynamics of mouse deaths after getting infected, immunization with BCG or MTB-mEp-5-1 ensured survival of 8 and 7 animals out of 10 for 111 days after the *M. tuberculosis* infection ($p < 0.001$; Table). Survival of mice in the control group was 20%. In the group of mice receiving circRNA-MTB-mEp, survival after getting infected showed no differences from that of the control. However, the number of survivors in this group was 50% (Fig. 2C).

Thus, only vaccination with MTB-mEp-5-1 yielded the protective immune response due to the decrease in bacterial load in the lung and spleen and increased survival of the infected mice. The reported protective effect of vaccination with this mRNA vaccine was comparable with that reported after BCG vaccination.

DISCUSSION

In this study, we assessed immunogenic and protective properties of the vaccines against *M. tuberculosis* based on the linear and circular RNAs compared to the BCG vaccine. The findings show that immunization with the MTB-mEp-5-1 linear mRNA-based vaccine yielded the adaptive and protective immune responses, reduced bacterial load in the lung and spleen, and increased survival rate of the infected mice. The MTB-mEp-5-1 immunogenicity was lower than that reported after BCG immunization, but protective efficacy was compared to that observed after BCG.

The circMTB-mEp-5-1 circular RNA-based vaccine turned out to be less effective. Our findings have shown

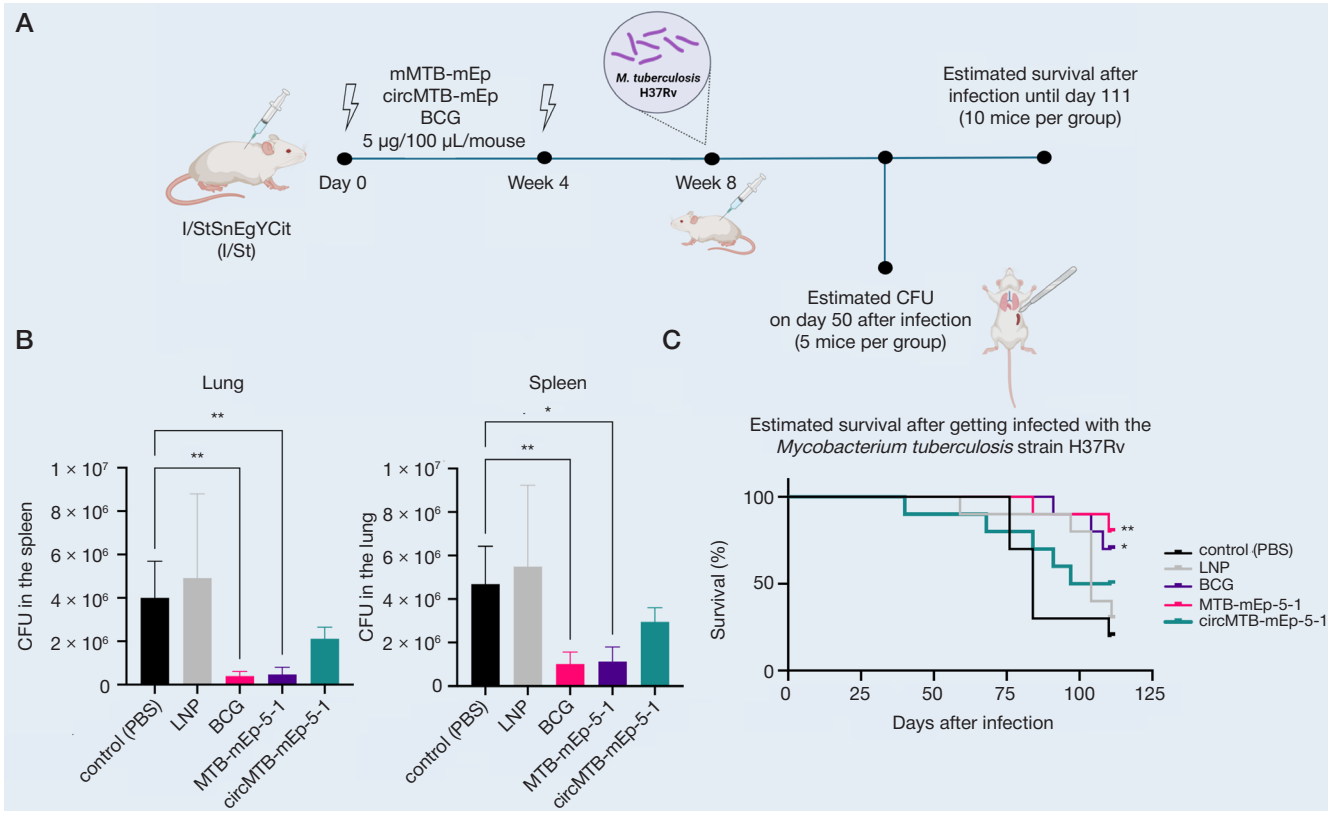

Fig. 2. Protective immune response assessment after immunization with mRNA vaccines **A**. Experimental design scheme. **B**. Bacterial load in the lung tissue and the spleen of immunized mice (n = 5) after the *M. tuberculosis* infection (H37Rv strain). The data are presented as the mean ± standard deviation. **C**. Survival dynamics of mice (n = 10) immunized with tuberculosis vaccines after the *M. tuberculosis* infection (H37Rv strain). * — $p < 0.05$; ** — $p < 0.01$.

that circMTB-mEp-5-1 can induce the pronounced humoral immune response comparable to that observed after BCG vaccination. However, immunization with circMTB-mEp-5-1 yielded no IFNγ-mediated T cell-mediated response and did not ensure protection of animals against the *M. tuberculosis* infection. These results show that yielding humoral immunity only is insufficient to ensure protection against pathogen, it is also necessary to yield T cell-mediated immunity, which is consistent with the results of the earlier studies of the nucleic acid-based tuberculosis vaccines [2].

Apparently, the differences in mRNA vaccine efficacy are associated with the mechanisms underlying the dynamics of antigen translation. Circular RNAs differ from linear RNAs by increased stability [7]. High circRNA stability contributes to prolonged antigen production, which can result in the pronounced humoral immune response, but due to IRES the antigen translation is considerably lower, than the speed of translation through the cap-dependent pathway with the linear RNA [18], which results in low antigen expression that can be not enough to develop the cell-mediated and protective immunity. The role of the T cell-mediated immunity is important to form granulomas. That is why in the earlier studies the main efforts were focused on the development of vaccines yielding the strong cell-mediated immune response [19]. However, a number of recent rodent, non-human primate, and human studies have shown that humoral immunity induction is important for vaccine efficacy [20]. This is in line with our data obtained for the linear RNA than induced moderate humoral

Table. Summary of study results

IgG titer (mean ± standard deviation, OD = A450)					
Dilution	BCG	MTB-mEp-5-1	circRNA-MTB-mEp	LNP	PBS
1 : 50	0.71 ± 0.08	0.50 ± 0.10	0.66 ± 0.11	0.39 ± 0.01	0.34 ± 0.05
1 : 100	0.58 ± 0.05	0.37 ± 0.11	0.56 ± 0.10	0.34 ± 0.02	0.30 ± 0.03
1 : 200	0.46 ± 0.05	0.30 ± 0.06	0.44 ± 0.08	0.28 ± 0.02	0.26 ± 0.03
1 : 400	0.39 ± 0.07	0.25 ± 0.03	0.42 ± 0.05	0.21 ± 0.02	0.20 ± 0.01
IPCs per well (mean ± standard deviation)					
	114.00 ± 38.99	27.60 ± 5.41	1.00 ± 1.00	1.00 ± 1.00	0.20 ± 0.45
CFU in the lung (mean ± standard deviation)					
	$4.00 \times 10^5 \pm 2.13 \times 10^5$	$4.72 \times 10^5 \pm 3.44 \times 10^5$	$2.12 \times 10^6 \pm 5.30 \times 10^5$	$4.91 \times 10^6 \pm 3.89 \times 10^6$	$4.01 \times 10^6 \pm 1.69 \times 10^6$
CFU in the spleen (mean ± standard deviation)					
	$1.01 \times 10^6 \pm 5.52 \times 10^5$	$1.13 \times 10^6 \pm 6.78 \times 10^5$	$2.95 \times 10^6 \pm 6.47 \times 10^5$	$5.49 \times 10^6 \pm 3.75 \times 10^6$	$4.69 \times 10^6 \pm 1.74 \times 10^6$
Median survival					
	–	–	94	104	84

and cell-mediated immune responses that ensured the development of protective response.

One more possible explanation of developing stronger humoral immunity after immunization with circMTB-mEp-5-1 is possible excess innate immunity activation after vaccination. Along with the viral IRES sequence, circular RNAs also comprise bacterial sequences: the intron-exon regions being the essential elements for RNA circularization, when the self-splicing group 1 introns are used [21]. The study conducted by Chen et al. has shown that the exogenous circular RNA obtained using the self-splicing group 1 introns can activate the innate immune response receptors (RIG-1, MDA5, OAS1, OASL), in contrast to the endogenous circular RNA obtained using human introns deprived of the capability of autocatalytic splicing, but capable of forming circular RNAs in the cell using a spliceosome. The endogenous circular RNA is not identified as a foreign one, since it is associated with many RNA-binding proteins [22]. Due to its structure, the exogenous circular RNA can activate the innate immunity receptors, thereby contributing to the higher reactogenicity of the circular RNA-based vaccines compared to the linear RNA-based one. Activation of the innate immunity receptors, such as RIG-1 and MDA5, results in IFN-I production [23], which can cause activation of humoral immunity, intense B cell-mediated response [24], as well as inhibition of the T cell-mediated response [25].

However, the circular RNA-based vaccine is likely to be effective against viral infections, in which it is more important to generate a strong humoral immune response. Perhaps in the future it will be a promising avenue to combine the circular RNA- and linear RNA-based vaccines in the same formulation or different prime-boost strategies in order to enhance both humoral and T cell-mediated immune responses and ensure more effective protective immunity.

When constructing vaccines against *M. tuberculosis*, it is necessary to consider one more problem: the microorganism complexity. Given the fact that *M. tuberculosis* comprises about 4000 genes [26], the use of strategies that are used to develop the viral mRNA-based vaccines can hardly be applicable to the mRNA-based tuberculosis vaccines. The M. tuberculosis genetic complexity is in contrast to the relatively simple structure of viral genomes and is further enhanced due to unusual genetic diversity reported for various mycobacterial strains [27]. Today, there is no evidence that one particular antigen or a limited number of antigens can play a crucial role in ensuring the protection.

It is likely that in the future the use of the heterologous boost immunization to enhance the BCG vaccine effect will become an important component of complex TB vaccination strategies. Heterologous revaccination can be performed in infancy or adolescence, when the BCG effect starts to wane. The mRNA-based vaccines are at the forefront of the research focused on assessing the possibility of enhancing cell-mediated immunity against TB through heterologous boost immunization. Despite the fact that the infection limitation for TB prevention can be a realistic first step, the ultimate goal should be to use the heterologous boost regimen to create either pre-exposure vaccines preventing the development of infection and disease, or post-exposure vaccine capable of stopping disease reactivation in individuals with latent infection.

CONCLUSIONS

The data obtained on immunogenicity and protective efficacy of the linear RNA-based vaccines confirm that these are promising in terms of using as vaccines against M. tuberculosis. The circMTB-mEp-5-1 circular RNA-based vaccine is still inferior in efficacy to that based on the linear RNA, despite the fact that it ensures a rather strong humoral immune response. Further optimization is required to enhance cell-mediated and protective immune responses.

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