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The adjuvant effect of polymuramil, a NOD1 and NOD2 agonist, differs when immunizing mice of different inbred lines with nonstructural hepatitis C virus (Flaviviridae: *Hepacivirus*) proteins and is synergistically enhanced in combination with pyrogenalum, a TLR4 agonist

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Introduction. Hepatitis C is a liver disease with high chronicity, the cause of cirrhosis and hepatocarcinoma. The main obstacle to controlling hepatitis C is the lack of vaccines.
The aim of the work was to compare the immunogenic activity of nonstructural recombinant proteins NS3, NS4 and NS5B of hepatitis C virus (HCV) as components of a subunit candidate vaccine and to analyze the adjuvant properties of two available commercial drugs, polymuramil and pyrogenalum.
Materials and methods. BALB/c, DBA/2J and C57BL/6 mice were immunized with nonstructural proteins without adjuvants or with polymuramyl (NOD1 and NOD2 agonist) and pyrogenalum (TLR-4 agonist). The activity of antibodies was determined in ELISA, the cellular response – by antigen-specific lymphocyte proliferation and by production of IFN-γ *in vitro*.
Results. Recombinant proteins showed different immunogenicity. NS4 induced antibodies more efficiently than NS3 and NS5B. Significant differences were found in the immune response of three inbred lines mice: the level of IFN-γ in BALB/c and DBA/2J mice induced by NS5B protein was 30 times higher than in C57Bl/6 mice. In contrast, the induction of antibodies in BALB/c mice was lower than in C57Bl/6 and DBA/2J. Polymuramil did not increase the humoral response to NS5B and enhanced the cellular response only in C57BL/6 mice. The combined use of polymuramil with pyrogenalum significantly increased both the humoral and cellular response of mice to all recombinant HCV proteins.
Conclusion. Different immunogenic properties and different functions of recombinant non-structural HCV proteins indicate the feasibility of their combined inclusion in subunit vaccines. It was established for the first time that immunization with HCV proteins with a complex adjuvant (polymuramyl + pyrogenalum) has a synergistic effect, significantly exceeding the effect of each of them separately.

Keywords: *hepatitis C; hepatitis C virus; non-structural hepatitis C virus proteins; BALB/c; DBA/2J and C57BL/6 mice; humoral and cellular immune responses; polymuramyl; pyrogenalum; synergistic adjuvant effect*

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Адъювантное действие агониста NOD1 и NOD2 полимурамила различается при иммунизации мышей разных инбредных линий неструктурными белками вируса гепатита С (Flaviviridae: *Hepacivirus*) и синергетически усиливается в комбинации с агонистом TLR4 пирогеналом

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Введение. Гепатит С – заболевание печени с высокой хронизацией, являющееся причиной цирроза и гепатокарциномы. Главное препятствие для контроля за гепатитом С – отсутствие вакцин.
Цель работы – сравнение иммуногенной активности неструктурных рекомбинантных белков NS3, NS4 и NS5B вируса гепатита С (ВГС) как компонентов субъединичной кандидатной вакцины и анализ адъювантных свойств двух отечественных коммерческих препаратов: полимурамила и пирогенала.
Материалы и методы. Мышей линий BALB/c, DBA/2J и C57BL/6 иммунизировали трижды с интервалом 2 недели белками NS3, NS4 и NS5B без адъювантов или с полимурамилом (агонист рецепторов иммунного ответа NOD1 и NOD2) и пирогеналом (липополисахарид, агонист TLR-4). Гуморальный ответ определяли по активности антител в ИФА, клеточный – по индексу стимуляции пролиферации лимфоцитов и способности продуцировать IFN-γ при антигенспецифической стимуляции *in vitro*.
Результаты. Рекомбинантные белки проявляли различную иммуногенность. NS4 индуцировал антитела эффективнее, чем NS3 и NS5B. Значительные различия обнаружены в иммунном ответе мышей трех инбредных линий: уровень секреции IFN-γ у мышей BALB/c и DBA/2J на белок NS5B был в 30 раз выше, чем у мышей C57Bl/6. Индукция антител, напротив, у мышей BALB/c была ниже, чем у C57Bl/6 и у DBA/2J. Полимурамил не увеличивал гуморальный ответ на NS5B и усиливал клеточный ответ только у мышей C57BL/6. Сочетанное применение полимурамила с пирогеналом значительно увеличивало как гуморальный, так и клеточный ответ мышей на все рекомбинантные белки ВГС.
Выводы. Различные иммуногенные свойства и разные функции неструктурных белков ВГС в репликации вируса свидетельствуют о целесообразности их сочетанного включения в состав субъединичных вакцин. Впервые установлено, что иммунизация белками ВГС с комплексным адъювантом (полимурамил + пирогенал) оказывает синергетический эффект, значительно превышая действие каждого из них по отдельности.

Ключевые слова: *гепатит С; вирус гепатита С; неструктурные белки вируса гепатита С; мыши BALB/c, DBA/2J и C57BL/6; гуморальный и клеточный иммунные ответы; полимурамил; пирогенал; синергетическое адъювантное действие*

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Introduction

Hepatitis C caused by the hepatitis C virus (HCV) has been recognized as a major cause of chronic liver disease, including end-stage liver disease – cirrhosis and hepatocellular carcinoma. Approximately 80% of people with acute hepatitis C develop chronic infection, chronic hepatitis C [1, 2]. Direct acting antivirals (DAAs), which were developed about 10 years ago, have helped over 90% of patients achieve sustained elimination of the virus from the peripheral blood, blocking HCV replication. However, over time, it has become obvious that the hepatitis C problem has not been solved. DAAs are extremely expensive, making treatment unaffordable for many patients. Their long-term effects are insufficiently studied, demonstrating that HCV can persist in liver and blood cells in some patients who achieved viral RNA clearance from plasma [3]. HCV-induced changes in the epigenetic status and signaling pathways persist following DAA treatment [4]. Resistance to DAAs has been reported [5]. Some known DAA treatment-associated side effects include HCV mutations and reactivation of other viruses [6]. Thus, studies on preventive and therapeutic vaccines against hepatitis C, which began after the discovery of HCV in 1989 and slowed down with the advent of DAAs, have intensified in recent years. Currently, it is assumed that the absence of vaccines poses a major challenge to prevention and control of hepatitis C [7].

Current approaches to the development of vaccines against hepatitis C are diverse, including the development of genetically engineered virus-specific components. Recombinant viral proteins as components of subunit vaccines are safer than attenuated or inactivated whole-virion vaccines; however, they elicit a weak immune response and require the addition of adjuvants to increase their efficacy. Licensed, commonly used adjuvants based on aluminum salts or oil-in-water emulsions mainly induce Th2-type immune responses, causing increased antibody production [8]. However, not only a humoral, but also a cell-mediated immune response is critical in most viral infections. In addition to neutralizing antibodies against surface proteins E1 and E2, the effective vaccine against hepatitis C must induce a robust, multi-epitope, and functional Th1 immune response targeting relatively conserved nonstructural proteins NS3, NS4A/B, NS5A, and NS5B of HCV [9]. The antigenic and immunogenic properties of individual HCV nonstructural proteins are poorly studied, though they are essential for development of specific vaccine components.

Pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) play a key role in the activation of the immune response. There are several families of PRRs, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Unlike TLRs – transmembrane receptors located on plasma membranes, NLRs are intracellular PRRs, thus providing the ability to recognize viral nucleic acids throughout the viral life cycle [10]. TLR4 interacts with lipopolysaccharides (LPS) of the bacterial cell wall and induces a strong immune response to antigens of different nature [11]. In Russia, the approved pharmaceutical product manufactured by the Medgamal Branch of the Gamaleya National Research Center of Epidemiology and Microbiology of the Ministry of Health of the Russian Federation is the TLR4 ligand Pyrogenal (PG) – LPS isolated from *Salmonella typhi* cells. NLRs recognize bacterial peptidoglycans and viral RNAs, including HCV genomic RNA, in the cytoplasm of an infected cell [12]. Muramyl dipeptide (MDP), the minimum structural unit, is responsible for the immunological activity of peptidoglycans. In addition to MDP, there are other NLR agonists, including different synthetic and compound MDP derivatives targeting the nucleotide-binding oligomerization domain containing either protein 1 (NOD1) or protein 2 (NOD2). One of the new products – Polymuramyl (PM) manufactured by the Combiotech Research and Production Company (Russia) – contains three fragments of cell wall peptidoglycan of *S. typhi*, thus having an advantage over other NLR agonists through the interaction with two receptors – NOD1 and NOD2 [13]. Different *in vitro* and *in vivo* models demonstrate that the interaction between TLR4 and NOD1 or NOD2 receptors can have a synergistic effect and trigger an immune response [14]. Based on the available data, PRRs can be seen as prospective therapeutic targets in viral infections and as vaccine adjuvants [15].

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The effects of combined agonists of TLR4 and NOD1 + NOD2 receptors on immunization with individual recombinant HCV nonstructural proteins have not been studied. At the same time, the above data suggest that the interaction of receptors leading to induction of immune processes can increase *in vivo* the immune response against HVC antigens.

The **purpose of the study** is to assess the ability of the available drug products PM and PG to enhance humoral and cell-mediated responses when used individually or in combination for immunization of mice of various inbred strains with recombinant HCV proteins NS3, NS4A/B, and NS5B to evaluate their potential application as a compound adjuvant for HCV vaccines.

Materials and methods

Recombinant proteins NS3 (1356–1459 aa), NS4 (1677–1754 aa), and NS5B (2420–2990 aa), HCV genotype 1b, were used as immunogens for immunization of mice, as antigens for *in vitro* activation of T-cell responses and as sorbents in the enzyme-linked immunosorbent assay (ELISA) to assess the production of antibodies. The proteins were purified using affinity chromatography; their properties were described earlier [16–18]. Protein concentrations were measured using Bradford assay (Merck Sigma-Aldrich, United States).

Drug products. Two immunomodulatory drugs were used as prospective adjuvants: PM – a highly purified mixture of three active components extracted from the cell wall peptidoglycan of *S. typhi* gram-negative bacteria (200 µg/0.5 ml, series 011-0219); PG – lipopolysaccharide extracted from *S. typhi* bacteria (100 µg/ml, series 164). The drugs were bought in a pharmacy chain store.

Mice. Female mice of three inbred mouse strains BALB/c (H-2d), DBA/2J (H-2d), and C57BL/6 (H-2b) (age 6–8 weeks, average weight 20±3 g; from the Stolbovaya nursery of laboratory animals) were used for *in vivo* tests.

Immunization of mice. The immune-stimulating activity of PM and PG was assessed using the model of recombinant HCV nonstructural proteins NS3, NS4, and NS5B during several series of tests. Proteins at a dose of 4 µg/mouse were injected subcutaneously into the root of the tail three times at 2-week intervals, without adjuvants, with saline or combined with PM and/or PG, as described in the "Results" section. The mice from control groups were inoculated with saline following the same schedule. Immune responses were assessed 9–13 days after the last immunization.

Assessment of the immune response. Humoral response. The activity of the interaction between antibodies to HCV proteins in the mouse sera and HCV antigens was measured using indirect ELISA. 96-well plates were sensitized with recombinant proteins diluted in phosphate-buffered saline at a concentration of 1 µg/ml. Mouse immunoglobulin IgG1 and IgG2a isotype antibodies conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, United States) were used as secondary antibodies; tetramethylbenzidine (Sigma, United States) was used as a peroxidase substrate; optical density was measured at 450 nm. In ELISA, the endpoint serum titer was defined as the highest serum dilution, at which the optical density value at A_{450} was 2 times greater than the value for the control sample (serum of non-immunized mice).

Antigen-specific T-cell responses *in vitro* were assessed by the lymphocyte transformation test (LTT) and by their secretion of the cytokine IFN-γ. To obtain lymphocytes, spleens were dissociated; cell suspensions were filtered through 100 µm cell strainers (BD Falcon Cell strainers), washed twice in the RPMI-1640 medium (PanEco, Russia). Lymphocytes were placed in 96-well cell culture plates, 5×10^6 cells per well in the RPMI-1640 medium containing 20% fetal bovine serum (Invitrogen, United States), 2 mM glutamine, 4.5 g/l glucose, 50 µg/ml gentamicin, 0.2 U/ml insulin. The cells were supplemented with stimulants – the respective recombinant proteins NS3, NS4, or NS5B at endpoint concentrations of 1 µg/ml – and were grown at 37 °C in 5% CO_2 for 5 days. Spleen cells of non-immunized mice, which were stimulated with the same antigens, and unstimulated spleen cells of immunized mice were used as negative controls. The LTT results were expressed as a stimulation index (SI) calculated as a ratio of the average number of lymphoblasts observed in the presence of stimulants to the average number of lymphoblasts in the absence of stimulants (wells with the medium).

Cytokine levels in cell culture supernatants obtained after the stimulation of lymphocytes were measured by ELISA using the Mouse IFN-γ ELISA development kit (HRP) (Mabtech, Sweden) in accordance with the manufacturer's instructions. Cytokine levels were quantified using calibration curves derived from standards specimens. The limit of detection of IFN-γ was 2 pg/ml.

The statistical analysis of the results was performed using Statistica 8 and GraphPadPrism 8 software. The data were presented as the mean (or the geometric mean for antibody titers) ± standard deviation (SD). Parametric quantitative data were compared using the t-test (Student's test) and non-parametric data were compared using the U-test (Mann-Whitney test). Differences between variables were considered statistically significant at $p < 0.05$. To assess the combined effect of two compounds on enhancement of the immune response in animals inoculated with recombinant HCV proteins, the synergy index (SI) was calculated by the formula:

$$SI = \text{Parameter (PM, PG)} / [\text{Parameter (PM)} + \text{Parameter (PG)}],$$

where Parameter (PM, PG) is the parameter of the immune response after PM and PG combined immunization; Parameter (PM) and Parameter (PG) are parameters of the immune response after immunization with PM and PG separately. The PM and PG interaction was considered synergistic at SI > 1 and $p < 0.05$ in the t-test.

The authors confirm the compliance with the institutional and national standards for the use of laboratory animals in accordance with the Consensus Author Guidelines for Animal Use (IAVES, July 23, 2010). The proto-

col of the study was approved by the Ethics Committee of the Gamaleya National Research Center of Epidemiology and Microbiology of the Ministry of Health of the Russian Federation (Protocol No 10 dated June 7, 2021).

Results

1. *Effect of Polymuramyl on immune responses in mice of different inbred strains after immunization with HCV NS5B protein*

To assess the adjuvant properties of PM after immunization with the recombinant NS5B protein, we used 3 mouse strains – BALB/c, DBA/2J, and C57BL/6. The protein was injected with saline and with PM at a concentration of 20 μg/mouse three times at a two-week interval. The optimal dose of PM was defined in the previous tests through the comparative analysis of immune responses in mice to PM effects when administered at doses of 4, 20 and 100 μg/mouse. The immune responses were assessed individually for each mouse (8 animals in each group) 9 days after the last injection. The results are summarized in **Table 1**.

In the groups of DBA/2J and C57BL/6 mice, immunized without adjuvants, the titers of IgG1 antibodies to the NS5B protein reached 1 : 10^4 and did not increase after PM was added; in the BALB/c mice, the antibody activity was 50–100 times as low ($p < 0.05$, Table 1) and also did not change when PM was added. The levels of IgG2a antibodies were significantly lower compared to the IgG1 levels (approximately 1 : 200) in mice of all the tested strains and showed no changes associated with PM. The highest lymphocyte proliferation induced by the NS5B stimulation was demonstrated by the BALB/c mice; however, no statistically significant differences compared with other strains were found. The immunization with NS5B combined with PM resulted in a five-fold increase in SI in C57BL/6 mice ($p < 0.05$) but had no effect on proliferation in other groups. The IFN-γ production induced by lymphocyte stimulation by NS5B was 20–50 times as high in the BALB/c and DBA/2J mice immunized with the protein without the adjuvant compared to the IFN-γ levels in C57BL/6 mice ($p < 0.05$); the use of PM in this group increased the secretion of cytokine 20 times. Thus, when the NS5B protein was used without the adjuvant, the activity of IgG1 antibodies in the BALB/c mice was significantly lower and the IFN-γ secretion was significantly higher than the respective activity and secretion in the C57BL/6 mice. The adjuvant properties of PM were observed only in the C57BL/6 mice, which demonstrated a significant increase only in the cell-mediated immune response. Further tests were performed using mice of the above strain.

In the control groups, the mice did not have any immune response to NS5B; all the variables remained at baseline levels (the results are not shown).

2. *Effect of Pyrogenal on immune responses in C57Bl/6 mice immunized with HCV NS5B protein*

The immune-stimulating ability of PG was assessed in C57BL/6 mice immunized with the NS5B protein. The protein was injected either without the adjuvant (saline) or with PG at doses of 1, 5, or 15 μg/mouse (8 animals in each group). The titers of NS5B-specific antibodies and IFN-γ production induced by the antigen-specific stimulation were compared **(Fig. 1)**.

It was found that PG increased the production of IgG1 antibodies to NS5B; the highest increase in production of antibodies resulted from the dose of 15 μg/mouse; the antibody titers increased 12 times (Fig. 1 *a*). The IFN-γ production by splenocytes demonstrated comparable results: PG significantly increased the IFN-γ secretion at all the doses; the highest levels of cytokines were observed at a dose of 15 μg/mouse – a 150-fold increase (Fig. 1 *b*).

Based on the obtained results, the dose of 15 μg/mouse or 0.75 mg/kg body weight was selected for PG used as an adjuvant for immunization of animals with recombinant HCV proteins.

Table 1. Immune response of mice of different lines to immunization with recombinant HCV NS5B protein with polymuramil

Таблица 1. Иммунный ответ мышей разных линий на иммунизацию рекомбинантным белком NS5B ВГС с полимурамилом

Parameter Показатель		BALB/c mice Мыши линии BALB/c		DBA/2J mice Мыши линии DBA/2J		C57BL/6 mice Мыши линии C57BL/6	
		saline ф/р	PM ПМ	saline ф/р	PM ПМ	saline ф/р	PM ПМ
Titer of antibodies to NS5B Титр антител к NS5B	IgG1	132 ± 80*	200 ± 66	10125 ± 3200*	9040 ± 2560	8226 ± 1850*	9979 ± 2340
	IgG2a	229 ± 78	174 ± 73	250 ± 100	720 ± 400	200 ± 56	150 ± 78
Lymphocyte proliferation (SI) Пролиферация лимфоцитов (ИСП)		3,0 ± 0,7	2,6 ± 0,5	1,8 ± 0,4	1,6 ± 0,3	2,0 ± 0,4**	10,1 ± 2,7**
IFN-γ secretion (pg/mL) Секреция IFN-γ (пг/мл)		1580 ± 223*	1700 ± 294	725 ± 120*	920 ± 185	34 ± 15*, **	781 ± 154**

Note. Saline – immunization with NS5B without adjuvant; PM – immunization with NS5B in a mixture with polymuramyl at a dose of 20 μg/mouse; SI – stimulation index; *$p < 0.05$ between mouse lines; **$p < 0.05$ between parameters in groups immunized with saline and with PM.

Примечание. ф/р – иммунизация NS5B без адъюванта; ПМ – иммунизация NS5B в смеси с полимурамилом в дозе 20 мкг/мышь; ИСП – индекс стимуляции пролиферации; *$p < 0{,}05$ между линиями мышей; **$p < 0{,}05$ между показателями в группах, иммунизированных с ф/р и с ПМ.

3. *Immune responses to recombinant HCV proteins NS3 and NS4 in mice immunized with Polymuramyl combined with Pyrogenal*

It was important to check whether PM and PG have immune-stimulating properties when mice are inoculated with other recombinant HCV proteins. The C57BL/6 mice were immunized three times subcutaneously with HCV NS3 and NS4 proteins (4 µg/mouse) combined with PM (20 µg/mouse), PG (15 µg/mouse), and both of them. The results were compared with the results in the group inoculated with the above proteins with saline.

In the mice immunized with the NS3 protein, this protein induced IgG1 titers that were 20 times as high as the titers induced by the NS5B protein when used without adjuvants (approximately 1 : 1.6×10^5, $p < 0.05$, Table 1 and **Fig. 2** *a*). PM and, especially, PG increased the activity of IgG1 antibodies ($p < 0.05$). IgG2a antibodies to NS3 demonstrated lower titers compared to IgG1 and increased when PG was used ($p < 0.05$, Fig. 2 *b*). Neither PM nor PG had any effect on cell-mediated responses (Fig. 2 *c*, *d*). When combined, PM and PG significantly increased humoral and cell-mediated responses to the NS3 protein ($p < 0.05$, Fig. 2 *a*–*d*).

The NS4 protein model demonstrates that IgG1 antibody titers were 3 times as high compared to those observed with NS3 and 50 times as high compared to those observed with NS5B (1 : 4.5×10^5, $p < 0.05$, Table 1, Fig. 2 *a* and **Fig. 3** *a*). PM had no effect on the immune response, while PG enhanced the cell-mediated response (Fig. 3 *c*, *d*). The immunization with NS4 combined with PM and PG significantly increased IgG2a antibody titers, proliferation of lymphocytes, and their IFN-γ secretion induced by the *in vitro* antigen-specific stimulation (Fig. 3 *b*–*d*).

The synergy index (SI) was calculated for assessment of the combined effect of two compounds on the immune response in the animals inoculated with recombinant HCV proteins. The analysis of the results presented in **Table 2** shows that in most cases, the enhanced immune response to recombinant HCV proteins is induced by the PM and PG synergy: Their combined effect is significantly greater than the effect of each of them separately.

Discussion

To study adjuvant properties of PM and PG, we used HCV nonstructural proteins NS3, NS4A/B, and NS5B as antigens. The analysis of the role these proteins have in the HCV life cycle has shown that nonstructural proteins form a replication complex and are required for viral genome amplification [19]. NS3 contains protease and helicase domains responsible for HCV polyprotein processing and unwinding of RNA secondary structures, respectively. NS4A is an NS3 protease cofactor; NS4B induces the formation of vesicles, which serve as replication organelles; NS5B functions as an RNA-dependent RNA polymerase. Clearly, HCV nonstructural proteins are attractive components for vaccine development. In the meantime, the antigenic and immunogenic properties of nonstructural proteins have been insufficiently studied. Earlier, we found that the immunization of C57Bl/6 mice with the NS5B recombinant protein without an adjuvant induced weak T-cell and B-cell responses [20]. These findings were confirmed by the comparative analysis of responses in the C57Bl/6 mice to recombinant proteins NS3 and NS4. It was interesting to assess the effect PM and PG on immunogenic properties of this protein – the key component of the replication complex. It has been found that this protein can activate one of NLRs – NOD1 [12]. We have found that the low level of the T-cell response to NS5B used without adjuvants is increased significantly when PM is used; however, the level of anti-NS5B antibodies remains unchanged (Table 1). When PG

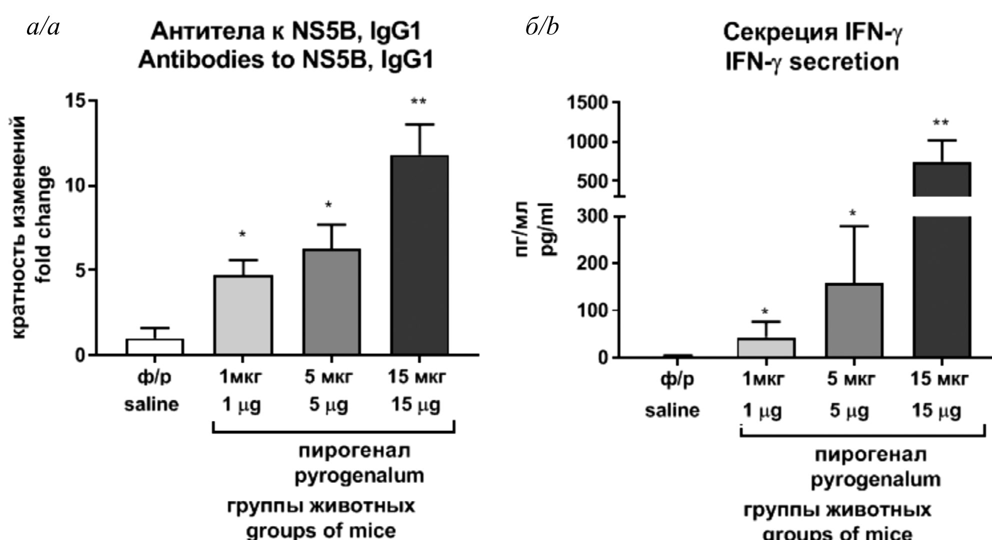

Fig. 1. Humoral (*a*) and cellular (*b*) response of C57BL/6 mice to recombinant HCV NS5B protein injected with pyrogenalum.
*$p < 0.05$ compared to the group to which NS5B was administered with saline solution (saline); **$p < 0.05$ compared to other groups of mice.

Рис. 1. Гуморальный (*а*) и клеточный (*б*) ответ мышей C57BL/6 на рекомбинантный белок NS5B ВГС, введенный с пирогеналом.
*$p < 0,05$ по сравнению с группой мышей, иммунизированных с ф/р; **$p < 0,05$ по сравнению с остальными группами мышей.

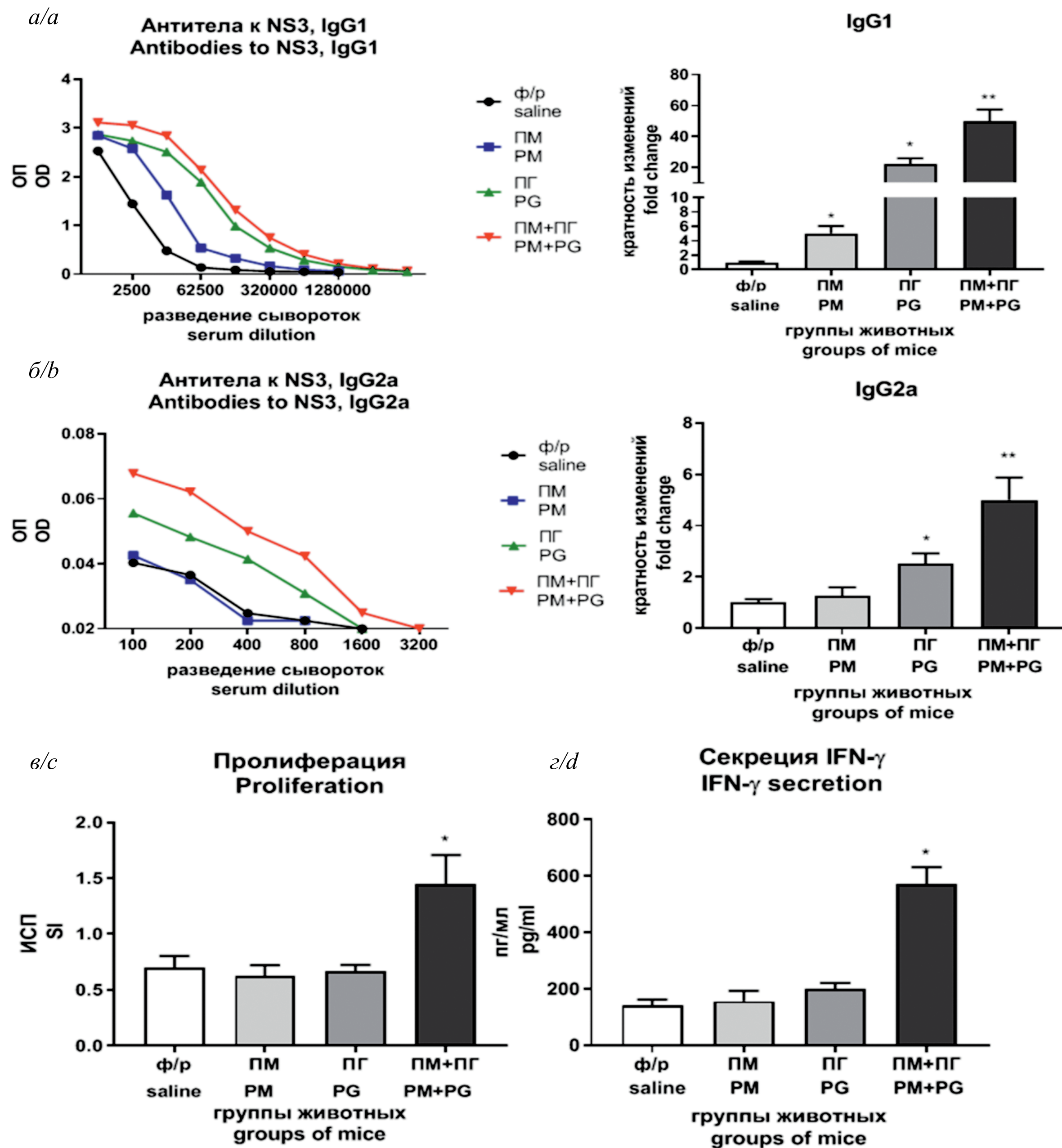

Fig. 2. Enhancement of the humoral (*a*, *b*) and cellular (*c*, *d*) response of mice to the recombinant HCV NS3 protein with the combined use of polymuramyl and pyrogenalum as adjuvants.

OD – optical density at 450 nm; SI – stimulation index. $*p < 0.05$ compared to the group to which NS3 was administered with saline solution (saline). $**p < 0.05$ compared to other groups of mice.

Рис. 2. Усиление гуморального (*а*, *б*) и клеточного (*в*, *г*) ответа мышей на рекомбинантный белок NS3 ВГС при сочетанном использовании полимурамила и пирогенала в качестве адъювантов.

ОП – оптическая плотность при 450 нм; ИСП – индекс стимуляции пролиферации. $*p < 0,05$ по сравнению с группой мышей, иммунизированных с ф/р; $**p < 0,05$ по сравнению с остальными группами мышей.

was used for immunization of C57Bl/6 mice, it increased the levels of both immunity components: The activity of antibodies to NS5B increased 12 times and the IFN-γ secretion levels increased more than 150 times (Fig. 1).

When immunized with the NS5B protein, the mice of other two frequently used strains BALB/c and DBA/2J demonstrated that PM had no adjuvant effect on any of the measured immunity components in these mice. It should be noted that the responses to the recombinant NS5B protein without the adjuvant differed significantly in mice of different strains. For example, in the BALB/c and DBA/2J mice, the IFN-γ secretion levels were on average 30 times as high as those in the C57Bl/6 mice (Table 1). The anti-NS5B IgG1 antibody levels, on the contrary, were 62 times as low in the BALB/c (H-2d) mice compared to the levels in the C57Bl/6 (H-2b) mice

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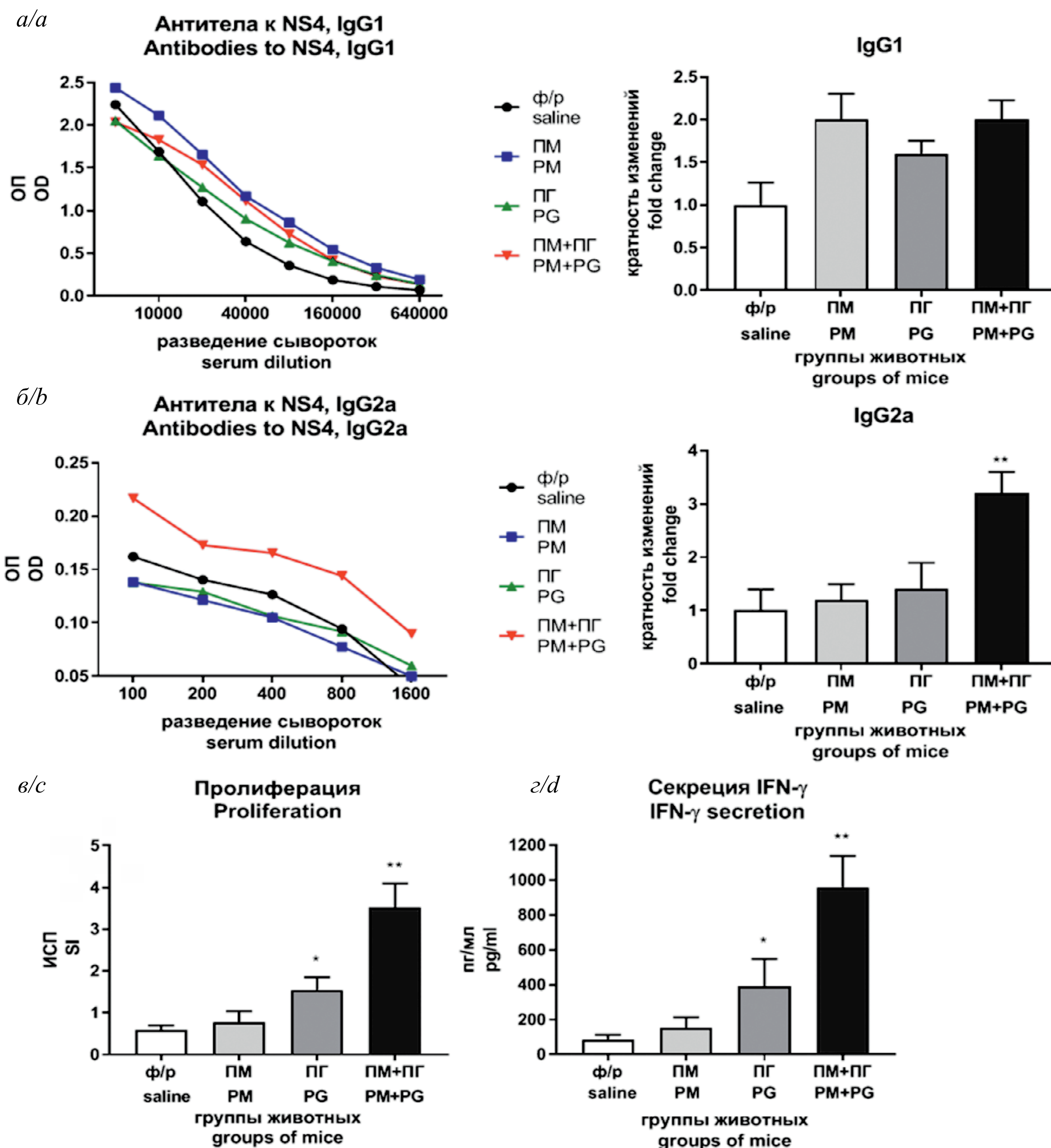

Fig. 3. Enhancement of the humoral (*a*, *b*) and cellular (*c*, *d*) response of mice to the recombinant HCV NS4 protein with the combined use of polymuramyl and pyrogenalum as adjuvants.

OD – optical density at 450 nm; SI – stimulation index. $*p < 0.05$ compared to the group to which NS4 was administered with saline solution (saline); $**p < 0.05$ compared to other groups of mice.

Рис. 3. Усиление гуморального (*а*, *б*) и клеточного (*в*, *г*) ответа мышей на рекомбинантный белок NS4 ВГС при сочетанном использовании полимурамила и пирогенала в качестве адъювантов.

ОП – оптическая плотность при 450 нм; ИСП – индекс стимуляции пролиферации. $*p < 0,05$ по сравнению с группой мышей, иммунизированных с ф/р; $**p < 0,05$ по сравнению с остальными группами мышей.

and 77 times as low as the levels in the DBA/2J mice belonging to the same haplotype (H-2d).

The difference in immune responses in mice of different inbred strains was observed for various bacterial and viral infections. Many researchers note that BALB/c or DBA mice are more susceptible to bacterial infections compared to C57BL/6 mice, for example, to infections with *Listeria monocytogenes* [21] or with *Mycoplasma pulmonis* [22]. On the other hand, C57BL/6 mice were more susceptible to the infection with *S. enterica Serovar Typhimurium*, while BALB/c mice demonstrated higher resistance to this bacterial infection [23]. Responses to viral infections also differed in laboratory mice of different strains. For example, DBA/2J mice were 1,000 times more susceptible to the A/Puerto Rico/8/1934 influenza virus than C57BL/6J mice [24, 25]. Very similar results were obtained by comparing immune responses in BALB/c and C57BL/6J mice to the respiratory syncytial

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Table 2. Synergistic enhancement of the immune response to recombinant HCV proteins with the combined action of polymuramyl and pyrogenalum

Таблица 2. Синергетическое усиление иммунного ответа на рекомбинантные белки ВГС при сочетанном действии полимурамила и пирогенала

HCV protein for mice immunization Белок ВГС для иммунизации мышей	Параметры иммунного ответа Parameters of the immune response			
	lymphocyte proliferation пролиферация лимфоцитов	IFN-γ secretion секреция IFN-γ	titer of antibodies, IgG1 титр антител изотипа IgG1	titer of antibodies, IgG2a титр антител изотипа IgG2a
NS3	1,12 ± 0,5[a]	1,62 ± 0,51*	1,85 ± 0,69*	1,33 ± 0,21*
NS4	1,38 ± 0,28*	1,75 ± 0,63*	0,60 ± 0,41	1,39 ± 0,27*

Note: [a] – values of the combination indices (M ± SD); * – difference from 1, $p < 0.05$ (for explanations see Materials and methods).

Примечание: [a] – индексы синергизма (M ± SD); * – отличие от 1, $p < 0,05$ (объяснения см. в разделе «Материалы и методы»).

virus [26], inactivated dengue virus [27], and adeno-associated virus type 6 (AAV6) containing the *ACE2* gene encoding the cell receptor of SARS-CoV-2 [28]. Interestingly, differences in immune responses were also demonstrated by BALB/c and DBA/2J mice belonging to the same haplotype 2d. For example, when infected with *M. pneumonia*, DBA/2 mice had a significantly more pronounced cell-mediated response than BALB/c mice [29]. The cells isolated from the lymph nodes of DBA/2 mice immunized with the KLH model antigen produced up to 30 times as many interleukins (IL) 12 as the cells in BALB/c mice, which were restimulated *in vitro* following the same procedure [30].

Differences in immune responses in mice of different strains are explained by the direction of T-cell responses in BALB/c mice biased towards the Th2-type, while C57BL/6 mice are biased towards the Th1-type [31, 32]. In the first case, primarily genes of anti-inflammatory cytokines (IL-4, IL-10, and IL-13) are induced, while genes of pro-inflammatory cytokines (TNF-α and IFN-γ) are induced in the second case. Different cytokine profiles are important determinants of susceptibility or resistance to infections or vaccines. The vaccine efficacy also depends on the subtype of induced antibodies, as IgG subclasses differ in their biological function. At the same time, mice of different strains can produce different levels and types of antibodies. This conclusion was made by the authors who used 28 species of inbred and non-inbred “wild-type” mice for immunization with the acellular pertussis vaccine [33]. Genetic differences in mice significantly contributed to the magnitude and duration of activity of antibodies of the IgG1, IgG2b, and IgG3 subtypes induced by the vaccine. Analyzing the factors contributing to immune heterogeneity, we can state that the exact genetic mechanism that governs immune responses and susceptibility to infections in different mouse strains remains essentially unclear. The results obtained in our studies and in the studies of other researchers highlight the need to assess the contribution of genetic factors both for selection of the optimum animal model and for evaluation of the effectiveness of a vaccine using different inbred or non-inbred mice.

The parameters of the innate immune response to infections largely depend on PRR interactions. The well-known synergistic interaction is the interaction between TLRs and members of the NOD-like receptor family (NOD1 and NOD2), which recognize certain fragments of bacterial peptidoglycan: NOD1 – γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP), NOD2 – L-Ala-D-isoGln [34, 35]. Synergistic interactions between NODs and TLRs can be used therapeutically to increase the host resistance to pathogens. For example, the combination of NOD1 and TLR5 agonists provided 80% resistance to the lethal dose of *S. typhimurium*, while each individual component did not exhibit any protective properties [36]. In another publication by the above authors, it was shown that the combined stimulation of PRRs from different families (NOD2 and TLR4) caused a stronger activation of the adaptive immunity compared to the stimulation of each individual PRR. The immunization with the model antigen ovalbumin combined with the TLR4 agonist (MPLA) and NOD2 agonist (MDP) synergistically enhanced humoral and cell-mediated immune responses [37]. In the mice immunized with the influenza virus antigen combined with TLR4 and NOD2 agonists, the adjuvant effect resulted in enhanced immunogenicity and protection of animals against the lethal viral infection [38].

At the same time, it has been found that combined effects of agonists of the TLR and NOD families can produce not only synergistic, but also additive induction of immune responses (for example, in macrophages *in vitro*), and under certain conditions, they can even suppress each other’s immune response [14, 39]. Therefore, combined effects of adjuvant candidates should be thoroughly assessed when developing vaccines against a specific pathogen, especially since the mechanisms of synergistic NOD-TLR interactions are still not completely understood. Researchers are discussing possible mechanisms of synergy [40]. One of the hypotheses focuses on PRR signaling pathways. Assumedly, synergy between PRR pairs occurs if the two receptors first use different proximal adapters and then, this time jointly, use distal parts of molecular pathways [41]. TLR4 and NOD1 agonists transmit signals in different ways: Rip2 for the NOD1 and NOD2 signaling pathway [42], MyD88 and TRIF for TLR4 [43]. Potentially, when combined, NOD1 and TLR4 agonists can enhance the expression of a wider set of genes, which mutually contribute to the transmission of signals. In their study, the researchers [14] thoroughly explored the stimulation of cells by NOD1 and TLR4 agonists in dynamics using

macrophages. The study of signaling pathways showed the complexity of the response of human macrophages to a combination of NOD1 and TLR4 agonists. It was found that synergy between the two receptors does not occur until the activation signal reaches the nucleus. However, synergistic activation of NOD1- and TLR4-induced genes is observed at the signal exit from the nucleus. The analysis of the results leads to the conclusion that the synergy of receptor agonists belonging to different families results from multiple processes that are still not fully understood. Thorough understanding of molecular pathways and gene interactions in the regulation of synergy is essential for creating highly effective compound adjuvants and development of subunit vaccines, which would be comparable with attenuated or inactivated whole-virion vaccines in terms of the induced immune response but would demonstrate higher levels of safety.

Conclusions

The combined use of recombinant nonstructural NS3, NS4, and NS5B proteins of the HCV replication complex is advisable for subunit vaccines, considering their antigenic and immunogenic properties as well as their different functions in virus replication.

Recombinant HCV nonstructural proteins demonstrate different immunogenicity when injected into mice of different inbred strains (DBA/2J, BALB/c, C57Bl/6) both in those being different by antigens of the major histocompatibility complex (H2b and H2d haplotypes) and in those belonging to the same haplotype. Therefore, animals of different inbred strains or non-inbred animals should be used for evaluation of the effectiveness of subunit vaccines.

The available Russian drugs PG and PM have a synergistic effect on the B-cell and T-cell-mediated immune response after immunization with nonstructural HCV proteins and open new opportunities for creating a compound adjuvant as a component of a subunit vaccine candidate against hepatitis C.

REFERENCES

1. Park S.H., Rehermann B. Immune responses to HCV and other hepatitis viruses. *Immunity*. 2014; 40(1): 13–24. https://doi.org/10.1016/j.immuni.2013.12.010
2. Dustin L.B. Innate and adaptive immune responses in chronic HCV infection. *Curr. Drug Targets*. 2017; 18(7): 826–43. https://doi.org/10.2174/1389450116666150825110532
3. Wang Y., Rao H., Chi X., Li B., Liu H., Wu L., et al. Detection of residual HCV-RNA in patients who have achieved sustained virological response is associated with persistent histological abnormality. *EBioMedicine*. 2019; 46: 227–35. https://doi.org/10.1016/j.ebiom.2019.07.043
4. Polyak S.J., Crispe I.N., Baumert T.F. Liver abnormalities after elimination of HCV infection: persistent epigenetic and immunological perturbations post-cure. *Pathogens*. 2021; 10(1): 44. https://doi.org/10.3390/pathogens10010044
5. Sun D., Dai M., Shen S., Li C., Yan X. Analysis of naturally occurring resistance-associated variants to NS3/4A protein inhibitors, NS5A protein inhibitors, and NS5B polymerase inhibitors in patients with chronic hepatitis C. *Gene Expr.* 2018; 18(1): 63–9. https://doi.org/10.3727/105221617X15100607143377
6. Ghweil A.A., Helal M.M. Reactivation of herpesvirus in patients with hepatitis C treated with direct-acting antiviral agents. *Infect. Drug Resist*. 2019; 12: 759–62. https://doi.org/10.2147/IDR.S184598
7. Todryk S.M., Bassendine M.F., Bridge S.H. Revisiting the elusive hepatitis C vaccine. *Vaccines (Basel)*. 2021; 9(2): 114. https://doi.org/10.3390/vaccines9020114
8. Kozak M., Hu J. The integrated consideration of vaccine platforms, adjuvants, and delivery routes for successful vaccine development. *Vaccines (Basel)*. 2023; 11(3): 695. https://doi.org/10.3390/vaccines11030695
9. Bailey J.R., Barnes E., Cox A.L. Approaches, progress, and challenges to hepatitis C vaccine development. *Gastroenterology*. 2019; 156(2): 418–30. https://doi.org/10.1053/j.gastro.2018.08.060
10. Carty M., Guy C., Bowie A.G. Detection of viral infections by innate immunity. *Biochem. Pharmacol*. 2021; 183: 114316. https://doi.org/10.1016/j.bcp.2020.114316
11. Caucheteux S.M., Hu-Li J., Mohammed R.N., Ager A., Paul W.E. Cytokine regulation of lung Th17 response to airway immunization using LPS adjuvant. *Mucosal Immunol*. 2017; 10(2): 361–72. https://doi.org/10.1038/mi.2016.54
12. Vegna S., Gregoire D., Moreau M., Lassus P., Durantel D., Assenat E., et al. NOD1 participates in the innate immune response triggered by hepatitis C virus polymerase. *J. Virol*. 2016; 90(13): 6022–35. https://doi.org/10.1128/JVI.03230-15
13. Guryanova S.V., Khaitov R.M. Strategies for using muramyl peptides – modulators of innate immunity of bacterial origin – in medicine. *Front. Immunol*. 2021; 12: 607178. https://doi.org/10.3389/fimmu.2021.607178
14. Budikhina A.S., Murugina N.E., Maximchik P.V., Dagil Y.A., Nikolaeva A.M., Balyasova L.S., et al. Interplay between NOD1 and TLR4 receptors in macrophages: nonsynergistic activation of signaling pathways results in synergistic induction of proinflammatory gene expression. *J. Immunol*. 2021; 206(9): 2206–20. https://doi.org/10.4049/jimmunol.2000692
15. Hennessy C., McKernan D.P. Anti-viral pattern recognition receptors as therapeutic targets. *Cells*. 2021; 10(9): 2258. https://doi.org/10.3390/cells10092258
16. Абдулмеджидова А.Г., Масалова О.В., Атанадзе С.Н., Уланова Т.И., Бурков А.Н., Худяков Ю.Е. и др. Характеристика панели моноклональных антител к рекомбинантному белку NS3 вируса гепатита С. *Вопросы вирусологии*. 2002; 47(1): 21–5.
17. Ivanov A.V., Korovina A.N., Tunitskaya V.L., Kostyuk D.A., Rechinsky V.O., Kukhanova M.K., et al. Development of the system ensuring a high-level expression of hepatitis C virus nonstructural NS5B and NS5A proteins. *Protein Expr. Purif.* 2006; 48(1): 14–23. https://doi.org/10.1016/j.pep.2006.02.011
18. Masalova O.V., Lakina E.I., Abdulmedzhidova A.G., Atanadze S.N., Semiletov Y.A., Shkurko T.V., et al. Characterization of monoclonal antibodies and epitope mapping of the NS4 protein of hepatitis C virus. *Immunol. Lett*. 2002; 83(3): 187–96. https://doi.org/10.1016/s0165-2478(02)00081-0
19. Li H.C., Yang C.H., Lo S.Y. Hepatitis C viral replication complex. *Viruses*. 2021; 13(3): 520. https://doi.org/10.3390/v13030520
20. Masalova O.V., Lesnova E.I., Andreev S.M., Shershakova N.N., Kozlov V.V., Permyakova K.Yu., et al. Adjuvant effect of dispersed fullerene C60 on the immune response to constructs harboring amino acid and nucleotide sequences of hepatitis C virus nonstructural NS5B protein. *Voprosy virusologii*. 2022; 67(6): 516–26. https://doi.org/10.36233/0507-4088-149 https://elibrary.ru/laaczg (in Russian)
21. Liu T., Matsuguchi T., Tsuboi N., Yajima T., Yoshikai Y. Differences in expression of toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. *Infect. Immun*. 2002; 70(12): 6638–45. https://doi.org/10.1128/IAI.70.12.6638-6645.2002
22. Boonyarattanasoonthorn T., Elewa Y.H.A., Tag-El-Din-Hassan H.T., Morimatsu M., Agui T. Profiling of cellular immune responses to Mycoplasma pulmonis infection in C57BL/6 and DBA/2 mice. *Infect. Genet. Evol.* 2019; 73: 55–65. https://doi.org/10.1016/j.meegid.2019.04.019
23. Huang H., Even Z., Wang Z., Li L., Erickson A., Golovko M., et al. Proteomics profiling reveals regulation of immune response to Salmonella enterica serovar Typhimurium infection in mice. *Infect. Immun*. 2023; 91(1): e0049922. https://doi.org/10.1128/iai.00499-22
24. Casanova T., Van de Paar E., Desmecht D., Garigliany M.M. Hyporeactivity of alveolar macrophages and higher respiratory cell permissivity characterize DBA/2J mice infected by influenza A virus. *J. Interferon Cytokine Res*. 2015; 35(10): 808–20. https://doi.org/10.1089/jir.2014.0237
25. Srivastava B., Błazejewska P., Hessmann M., Bruder D., Geffers R., Mauel S., et al. Host genetic background strongly influences the response to influenza a virus infections. *PLoS One*. 2009; 4(3): e4857. https://doi.org/10.1371/journal.pone.0004857
26. Marzec J., Cho H.Y., High M., McCaw Z.R., Polack F., Kleeberger S.R. Toll-like receptor 4-mediated respiratory syncytial virus disease and lung transcriptomics in differentially susceptible inbred mouse strains. *Physiol. Genomics*. 2019; 51(12): 630–43. https://doi.org/10.1152/physiolgenomics.00101.2019

27. Leclerc C., Dériaud E., Megret F., Briand J.P., Van Regenmortel M.H., Deubel V. Identification of helper T cell epitopes of dengue virus E-protein. *Mol. Immunol*. 1993; 30(7): 613–25. https://doi.org/10.1016/0161-5890(93)90072-j
28. Tailor N., Warner B.M., Griffin B.D., Tierney K., Moffat E., Frost K., et al. Generation and characterization of a SARS-CoV-2-susceptible mouse model using adeno-associated virus (AAV6.2FF)-mediated respiratory delivery of the human ACE2 gene. *Viruses*. 2022; 15(1): 85. https://doi.org/10.3390/v15010085
29. Tamiya S., Yoshikawa E., Suzuki K., Yoshioka Y. Susceptibility analysis in several mouse strains reveals robust T-cell responses after mycoplasma pneumoniae infection in DBA/2 mice. *Front Cell Infect. Microbiol*. 2021; 10: 602453. https://doi.org/10.3389/fcimb.2020.602453
30. Gieni R.S., Fang Y., Trinchieri G., Umetsu D.T., DeKruyff R.H. Differential production of IL-12 in BALB/c and DBA/2 mice controls IL-4 versus IFN-gamma synthesis in primed CD4 lymphocytes. *Int. Immunol*. 1996; 8(10): 1511–20. https://doi.org/10.1093/intimm/8.10.1511
31. Saha P., Xiao X., Li Y., Golonka R.M., Abokor A.A., Yeoh B.S., et al. Distinct iron homeostasis in C57BL/6 and Balb/c mouse strains. *Physiol. Rep.* 2020; 8(9): e14441. https://doi.org/10.14814/phy2.14441
32. Watanabe H., Numata K., Ito T., Takagi K., Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock*. 2004; 22(5): 460–6. https://doi.org/10.1097/01.shk.0000142249.08135.e9
33. Mosley Y.C., Radder J.E., HogenEsch H. Genetic variation in the magnitude and longevity of the IgG subclass response to a diphtheria-tetanus-acellular pertussis (DTaP) vaccine in mice. *Vaccines (Basel)*. 2019; 7(4): 124. https://doi.org/10.3390/vaccines7040124
34. Girardin S.E., Boneca I.G., Viala J., Chamaillard M., Labigne A., Thomas G., et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem*. 2003; 278(11): 8869–72. https://doi.org/10.1074/jbc.C200651200
35. Girardin S.E., Boneca I.G., Carneiro L.A., Antignac A., Jéhanno M., Viala J., et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science*. 2003; 300(5625): 1584–7. https://doi.org/10.1126/science.1084677
36. Tukhvatulin A.I., Gitlin I.I., Shcheblyakov D.V., Artemicheva N.M., Burdelya L.G., Shmarov M.M., et al. Combined stimulation of Toll-like receptor 5 and NOD1 strongly potentiates activity of NF-κB, resulting in enhanced innate immune reactions and resistance to Salmonella enterica serovar Typhimurium infection. *Infect. Immun*. 2013; 81(10): 3855–64. https://doi.org/10.1128/IAI.00525-13
37. Tukhvatulin A.I., Dzharullaeva A.S., Tukhvatulina N.M., Shcheblyakov D.V., Shmarov M.M., Dolzhikova I.V., et al. Powerful complex immunoadjuvant based on synergistic effect of combined TLR4 and NOD2 activation significantly enhances magnitude of humoral and cellular adaptive immune responses. *PLoS One*. 2016; 11(5): e0155650. https://doi.org/10.1371/journal.pone.0155650
38. Tukhvatulin A., Dzharullaeva A., Erokhova A., Zemskaya A., Balyasin M., Ozharovskaia T., et al. Adjuvantation of an influenza hemagglutinin antigen with TLR4 and NOD2 agonists encapsulated in poly(D,L-Lactide-Co-Glycolide) nanoparticles enhances immunogenicity and protection against lethal influenza virus infection in mice. *Vaccines (Basel)*. 2020; 8(3): 519. https://doi.org/10.3390/vaccines8030519
39. Hedl M., Li J., Cho J.H., Abraham C. Chronic stimulation of Nod2 mediates tolerance to bacterial products. *Proc. Natl Acad. Sci. USA*. 2007; 104(49): 19440–5. https://doi.org/10.1073/pnas.0706097104
40. Pashenkov M.V., Murugina N.E., Budikhina A.S., Pinegin B.V. Synergistic interactions between NOD receptors and TLRs: Mechanisms and clinical implications. *J. Leukoc. Biol.* 2019; 105(4): 669–80. https://doi.org/10.1002/JLB.2RU0718-290R
41. Bagchi A., Herrup E.A., Warren H.S., Trigilio J., Shin H.S., Valentine C., et al. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J. Immunol*. 2007; 178(2): 1164–71. https://doi.org/10.4049/jimmunol.178.2.1164
42. Magalhaes J.G., Lee J., Geddes K., Rubino S., Philpott D.J., Girardin S.E. Essential role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands. *Eur. J. Immunol*. 2011; 41(5): 1445–55. https://doi.org/10.1002/eji.201040827
43. Owen A.M., Luan L., Burelbach K.R., McBride M.A., Stothers C.L., Boykin O.A., et al. MyD88-dependent signaling drives toll-like receptor-induced trained immunity in macrophages. *Front. Immunol*. 2022; 13: 1044662. https://doi.org/10.3389/fimmu.2022.1044662

ЛИТЕРАТУРА

1. Park S.H., Rehermann B. Immune responses to HCV and other hepatitis viruses. *Immunity*. 2014; 40(1): 13–24. https://doi.org/10.1016/j.immuni.2013.12.010
2. Dustin L.B. Innate and adaptive immune responses in chronic HCV infection. *Curr. Drug Targets*. 2017; 18(7): 826–43. https://doi.org/10.2174/1389450116666150825110532
3. Wang Y., Rao H., Chi X., Li B., Liu H., Wu L., et al. Detection of residual HCV-RNA in patients who have achieved sustained virological response is associated with persistent histological abnormality. *EBioMedicine*. 2019; 46: 227–35. https://doi.org/10.1016/j.ebiom.2019.07.043
4. Polyak S.J., Crispe I.N., Baumert T.F. Liver abnormalities after elimination of HCV infection: persistent epigenetic and immunological perturbations post-cure. *Pathogens*. 2021; 10(1): 44. https://doi.org/10.3390/pathogens10010044
5. Sun D., Dai M., Shen S., Li C., Yan X. Analysis of naturally occurring resistance-associated variants to NS3/4A protein inhibitors, NS5A protein inhibitors, and NS5B polymerase inhibitors in patients with chronic hepatitis C. *Gene Expr.* 2018; 18(1): 63–9. https://doi.org/10.3727/105221617X15100607143377
6. Ghweil A.A., Helal M.M. Reactivation of herpesvirus in patients with hepatitis C treated with direct-acting antiviral agents. *Infect. Drug Resist*. 2019; 12: 759–62. https://doi.org/10.2147/IDR.S184598
7. Todryk S.M., Bassendine M.F., Bridge S.H. Revisiting the elusive hepatitis C vaccine. *Vaccines (Basel)*. 2021; 9(2): 114. https://doi.org/10.3390/vaccines9020114
8. Kozak M., Hu J. The integrated consideration of vaccine platforms, adjuvants, and delivery routes for successful vaccine development. *Vaccines (Basel)*. 2023; 11(3): 695. https://doi.org/10.3390/vaccines11030695
9. Bailey J.R., Barnes E., Cox A.L. Approaches, progress, and challenges to hepatitis C vaccine development. *Gastroenterology*. 2019; 156(2): 418–30. https://doi.org/10.1053/j.gastro.2018.08.060
10. Carty M., Guy C., Bowie A.G. Detection of viral infections by innate immunity. *Biochem. Pharmacol*. 2021; 183: 114316. https://doi.org/10.1016/j.bcp.2020.114316
11. Caucheteux S.M., Hu-Li J., Mohammed R.N., Ager A., Paul W.E. Cytokine regulation of lung Th17 response to airway immunization using LPS adjuvant. *Mucosal Immunol*. 2017; 10(2): 361–72. https://doi.org/10.1038/mi.2016.54
12. Vegna S., Gregoire D., Moreau M., Lassus P., Durantel D., Assenat E., et al. NOD1 participates in the innate immune response triggered by hepatitis C virus polymerase. *J. Virol*. 2016; 90(13): 6022–35. https://doi.org/10.1128/JVI.03230-15
13. Guryanova S.V., Khaitov R.M. Strategies for using muramyl peptides – modulators of innate immunity of bacterial origin – in medicine. *Front. Immunol*. 2021; 12: 607178. https://doi.org/10.3389/fimmu.2021.607178
14. Budikhina A.S., Murugina N.E., Maximchik P.V., Dagil Y.A., Nikolaeva A.M., Balyasova L.S., et al. Interplay between NOD1 and TLR4 receptors in macrophages: nonsynergistic activation of signaling pathways results in synergistic induction of proinflammatory gene expression. *J. Immunol*. 2021; 206(9): 2206–20. https://doi.org/10.4049/jimmunol.2000692
15. Hennessy C., McKernan D.P. Anti-viral pattern recognition receptors as therapeutic targets. *Cells*. 2021; 10(9): 2258. https://doi.org/10.3390/cells10092258
16. Абдулмеджидова А.Г., Масалова О.В., Атанадзе С.Н., Уланова Т.И., Бурков А.Н., Худяков Ю.Е. и др. Характеристика панели моноклональных антител к рекомбинантному белку NS3 вируса гепатита С. *Вопросы вирусологии*. 2002; 47(1): 21–5.
17. Ivanov A.V., Korovina A.N., Tunitskaya V.L., Kostyuk D.A., Rechinsky V.O., Kukhanova M.K., et al. Development of the system ensuring a high-level expression of hepatitis C virus nonstructural NS5B and NS5A proteins. *Protein Expr. Purif*. 2006; 48(1): 14–23. https://doi.org/10.1016/j.pep.2006.02.011
18. Masalova O.V., Lakina E.I., Abdulmedzhidova A.G., Atanadze S.N., Semiletov Y.A., Shkurko T.V., et al. Characterization of monoclonal antibodies and epitope mapping of the NS4 protein of hepatitis C virus. *Immunol. Lett*. 2002; 83(3): 187–96. https://doi.org/10.1016/s0165-2478(02)00081-0
19. Li H.C., Yang C.H., Lo S.Y. Hepatitis C viral replication complex. *Viruses*. 2021; 13(3): 520. https://doi.org/10.3390/v13030520
20. Масалова О.В., Леснова Е.И., Андреев С.М., Шершакова Н.Н., Козлов В.В., Пермякова К.Ю. и др. Адъювантное действие дисперсного фуллерена С60 на иммунный ответ против конструкций, имитирующих аминокислотные и нуклеотидные последовательности неструктурного белка NS5B вируса гепа-

тита С. *Вопросы вирусологии*. 2022; 67(6): 516–26. https://doi.org/10.36233/0507-4088-149 https://elibrary.ru/laaczg
21. Liu T., Matsuguchi T., Tsuboi N., Yajima T., Yoshikai Y. Differences in expression of toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. *Infect. Immun*. 2002; 70(12): 6638–45. https://doi.org/10.1128/IAI.70.12.6638-6645.2002
22. Boonyarattanasoonthorn T., Elewa Y.H.A., Tag-El-Din-Hassan H.T., Morimatsu M., Agui T. Profiling of cellular immune responses to Mycoplasma pulmonis infection in C57BL/6 and DBA/2 mice. *Infect. Genet. Evol.* 2019; 73: 55–65. https://doi.org/10.1016/j.meegid.2019.04.019
23. Huang H., Even Z., Wang Z., Li L., Erickson A., Golovko M., et al. Proteomics profiling reveals regulation of immune response to Salmonella enterica serovar Typhimurium infection in mice. *Infect. Immun*. 2023; 91(1): e0049922. https://doi.org/10.1128/iai.00499-22
24. Casanova T., Van de Paar E., Desmecht D., Garigliany M.M. Hyporeactivity of alveolar macrophages and higher respiratory cell permissivity characterize DBA/2J mice infected by influenza A virus. *J. Interferon Cytokine Res.* 2015; 35(10): 808–20. https://doi.org/10.1089/jir.2014.0237
25. Srivastava B., Błazejewska P., Hessmann M., Bruder D., Geffers R., Mauel S., et al. Host genetic background strongly influences the response to influenza a virus infections. *PLoS One*. 2009; 4(3): e4857. https://doi.org/10.1371/journal.pone.0004857
26. Marzec J., Cho H.Y., High M., McCaw Z.R., Polack F., Kleeberger S.R. Toll-like receptor 4-mediated respiratory syncytial virus disease and lung transcriptomics in differentially susceptible inbred mouse strains. *Physiol. Genomics*. 2019; 51(12): 630–43. https://doi.org/10.1152/physiolgenomics.00101.2019
27. Leclerc C., Dériaud E., Megret F., Briand J.P., Van Regenmortel M.H., Deubel V. Identification of helper T cell epitopes of dengue virus E-protein. *Mol. Immunol*. 1993; 30(7): 613–25. https://doi.org/10.1016/0161-5890(93)90072-j
28. Tailor N., Warner B.M., Griffin B.D., Tierney K., Moffat E., Frost K., et al. Generation and characterization of a SARS-CoV-2-susceptible mouse model using adeno-associated virus (AAV6.2FF)-mediated respiratory delivery of the human ACE2 gene. *Viruses*. 2022; 15(1): 85. https://doi.org/10.3390/v15010085
29. Tamiya S., Yoshikawa E., Suzuki K., Yoshioka Y. Susceptibility analysis in several mouse strains reveals robust T-cell responses after mycoplasma pneumoniae infection in DBA/2 mice. *Front Cell Infect. Microbiol*. 2021; 10: 602453. https://doi.org/10.3389/fcimb.2020.602453
30. Gieni R.S., Fang Y., Trinchieri G., Umetsu D.T., DeKruyff R.H. Differential production of IL-12 in BALB/c and DBA/2 mice controls IL-4 versus IFN-gamma synthesis in primed CD4 lymphocytes. *Int. Immunol*. 1996; 8(10): 1511–20. https://doi.org/10.1093/intimm/8.10.1511
31. Saha P., Xiao X., Li Y., Golonka R.M., Abokor A.A., Yeoh B.S., et al. Distinct iron homeostasis in C57BL/6 and Balb/c mouse strains. *Physiol. Rep.* 2020; 8(9): e14441. https://doi.org/10.14814/phy2.14441
32. Watanabe H., Numata K., Ito T., Takagi K., Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock*. 2004; 22(5): 460–6. https://doi.org/10.1097/01.shk.0000142249.08135.e9
33. Mosley Y.C., Radder J.E., HogenEsch H. Genetic variation in the magnitude and longevity of the IgG subclass response to a diphtheria-tetanus-acellular pertussis (DTaP) vaccine in mice. *Vaccines (Basel)*. 2019; 7(4): 124. https://doi.org/10.3390/vaccines7040124
34. Girardin S.E., Boneca I.G., Viala J., Chamaillard M., Labigne A., Thomas G., et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem*. 2003; 278(11): 8869–72. https://doi.org/10.1074/jbc.C200651200
35. Girardin S.E., Boneca I.G., Carneiro L.A., Antignac A., Jéhanno M., Viala J., et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science*. 2003; 300(5625): 1584 7. https://doi.org/10.1126/science.1084677
36. Tukhvatulin A.I., Gitlin I.I., Shcheblyakov D.V., Artemicheva N.M., Burdelya L.G., Shmarov M.M., et al. Combined stimulation of Toll-like receptor 5 and NOD1 strongly potentiates activity of NF-κB, resulting in enhanced innate immune reactions and resistance to Salmonella enterica serovar Typhimurium infection. *Infect. Immun*. 2013; 81(10): 3855–64. https://doi.org/10.1128/IAI.00525-13
37. Tukhvatulin A.I., Dzharullaeva A.S., Tukhvatulina N.M., Shcheblyakov D.V., Shmarov M.M., Dolzhikova I.V., et al. Powerful complex immunoadjuvant based on synergistic effect of combined TLR4 and NOD2 activation significantly enhances magnitude of humoral and cellular adaptive immune responses. *PLoS One*. 2016; 11(5): e0155650. https://doi.org/10.1371/journal.pone.0155650
38. Tukhvatulin A., Dzharullaeva A., Erokhova A., Zemskaya A., Balyasin M., Ozharovskaia T., et al. Adjuvantation of an influenza hemagglutinin antigen with TLR4 and NOD2 agonists encapsulated in poly(D,L-Lactide-Co-Glycolide) nanoparticles enhances immunogenicity and protection against lethal influenza virus infection in mice. *Vaccines (Basel)*. 2020; 8(3): 519. https://doi.org/10.3390/vaccines8030519
39. Hedl M., Li J., Cho J.H., Abraham C. Chronic stimulation of Nod2 mediates tolerance to bacterial products. *Proc. Natl Acad. Sci. USA*. 2007; 104(49): 19440–5. https://doi.org/10.1073/pnas.0706097104
40. Pashenkov M.V., Murugina N.E., Budikhina A.S., Pinegin B.V. Synergistic interactions between NOD receptors and TLRs: Mechanisms and clinical implications. *J. Leukoc. Biol.* 2019; 105(4): 669–80. https://doi.org/10.1002/JLB.2RU0718-290R
41. Bagchi A., Herrup E.A., Warren H.S., Trigilio J., Shin H.S., Valentine C., et al. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J. Immunol*. 2007; 178(2): 1164–71. https://doi.org/10.4049/jimmunol.178.2.1164
42. Magalhaes J.G., Lee J., Geddes K., Rubino S., Philpott D.J., Girardin S.E. Essential role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands. *Eur. J. Immunol.* 2011; 41(5): 1445–55. https://doi.org/10.1002/eji.201040827
43. Owen A.M., Luan L., Burelbach K.R., McBride M.A., Stothers C.L., Boykin O.A., et al. MyD88-dependent signaling drives toll-like receptor-induced trained immunity in macrophages. *Front. Immunol*. 2022; 13: 1044662. https://doi.org/10.3389/fimmu.2022.1044662