IMPACT OF UNTRANSLATED mRNA SEQUENCES ON IMMUNOGENICITY OF mRNA VACCINES AGAINST *M. TUBERCULOSIS* IN MICE

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Vaccination is among the most effective measures to reduce tuberculosis morbidity and mortality. In 1974, BCG vaccination was included in the Expanded Program on Immunization. Today, it covers 80% of all children around the globe. Unfortunately, BCG vaccine provides no protection against pulmonary tuberculosis, the most prevalent form of tuberculosis. It is necessary to urgently develop new vaccination strategies to stop large-scale dissemination of infection caused by the multidrug-resistant pathogen. The study was aimed to compare the capabilities of three variants of mRNA vaccines encoding ESAT6 epitopes of stimulating adaptive immune response formation in C57BL/6 mice (ELISpot, delayed hypersensitivity, IgG titers), as well as of protecting I/St mice against *M. tuberculosis* infection. Efficacy of mRNA vaccines comprising different untranslated regions packaged in lipid nanoparticles was compared with that of BCG vaccine. The 5'-TPL-Esat6-3'-Mod vaccine demonstrated the highest efficacy in our experimental model. Thus, the 5'-TPL-Esat6-3'-Mod mRNA vaccine can be considered as a candidate vaccine for further optimization, improving efficacy and subsequent use for prevention of tuberculosis.

Keywords: mRNA vaccine, BCG, adaptive immune response, tuberculosis

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ВЛИЯНИЕ НЕТРАНСЛИРУЕМЫХ ПОСЛЕДОВАТЕЛЬНОСТЕЙ мРНК НА ИММУНОГЕННОСТЬ мРНК-ВАКЦИН ПРОТИВ *M. TUBERCULOSIS* У МЫШЕЙ

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Вакцинация является одним из наиболее успешных медицинских мероприятий по снижению заболеваемости и смертности от туберкулеза. В 1974 г. вакцинация БЦЖ была включена в Расширенную программу вакцинации, и на сегодня охватывает 80% всех детей на земном шаре. К сожалению, вакцина БЦЖ не защищает от наиболее распространенной формы туберкулеза — туберкулеза легких. Требуется срочно разработать новые стратегии вакцинации, чтобы остановить широкомасштабное распространение инфекции с множественной лекарственной устойчивостью возбудителя. Целью исследования было сравнить способность трех вариантов мРНК-вакцин, кодирующих эпитопы ESAT6, стимулировать формирование адаптивного иммунитета у мышей C57BL/6 (ELISpot, ГЗТ, титры IgG), а также защищать мышей I/St от заражения *M. tuberculosis*. Эффективность упакованных в нанолипидные частицы мРНК-вакцин, различающихся последовательностями нетранслируемых регионов, сравнивали с эффективностью БЦЖ. В полученной нами экспериментальной модели максимальную эффективность по большинству показателей продемонстрировала вакцина 5'-TPL-Esat6-3'-Mod. Таким образом, мРНК-вакцина 5'-TPL-Esat6-3'-Mod может быть рассмотрена в качестве кандидатной для дальнейшей оптимизации, повышения ее эффективности и последующего применения для профилактики туберкулеза.

Ключевые слова: мРНК-вакцины, БЦЖ, адаптивный иммунный ответ, туберкулез

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About 10 million cases of active tuberculosis (TB) and about 1.5 million TB deaths are revealed annually all over the world [1]. The search for new TB vaccine frustrated many generations of enthusiastic researchers. Numerous painstaking attempts to understand the fundamental mechanisms underlying protective immunity in mycobacterial infection resulting only in understanding its complexity and not allowing to determine reliable immunological correlates of protection and form the basis for rational selection of promising vaccines. It is clear that unique features of effective immune response to the appropriate pathogen should be considered when developing the new generation vaccines [2]. The requirements for such vaccines should include: 1) induction of the "correct" ratio of T cell subpopulations and cytokine spectrum associated with the response to infection in combination with induction of protective and long-term (immunological memory) immunity; 2) activation of the "correct" effector mechanisms aimed at prevention of infection or elimination of the infectious agent; 3) high specificity for the infectious agent allowing to avoid the risk of autoaggression on account of cross-reacting antigens; 4) the use of bacterial antigens expressed in the host by all isolates and strains; 5) immunogenicity for all major histocompatibility complex (MHC) haplotypes in the human population.

The aspects most important for development of new effective TB vaccine are as follows: a) selection and combination of antigens, selection of the antigen physical and chemical properties (intracellular, surface-associates or secreted proteins, glycolipids, phospholigands); b) vaccine type (whole proteins or peptides, whole live attenuated or heat-killed bacteria, recombinant bacteria, DNA vaccines); c) vaccine form and administration route (high/low doses, adjuvants, immunomodulatory cytokines, immunostimulant DNA sequences).

The use of mRNA-based vaccines is a relatively new direction of vaccinology [3, 4]. The first experimental mRNA vaccines were developed in early 1990s. It has been shown that these induce both humoral and cell-based immunity *in vivo* [5, 6]. However, the first carriers used for transfer of mRNA molecules had poor safety profiles, while the per se use of mRNA led to the nucleic acid recognition by the immune system and degradation by RNAses [6]. These problems were partially overcome by using modified nucleosides in the mRNA molecule (replacement of uridine with pseudouridine or other analogues) making it possible to avoid induction of interferon-mediated antiviral pathways resulting in disruption of mRNA molecules [7]. At the same time, the toll-like receptor mediated activation of the innate immunity mechanisms by mRNA molecules can improve vaccination efficacy [8]. The development of carriers in the form of lipid nanoparticles (containing PEGylated lipids, cholesterol, ionizable lipids, and phospholipids) with improved safety profile increased the effectiveness of mRNA delivery. In general, these advances resulted in the growth and a constant interest in the use of mRNA vaccines for prevention of various infectious diseases, mostly viral. However, the experience of using mRNA vaccines for infectious diseases caused by bacteria is extremely limited.

Nevertheless, not all mRNA-based agents show high efficacy. Low RNA stability in the cell results in premature RNA degradation, low translation efficacy, decreased levels and expression duration of the target protein [9]. One of the key roles in ensuring stability of mRNA molecules and effectiveness of their translation is played by regulatory sequences of untranslated regions (5'-UTR and 3'-UTR). It should be noted that, despite extensive studies of the UTR properties, the number of studies focused on assessing the contribution of distinct UTRs to translation of heterologous RNA is limited [10, 11]. The study was aimed to assess immunogenicity and protective effects of the TB mRNA vaccines comprising various combinations of 5'-UTR and 3'-UTR sequences in the experimental murine model.

METHODS

Experimental design

The experiment involved 65 female C57BL/6Cit (B6) mice and 65 female I/StSnEgYCit (I/St) mice (body weight 20–25 g, age 2–4 months) taken from the Central Tuberculosis Research Institute breeding nursery. The animals were kept in a conventional vivarium with the fixed 12.00 : 12.00 h light/dark cycle and *ad libitum* access to water and food. B6 mice were intramuscularly immunized twice at a 3-week interval using three different mRNA vaccine variants: mRNA 5'-TPL-Esat6-3'Mod, 5'-Rabb-Esat6-3'EMCV and 5'-Mod- Esat6-3'Mod, 50 μg of RNA per injection (Fig. 1). Control animals were administered phosphate-buffered saline (PBS). BCG vaccination (BCG Pasteur) was performed once by subcutaneous injection with 100,000 CFU/mouse five weeks before tissue harvesting (B6)/ infection (I/St).

Eight animals from each group of B6 mice were used to assess delayed hypersensitivity. The remaining mice (five per group) were used to estimate T cell response (ELISpot) and titers of IgG and IgM against *M. tuberculosis* antigens. In I/St mice (five per group), mycobacterial load in the spleen and lung was determined 50 days after infection, along with the dynamic changes in mouse death rate after infection.

Cloning

To obtain constructs for further *in vitro* RNA transcription, a cassette comprising 5'-UTR, 3'-UTR and the earlier reported sequence encoding various ESAT6 protein epitopes [12] was inserted in the pSmart commercially available vector (Lucigen; USA). The 5'-UTR sequence of vaccine against SARS-CoV2 (Moderna; USA), sequence of the late adenoviral tripartite leader (TPL) or Rabb rabbit β-globin (Appendix 1) were used as 5'-UTR. The 3'-UTR sequence of vaccine against SARS-CoV2 (Moderna; USA) or 3'-UTR sequence of the encephalomyocarditis virus (EMCV) was used as 3'-UTR. Fragments were linked together by PCR involving overlapping primers. The EcoRI and BglII restriction sites were introduced into the 5'-UTR-Esat6-3'-UTR construct that were used for cloning into the pSmart vector. The NEB-stable strain was used for culturing (New England Biolabs; UK).

In vitro mRNA transcription

In vitro transcription was performed as described earlier [12]. RNA was precipitated by adding LiCl to the concentration of 0.32 M and EDTA (pH 8.0) to the concentration of 20 mM with subsequent incubation on ice for an hour. Then the solution was centrifuged for 15 min (25,000 g, 4 °C). The RNA precipitate was washed with 70% ethanol, dissolved in ultrapure water and then precipitated again with alcohol in accordance with the standard procedure. RNA concentration was determined by spectrophotometry based on absorption at 260 nm.

Formulation of mRNA into lipid nanoparticles

Formulation of mRNA into lipid nanoparticles was performed in the NanoAssemblr™ Benchtop system (Precision Nanosystems;

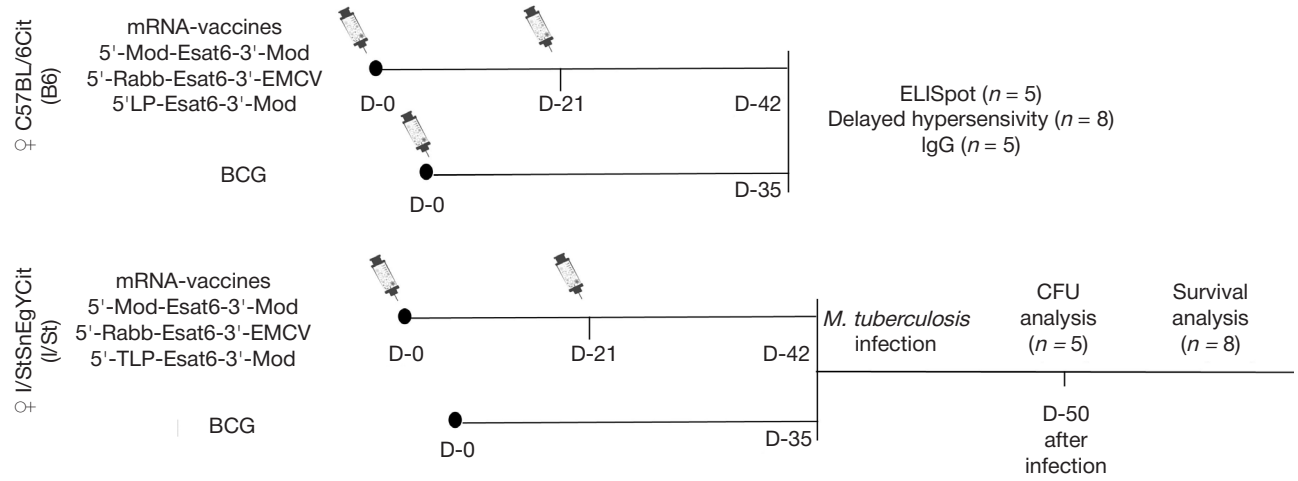

Fig. 1. Experimental design

USA) as described earlier [13]. The lipid mixture components were as follows: 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 concentration of mRNA loaded onto lipid nanoparticles was determined based on the difference in the fluorescent signal levels upon RiboGreen (Thermo Fischer Scientific; USA) staining of suspended particles before and after their destruction. The Triton X-100 detergent (Sigma-Aldrich; USA) was used to destroy the particles. The amount of encapsulated RNA in the particles in all samples constituted more than 95% of the total amount of RNA (encapsulated + free). Particle size was 80–90 nm, and polydispersity index was below 0.15.

Delayed hypersensitivity reaction measurement

Delayed hypersensitivity reaction in mice of each group (eight mice per group) was assessed four weeks after vaccination based on the left hind paw swelling in response to administration of 40 μL of PBS containing 50 IU of tuberculin purified protein derivative (SCEEMP; Russia) 48 h after injection. The data were presented as Δ (difference in thickness of the left and right paws in mm).

Antigens

The H37Rv *M. tuberculosis* sonicate, the soluble fraction of *M. tuberculosis* H37Rv disrupted by ultrasound, was used as antigen in *ex vivo* experiments [14].

Determination of titers of IgG and IgM against *M. tuberculosis* antigens

Murine blood serum was used to determine titers of specific antibodies (IgG and IgM) against mycobacterial antigens by enzyme immunoassay routinely used in laboratory settings [15, 16]. Serum dilutions between 1 : 50 and 1 : 400 were used for antibody determination.

Quantification of IFNγ-producing cells

Protective T cell immune response was estimated based on the counts of splenocytes secreting IFNγ in response to stimulation with mycobacterial antigens using the Mouse IFNγ ELISpot Set (BD NJ; USA) and AEC Substrate Set (BD NJ; USA) in accordance with the manufacturer's instructions.

Evaluation of protective response

The I/St mice were immunized twice (42 and 21 days before infection) with mRNA-based vaccines. BCG immunization was performed 35 days before infection. Mice were infected with the *M. tuberculosis* virulent strain in a dose of 500,000 CFU/mouse. Mycobacteria were quantified in the internal organs of infected mice on day 50 after infection. For that lungs and spleens of infected animals were isolated in the sterile environment and homogenized in 2 mL of saline. Then 10-fold serial dilutions of organ homogenates were prepared and sown on Petri dishes with Middlebrook 7H10 agar, 50 μL per dish. The Petri dishes with applied suspensions were incubated at 37 °C. Then, 21 days later the *M. tuberculosis* H37Rv macrocolonies were enumerated in the dish and their number was recalculated with reference to the organ.

Statistical analysis

Statistical processing of the results was performed using the Student's t-test; Bonferroni correction was applied when comparing more than two groups. The differences were considered significant at $p < 0.05$. The data provided in figures are presented as means ± SEM. The Gehan–Breslow–Wilcoxon method was used for survival curves.

RESULTS

Immunogenicity of the tested vaccines was assessed based on their ability to induce specific cellular immune response *ex vivo* (ELIspot) and *in vivo* (delayed hypersensitivity), as well as on the specific antibody response in blood of vaccinated mice (Fig. 2). Fig. 2A presents the results of IFNγ-producing cell quantification in the spleens of experimental animals. Significant differences from controls were obtained for the 5'-Mod-Esat6-3'-Mod and 5'-TPL-Esat6-3'-Mod mRNA vaccines ($p < 0.004$ and $p < 0.0008$, respectively). Among groups of animals immunized with mRNA vaccines, the group that received 5'-TPL-Esat6-3'-Mod was the leader based on the IFN-producing cell counts ($p < 0.004$ and $p < 0.0004$ vs. 5'-Mod-Esat6-3'-Mod, 5'-Rabb-Esat6-3'-EMCV mRNA vaccines, respectively). At the same time, the highest IFN-producing cell counts were found in the group of BCG-vaccinated mice ($p < 0.006$, $p < 0.006$ and $p < 0.03$ vs. 5'-Mod-Esat6-3'-Mod, 5'-Rabb-Esat6-3'-EMCV and 5'-TPL-Esat6-3'-Mod mRNA vaccines, respectively).

The delayed hypersensitivity reaction of mice immunized with the 5'-Rabb-Esat6-3'EMCV and 5'-TPL-Esat6-3'-Mod

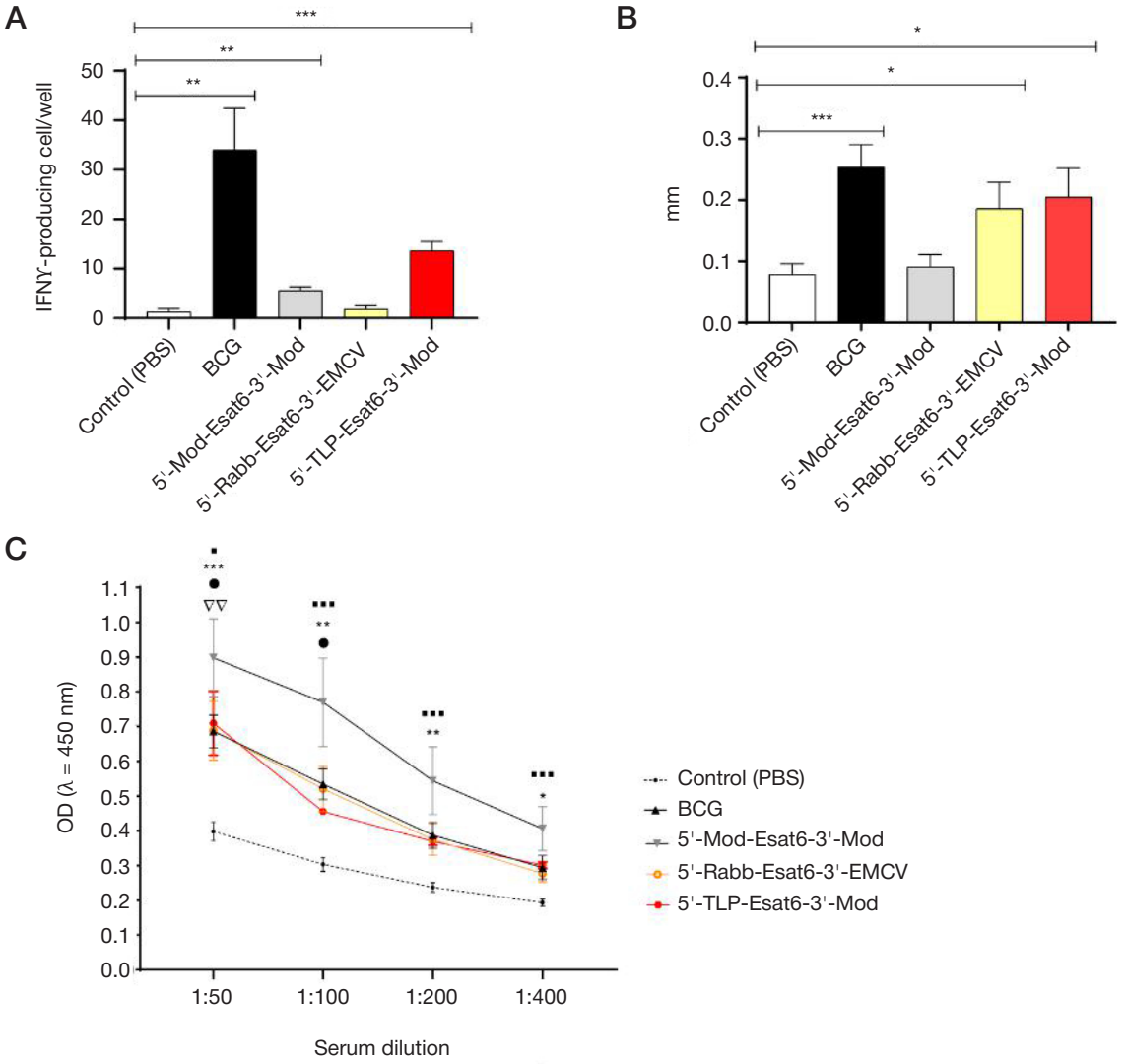

Fig. 2. Adaptive immune response induction by mRNA vaccines. B6 mice were immunized twice (42 and 21 days before the experiment) with mRNA vaccines. BCG immunization was performed 35 days before the experiment. On the day of experiment, IFN-producing cell counts were determined in responders to mycobacterial antigens **(A)**, as well as the delayed hypersensitivity reaction **(B)** and production of specific antibodies against mycobacterial antigens **(C)**. Means ± SEM are presented, where *n* — five mice per group for **A** and **C**, eight mice per group for B. A, B. * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$ compared to control. C. * — significant difference from control for BCG, Δ — significant difference from control for 5'-Mod-Esat6-3'-Mod vaccine, ● — significant difference from control for 5'-Rabb-Esat6-3'-EMCV vaccine, ■ — significant difference from control for 5'-TLP-Esat6-3'-Mod vaccine

mRNA vaccines was significantly different from that of controls ($p < 0.04$ for these groups) and similar to the delayed hypersensitivity reaction of BCG-vaccinated mice (Fig. 2B). 2B). There were no significant differences between the 5'-Rabb-Esat6-3'EMCV, 5'-TPL-Esat6-3'-Mod and BCG groups.

The studies focused on assessing the development of humoral immune response to the TB mRNA vaccines also showed differences from controls. The results of determining (titration curves) the antigen-specific antibodies (IgG) are presented in Fig. 2C. All the tested vaccines, including BCG, stimulated production of anti-mycobacterial IgG in immunized mice. Furthermore, among mRNA vaccines, the maximum production of antigen-specific IgG was reported in response to immunization with 5'-Rabb-Esat6-3'EMCV and 5'-TPL-Esat6-3'-Mod (compared to controls). There were no significant differences between the 5'-Rabb-Esat6-3'EMCV, 5'-TPL-Esat6-3'-Mod and BCG groups. The levels of antigen-specific IgM production in vaccinated mice were similar to that of controls (Appendix 2). Thus, our findings have shown that the Esat6 epitope-based mRNA vaccines cause the adaptive immune response induction. Despite the fact that all mRNA vaccine variants were less effective than BCG, we observed significant differences in their efficacy depending on the UTR sequences. The best results of testing the vaccine for both cellular and humoral immune response were obtained for the 5'-TPL-Esat6-3'-Mod mRNA vaccine.

The development of protective immune response was studied in the I/St mice susceptible to TB infection. Fig. 3 presents the results of determining mycobacterial load in infected animals, as well as the dynamic changes in death rate of immunized animals after infection. A significant decrease in the lung tissue mycobacterial counts compared to unvaccinated controls was revealed in mice immunized with the 5'-Rabb-Esat6-3'-EMCV, 5'-TPL-Esat6-3'-Mod mRNA vaccines and BCG (Fig. 3B). Furthermore mycobacterial load in the lung tissue of the groups of mice vaccinated with the 5'-TPL-Esat6-3'-Mod mRNA vaccine and BCG showed no significant differences. As for the spleen, significant differences from non-immunized controls were reported for BCG-vaccinated mice only (Fig. 3A).

The animals' extended lifespan after infection is among major criteria of the vaccine protective effect. That is why we compared the dynamic changes in death rate in the control and experimental groups of mice after infection. Significant

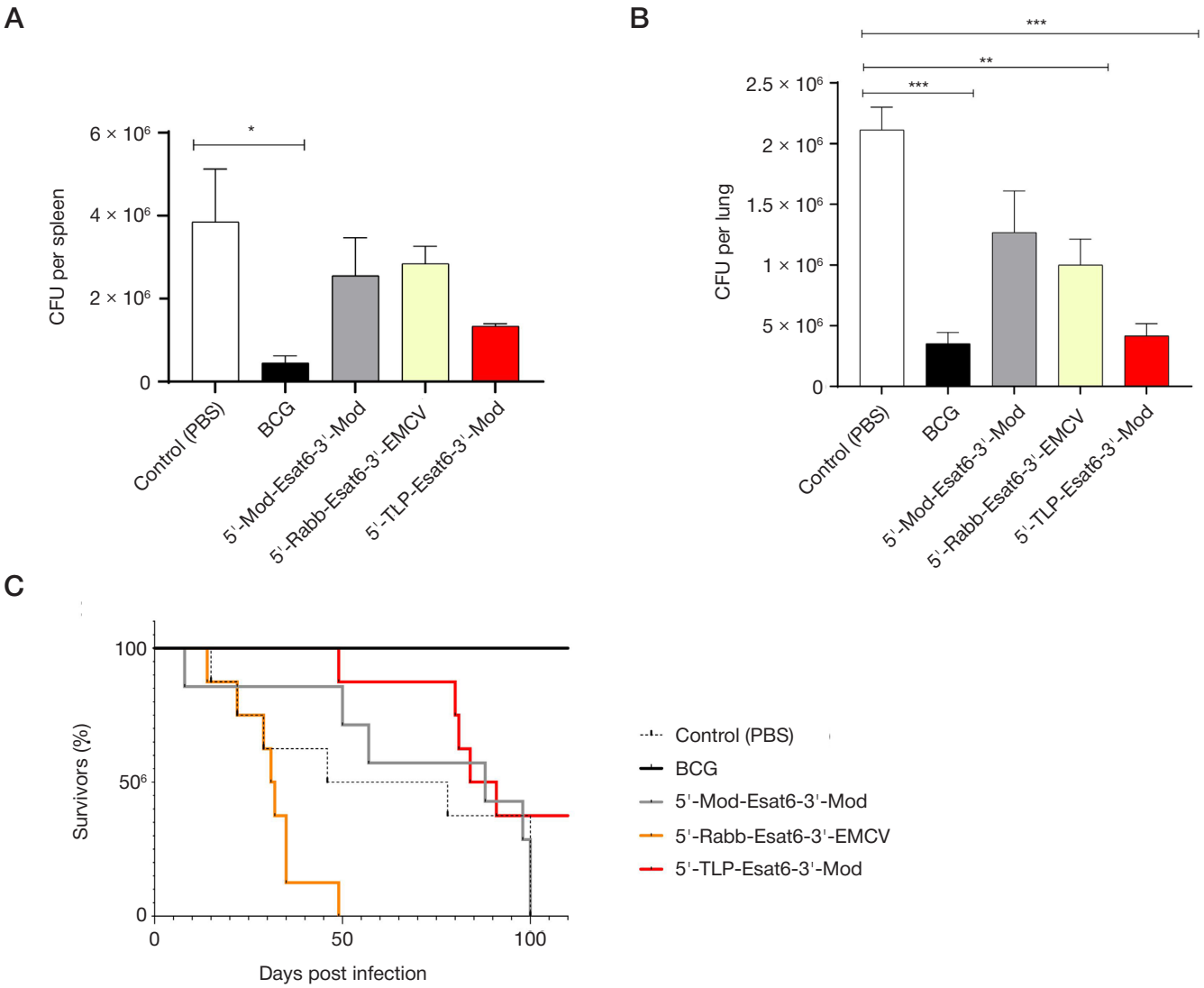

Fig. 3. Protective immune response associated with immunization with mRNA vaccines. I/St mice were immunized twice (42 and 21 days before the experiment) with mRNA vaccines. BCG immunization was performed 35 days before the experiment. Mice were infected with the *M. tuberculosis* virulent strain in a dose of 500,000 CFU/mouse. Mycobacterial load in the spleen **(A)** and the lungs **(B)** of infected animals was assessed 50 days later, along with the dynamic changes in the mouse death rate after infection **(C)**. Means ± SEM are presented, where n – five **(A, B)** and eight **(C)** mice per group; * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$

differences from unvaccinated controls (Gehan–Breslow–Wilcoxon method for survival curves) were reported for BCG-immunized mice and mice immunized with the 5'-TPL-Esat6-3'-Mod mRNA vaccine ($p = 0.0005$ and $p = 0.04$, respectively). Significant differences in the death rate of I/St mice vaccinated with BCG and 5'-TPL-Esat6-3'-Mod mRNA vaccine were also revealed ($p = 0.01$) (Fig. 3C). Similar to the adaptive immune response induction results, the 5'-TPL-Esat6-3'-Mod mRNA vaccine showed the highest efficacy among mRNA vaccines; it was the only vaccine that ensured reduced mortality relative to unvaccinated animals.

DISCUSSION

In this study involving the murine model of TB infection we have shown that administration of two doses of mRNA vaccines consisting of mRNA encoding some ESAT6 mycobacterial protein epitopes encapsulated in lipid particles yields protective immune response, the efficacy of which depends, inter alia, on the mRNA untranslated sequences comprised in the vaccine. Furthermore, stimulation of humoral immune response by various vaccine variants determined based on production of specific antibodies after vaccination is significantly different from activation of cell-based immunity assessed based on the delayed hypersensitivity reaction intensity and IFNγ production (based on ELIspot).

Historically, the stidues of immune response to infection caused by *M. tuberculosis* were focused mainly on T cells and macrophages, since their role in granuloma formation was rather well understood. In contrast, the role of B cells in the TB infection pathogenesis was relatively understudied. Therefore, the majority of newly developed TB vaccines were focused on the cellular immune response induction [2]. However, a number of recent studies of TB vaccine efficacy in mice, nonhuman primates and humans revealed minor induction of antibodies against *M. tuberculosis*, which could be associated with the observed vaccine efficacy [17]. In our study, among all mRNA vaccines, maximum protection (based on the lung-derived mycobacterial culture and survival of infected mice) was achieved by using the 5'-TPL-Esat6-3'-Mod mRNA vaccine. Immunization with the 5'-TPL-Esat6-3'-Mod mRNA vaccine results in formation of pronounced cellular immune response and moderate production of specific anti-mycibacterial IgG. At the same time, immunization of mice with the 5'-Mod-Esat6-3'-Mod mRNA vaccine stimulates active production of specific antibodies, but it is not associated with generation of protection

and does not activate the adaptive immune response cellular component.

We assume that the differences between three mRNA vaccines differing only by untranslated sequences can be associated with the mRNA translation duration and intensity and, therefore, with different antigen presentation effectiveness. The adenovirus tripartite leader (TPL) sequence acts as an enchancer of the viral late gene mRNA translation and is believed to be capable of initiating cap-independent translation of the adenovirus late mRNA [18, 19]. The 5'-UTR TPL consists of 245 nucleotides and has a complex secondary structure. It has been shown that TPL comprises IRES, through which it can recruit the ribosome regardless of interactions with cap [18, 19]. However, since cap-independent translation initiation is activated only under conditions of cellular stress, we assume that our data on TRL are not related to the cap-independent translation initiation. In addition to longer length, the TPL sequence has a higher GC content and a very negative minimum Gibbs free energy (ΔG), which is due to translation inhibition [20]. In contrast, 5'-UTR of rabbit β-globin (Rabb) and 5'-UTR of the mRNA-1273 vaccine (Moderna) have the length of about 50 nucleotides and comprise no strong secondary structures [21]. Apparently, the secondary structures emerging in the 5'-UTR without any RNA-binding protein involvement have no pronounced inhibitory effect on translation, since these structures can be uncoiled by the eIF4A factor immediately before the translation initiation [22]. At the same time, the TPL strong secondary structure can contribute to engaging RNA-binding proteins positively affecting translation.

Thus, the 5'-TPL-Esat6-3'-Mod mRNA vaccine seems to be the most promising for further research. Despite the fact that it is inferior to BCG, further optimization, including increasing the dose of vaccine, cap or using the uridine analogues, can improve its efficacy. Assessment of prospects of using the 5'-TPL-Esat6-3'-Mod mRNA vaccine for revaccination after primary BCG vaccination can constitute the next phase of vaccine testing.

CONCLUSIONS

The mRNA-based multi-epitope vaccines can be considered as independent preventive vaccines or booster vaccines against *M. tuberculosis*. Despite the fact that the studied vaccine variants have lower efficacy compared to BCG, the relationship between the efficacy and the sequence of regulatory regions has been revealed. Our findings have made it possible to determine the optimal combination of the expression cassette regulatory elements. Further development of mRNA vaccine against *M. tuberculosis* will be focused on improving its efficacy.

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