ОЦЕНКА ДЛИТЕЛЬНОСТИ СОХРАНЕНИЯ Т-КЛЕТОК ПАМЯТИ У МЫШЕЙ ПОСЛЕ ИММУНИЗАЦИИ ЖИВОЙ ТУЛЯРЕМИЙНОЙ ВАКЦИНОЙ

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Резюме. Вакцинный штамм *F. tularensis* 15 НИИЭГ индуцирует длительный клеточный иммунитет, однако проявляет определенную реактогенность и генетическую нестабильность. Прогресс в разработке новых противотуляремийных вакцин с улучшенными характеристиками затруднен из-за недостатка знаний о механизмах формирования и поддержания протективного иммунитета. Мыши линии BALB/c являются наиболее подходящей моделью при экспериментальной туляремии из-за относительно низкой стоимости, хорошо охарактеризованной генетики, доступных иммунологических инструментов и наиболее близко имитируют инфекционный процесс при заражении вирулентными штаммами *F. tularensis*.

Известно, что $CD4^+$ и $CD8^+$Т-клетки необходимы для формирования защитного иммунитета, однако роль определенных субпопуляций Т-клеток памяти в длительной защите от вирулентных штаммов *F. tularensis* не установлена. Мы предположили, что защитный иммунитет зависит от центральных (T_{CM}) и эффекторных Т-клеток памяти (T_{EM}) и их функциональной активности. В данной работе был изучен Т-клеточный иммунный ответ у мышей BALB/c через 30, 60 и 90 дней после подкожной вакцинации *F. tularensis* 15 НИИЭГ.

Анализ иммунного ответа спленоцитов проводили методом многопараметрической проточной цитометрии, стимулируя клетки *in vitro* антигеном *F. tularensis*. T_{EM} клетки идентифицировали как $CD3^+CD4^+CD44^+CD62L^-$ и $CD3^+CD8^+CD44^+CD62L^-$, T_{CM} клетки как $CD3^+CD4^+CD44^+CD62L^+$ и $CD3^+CD8^+CD44^+CD62L^+$, соответственно. Функциональную активность Т-клеток памяти оценивали по следующим параметрам: уровню экспрессии маркера активации CD69 и цитокин-продуцирующей активности путем окрашивания внутриклеточных цитокинов IFNγ и TNFα.

Таким образом, для разработки новой вакцины требуется выявление иммунологических критериев оценки протективного иммунитета, которые присутствуют не только в раннюю фазу после вакцина-

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ции, но после окончания эффекторной фазы иммунного ответа. Показано, что для поддержания длительного протективного иммунитета, инициированного вакцинацией *F. tularensis* 15 НИИЭГ, требуется наличие антиген-специфических $CD4^+$ и $CD8^+$Т-клеток памяти, продуцирующих IFNγ и TNFα и экспрессирующих маркер активации CD69. В отдаленные сроки после вакцинации было выявлено снижение количества и угасание функциональной активности субпопуляций $CD8^+T_{EM}$ и $CD8^+T_{CM}$. Выявленные параметры функциональной активности Т-клеток памяти могут служить критериями оценки протективного иммунитета в отношении вирулентных штаммов *F. tularensis*.

Ключевые слова: Fransicella tularensis, вакцинный штамм, Т-клетки памяти, CD69, IFNγ, TNFα, клеточный иммунитет

EVALUATION OF THE LONG-TERM MEMORY T CELL IN MICE AFTER IMMUNIZATION WITH A LIVE TULAREMIA VACCINE

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Abstract. The vaccine strain *F. tularensis* 15 NIIEG induces long-lived cell-mediated immunity but exhibits a certain reactogenicity and genetic instability. Progress in development of a vaccine against tularemia has been limited by a lack of information regarding the mechanisms required to protect against this disease. The BALB/c mouse is the most commonly used animal to study tularemia due to its relatively low cost, well-characterized genetics, available immunological tools and mouse infection with virulent *F. tularensis* recapitulates human disease.

$CD4^+$ and $CD8^+$T cells are known to be critical for the formation of protective immunity but the relative roles of memory T cell subpopulations in long lived protection against virulent strains of *F. tularensis* are not well established. We hypothesized that this immunity depends on central (T_{CM}) and effector memory (T_{EM}) T cells and their functional activity. In this study we have dissected the T cell immune response in BALB/c mice 30, 60 and 90 days after subcutaneous vaccination with 15 NIIEG.

Multiparametric flow cytometry were used to characterize *in vitro* recall responses of splenocytes to *F. tularensis* antigen. T_{EM} cells were identified as $CD3^+CD4^+CD44^+CD62L^-$ and $CD3^+CD8^+CD44^+CD62L^-$, T_{CM} cells as $CD3^+CD4^+CD44^+CD62L^+$ and $CD3^+CD8^+CD44^+CD62L^+$, respectively. The functional activity of memory T cells was assessed by the following parameters: the level of expression of the activation marker CD69 and cytokine-producing activity by staining with the intracellular cytokines IFNγ and TNFα.

Thus, development of a long-lived vaccine directed against *F. tularensis* is dependent on identifying not only the correlates of immunity present early after vaccination, but also those that persist in the host after the effector phase has ended. The maintenance of long-term protective immunity initiated by vaccination with *F. tularensis* strain 15 NIIEG has been shown to require the presence of antigen-specific $CD4^+$ and $CD8^+$ memory T cells producing IFNγ and TNFα and expressing the activation marker CD69. A decrease in count and functional activity of $CD8^+T_{CM}$ and $CD8^+T_{EM}$ was detected in the long term after vaccination. The detected parameters of functional activity of memory T cells can be used as criteria for evaluation of protective immunity against virulent strains of *F. tularensis*.

Keywords: Fransicella tularensis, vaccine strain, memory T cells, CD69, IFNγ, TNFα, cellular immunity

Introduction

The live tularemia vaccine used in the Russian Federation based on the *F. tularensis* 15 strain NIIEG was developed empirically with limited understanding of its immunological mechanisms. The *F. tularensis* 15 NIIEG vaccine strain provides effective immunity against tularemia but exhibits a certain reactogenicity and genetic instability [2]. $CD4^+$ and $CD8^+$T cells are known to play a key role in the formation of protective immunity against tularemia [5] but the contribution of central (T_{CM}) and effector (T_{EM}) memory T cells subpopulation in the formation of long-term protective immunity remains poorly understood. The detailed knowledge of the mechanisms of long-term

immunity would allow an objective assessment of the immunogenic and protective properties of the new tularemia vaccine strains under development with improved properties.

The mouse is the most commonly used animal to study tularemia due to available immunological tools that allow a detailed study of the T cell immune response [5]. The study of the mechanisms of protective immunity to *F. tularensis* and the identification of immunological criteria for its assessment in a mouse model is an important step in research on the development of new vaccine strains and the improvement of laboratory methods for the evaluation of the T cell immunity.

The purpose of the present study was to investigate the subpopulation composition and functional activity of memory T cells in BALB/c mice in the long time after vaccination with *F. tularensis* strain 15 NIIEG.

Materials and methods

All animal experiments were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Specific-pathogen-free, 6-8-week-old BALB/c mice ($H2^d$) were purchased from Laboratory Animal Breeding Facility (Pushchino, Moscow Region).

Vaccine strain of F. tularensis 15 NIIEG was obtained in the SRCAMB collection (Obolensk, Moscow Region). Mice were vaccinated subcutaneously with *F. tularensis* 15 NIIEG cells in PBS (30 CFU per mouse) in the inner part of the upper thigh. A group of unvaccinated mice served as a control.

At 30, 60 and 90 days after immunization animals were euthanized, spleens were sterilely extracted and lymphocyte cultures were obtained according for evaluation of T cell immunological memory as previously described [4]. Lymphocytes were plated at 2×10^5 cells/well into a 96-well plate and incubated with 10 μg/mL *F. tularensis* antigen obtained by the published procedure [7].

Subpopulation composition and functional activity of memory T cells were detected by flow cytometry using commercial monoclonal antibodies (MAbs) (BD Bioscience, USA) conjugated with the different fluorochromes: CD3 APC and CD3 BV421 (clone 17A2); CD4 BB700 (clone RM4-5); CD8 APC (clone 53-6.7); CD62L FITC (clone MEL14); CD44 PE (clone IM7); CD69 BV421 (clone H1.2F3).

Central ($CD4^+T_{CM}$) and effector ($CD4^+T_{EM}$) memory T helper cells were identified as $CD3^+CD4^+CD44^+CD62L^+$ and $CD3^+CD4^+CD44^+CD62L^-$ subpopulations, respectively; cells with $CD3^+CD8^+CD44^+CD62L^+$ and $CD3^+CD8^+CD44^+CD62L^-$ phenotypes were identified as central ($CD8^+T_{CM}$) and effector ($CD8^+T_{EM}$) memory T cells. The functional activity of the above memory T cell subpopulations was assessed based on the expression level of the CD69 molecule on their surface.

Memory T cells were stained intracellular MAbs against IFNγ APC (clone XMG1.2) and TNFα APC (clone MP6-XT22) to analyze cytokine-producing activity as previously described [3]. Cells were acquired and analyzed using a FACSAria III (BD Biosciences, USA) flow cytometer and BD FACSDiva Software v. 8.0.1.

Statistical processing of the results was performed using GraphPad Prism 6.00 for Windows (GraphPad Prism Software Inc., USA). Cytometry data were presented as a percentage of the target T cell subpopulation and provided as a median and interquantile range (Me ($Q_{0.25}$-$Q_{0.75}$)). The two experimental groups were compared using the Mann–Whitney U test with the significance set at $p < 0.05$.

Results and discussion

In our earlier study, we demonstrated that the long-term protective immunity against tularemia in vaccinated mice depends on the subspecies infecting strain: infection with the virulent *F. tularensis* 503 of the same *holarctica* subspecies as the vaccine strain *F. tularensis* 15 NIIEG provides 100 % protection for 180 days after vaccination; infection with the virulent *F. tularensis* Schu subspecies of *tularensis* leads to a decrease in protection with an increase in the postvaccination period to 60 days [4].

The major objective of this study was to identify immunological parameters characterizing the duration of protection against virulent *F. tularensis* strains. Antigen-specific $CD4^+$ and $CD8^+$ memory T cells are heterogeneous in composition and during immune response are differentiated in T_{EM} and T_{CM} [6]. We performed a comparative analysis of the dynamics of changes in subpopulation composition and functional activity of $CD4^+$ and $CD8^+$ memory T cells by flow cytometry at 30, 60 and 90 days after immunization with *F. tularensis* 15 NIIEG vaccine strain. A fluorescent-labelled monoclonal antibody panel to CD4, CD8, CD44 and CD62L surface antigens allows the identification of T_{EM} ($CD44^+CD62L^-$) and T_{CM} ($CD44^+CD62L^+$) in the $CD4^+$ and $CD8^+$ lymphocyte pool. Expression levels of the lymphocyte activation marker CD69 [8] and cytokine production of IFNγ and TNFα were analyzed to assess the antigen reactivity of memory T cell subpopulations. The dynamics of changes in the subpopulation composition and functional activity of memory T cells as a function of the time after vaccination is presented in Figure 1.

Comparative analysis of the data has shown that the percentage of functionally active $CD4^+T_{CM}$ did not change and was comparable in all studied periods after immunization: the cells expressed on their surface the

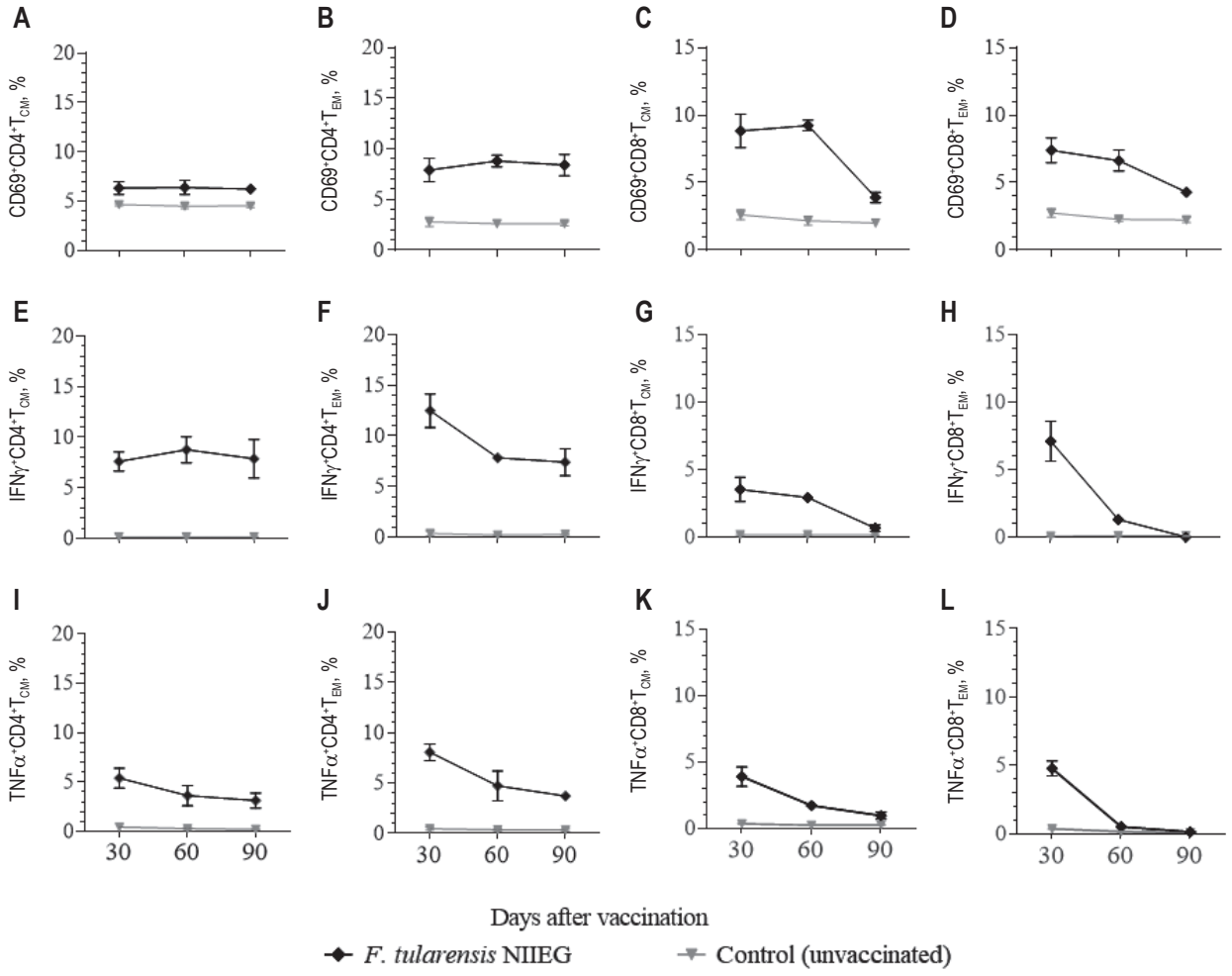

Figure 1. Subpopulation composition and functional activity of T_{CM} and T_{EM} in the pool of $CD4^+$ and $CD8^+$T cells in the spleen of BALB/c mice during early and late time points after immunization with live tularemia vaccine

Note. (A, B, C, D) T cell subpopulations expressing the activation marker CD69. (E, F, G, H) IFNγ-producing memory T cell subpopulations. (I, K, L, M) TNFα-producing memory T cell subpopulations.

activation marker CD69 (Figure 1A) and produced IFNγ (Figure 1E) and TNFα (Figure 1I). After the effector phase has ended (30 days) we observed a significant decrease in the number of cells with $IFN\gamma^+CD4^+T_{EM}$ (Figure 1F) and $TNF\alpha^+CD4^+T_{EM}$ (Figure 1J) phenotype at day 60 after vaccination; at day 90 no change was detected compared to the previous post-vaccination period. Therefore, no significant differences in the percentage and functional activity of $CD4^+$ memory T cells were detected in the long-term after vaccination with the studied *F. tularensis* strains. Antigen-specific $CD4^+$ memory T cells with T_{CM} and T_{EM} phenotypes produced cytokines TNFα and IFNγ and expressed activation marker CD69 even 90 days after immunization with the tularemia vaccine strain.

An important difference was found for T_{CM} and T_{EM} $CD8^+$ with phenotypes memory T cells at a long-term post-vaccination period. Comparative analysis of $CD69^+$ memory T cell subpopulations has shown to increasing post-vaccination time from 60 to 90 days was observed a decrease in $CD8^+T_{CM}$ (Figure 1C) and $CD8^+T_{EM}$ (Figure 1D) in immunized mice. A similar tendency was observed in the $CD8^+T_{CM}$ (Figure 1G, K) and $CD8^+T_{EM}$ (Figure 1H, L) cytokine-producing activities: at 60 days after immunization with *F. tularensis* 15 NIIEG strain, the cytokine-producing activity of the $CD8^+T_{CM}$ and $CD8^+T_{EM}$ subpopulations was of a low profile; at 90 days after immunization there was no statistically significant difference in the number of all cytokine-producing ($IFN\gamma^+$ and $TNF\alpha^+$) $CD8^+$ memory cells compared to the intact group.

One explanation for the waning of protective immunity over time against intracellular infections is the decreasing pool of long-lived T_{CM} [1]. Our results are consonant with earlier published data about to the end of the effector phase of the T cell immune response most T_{EM} undergo apoptosis and for formation long-term protection it is necessary for the vaccine to induce the generation of T_{CM}

capable of rapidly proliferating and differentiating into T_{EM}, which, in turn, successfully eliminate the pathogen [6]. Therefore, it is possibly that exhaustion of the $CD8^+T_{CM}$ pool with increasing post-vaccination period results in decreased functional activity of $CD8^+T_{EM}$ on days 60 and 90 after vaccination with *F. tularensis* 15 NIIEG.

Conclusion

In summary, antigen-specific T_{CM} and T_{EM} with $CD4^+$ and $CD8^+$T cell phenotypes are producing IFNγ and TNFα and expressing CD69 are required to maintain long-term protective immunity initiated by vaccination with live tularemia vaccine. The obtained data on the significant functional activity of T_{CM} and T_{EM} $CD4^+$ memory T cells at all post-vaccination times explain the mechanism of protection up to 90 days after vaccination of mice who have been infected with the virulent strain *F. tularensis* 503 subsp. *holarctica* [4]. In turn, the decreased of functional activity of the T_{CM} and T_{EM} $CD8^+$ memory T cells is correlated with the weakening of protection against virulent *F. tularensis* Schu subsp. tularensis during late time points after vaccination with *F. tularensis* 15 NIIEG [4].

We recommend the functional activity of T_{EM} and T_{CM} by the expression level of the activation marker CD69 and the synthesis of cytokines IFNγ and TNFα as immunological criteria to evaluate the effectiveness of immunization with candidate vaccine strains of *F. tularensis*.

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