Original research article

MODULATION OF THE INFLAMMATORY RESPONSE IN EXPERIMENTAL MODELS OF MICE OF DIFFERENT STRAINS USING SYNTHETIC TLR9 LIGANDS

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Abstract: Cell-free DNA (cfDNA) from animals and humans possesses immunomodulatory properties, and the underlying mechanism of its action may involve binding to pattern recognition receptors such as TLR9. Activation of these receptors initiates inflammation and increases the production of proinflammatory cytokines. It is therefore relevant to study the effect of TLR9 receptor activation by the specific agonist ODN 1826 on changes in blood cfDNA levels and TNF-α production in response to an exogenous proinflammatory stimulus in C57BL/6 and Balb/c mice — strains known to differ in their response to the development of inflammation.

It was found that administration of the TLR9 receptor agonist ODN 1826 increased inflammation intensity in LPS-treated mice, as characterized by increased TNF-α and cfDNA concentrations in the blood and a decrease in the content of cfDNA covalently bound to proteins on the cell surface. At the early stage of inflammation development, the response of Balb/c mice to LPS was not qualitatively different from that of C57BL/6 mice.

These results suggest that agents capable of activating TLR9 may prove to be effective pharmacological tools for the treatment of immunopathological processes requiring correction of the Th1/Th2 balance.

Keywords: cell-free DNA, TLR9, oligonucleotides, inflammation, TNF-α, LPS

Оригинальная исследовательская статья

МОДУЛИРОВАНИЕ ВОСПАЛИТЕЛЬНОЙ РЕАКЦИИ В ЭКСПЕРИМЕНТАЛЬНЫХ МОДЕЛЯХ У МЫШЕЙ РАЗЛИЧНЫХ ЛИНИЙ С ИСПОЛЬЗОВАНИЕМ СИНТЕТИЧЕСКИХ ЛИГАНДОВ TLR9

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Резюме: Внеклеточная ДНК животных и человека обладает иммуномодулирующими свойствами и в основе механизма ее действия может лежать связывание с рецепторами распознавания образов, например TLR9. В результате активации этих рецепторов инициируется воспаление и увеличивается продукция провоспалительных цитокинов. Представляется актуальным изучение влияния активации TLR9-рецептора специфическим агонистом ODN 1826 на изменение уровней вкДНК и продукцию TNF-α в ответ на экзогенный провоспалительный стимул у мышей C57BL/6 и Balb/c, оппозитных линий — в ответ на развитие воспаления и продукцию TNF-α — в ответ на экзогенный провоспалительный стимул.

Установлено, что введение агониста рецептора TLR9 — олигонуклеотида ODN 1826 — увеличивает у мышей показатели интенсивности воспаления при введении LPS, характеризующиеся повышением концентрации TNFα и вкДНК в крови и уменьшением содержания вкДНК, ковалентно связанной с белками на поверхности клеток. На раннем этапе развития воспаления реакция мышей Balb/c на LPS качественно не отличалась от аналогичной реакции у мышей линии C57BL/6. Результаты дают основание предполагать, что способные активировать TLR9 препараты могут оказаться эффективными фармакологическими средствами при лечении иммунопатологических процессов, при которых требуется коррекция Th1/Th2-баланса.

Ключевые слова: внеклеточная ДНК, TLR9, олигонуклеотиды, воспаление, TNF-α, LPS

Introduction

General biological processes (apoptosis, NETosis) and epigenetic regulation may contribute to the development and maintenance of immune inflammation and pathological autoimmune responses [1—3]. To date, a substantial body of research has demonstrated changes in the content of cell-free DNA (cfDNA) in blood during certain pathological processes, as well as the use of this parameter as a diagnostic criterion for several diseases [1; 4—7]. Furthermore, some studies [8; 9] suggest that animal and human cfDNA possesses immunomodulatory properties, and that the underlying mechanism of its action may involve binding to pattern recognition receptors such as TLR9 [1]. TLR9 belongs to the extensive family of toll-like receptors, whose natural ligands are conserved regions within the molecules of pathogenic bacteria and viruses, collectively referred to as pathogen-associated molecular patterns [10]. Owing to their ability to recognize such patterns, proteins of this family play an important role in forming the first line of defense against infectious agents by initiating innate immune responses and nonspecific inflammation upon the entry of infectious agents into the body. In parallel, TLR proteins can activate the mechanisms involved in the subsequent pathogen-specific immune response, thereby participating in regulating the direction and intensity of immune reactions [10; 11].

TLR9 is expressed mainly in cells of the immune system, such as B cells, dendritic cells, and monocytes / macrophages, and is activated in response to DNA containing unmethylated CpG motifs characteristic of bacterial DNA, thereby participating in the regulation of innate antimicrobial immune responses [11—13]. Under certain conditions, TLR9 can also recognize self-DNA, leading to receptor activation, the initiation of inflammation, and increased production of proinflammatory cytokines [14]. As a result, TLR9 proteins may be involved in the development of autoimmune reactions. Knockout of the TLR9 gene in mice with immune complex-mediated lupus-like nephritis has been shown to markedly exacerbate disease manifestations, whereas overexpression of this gene in B cells substantially inhibits the development of nephritis [15].

Based on these data, we therefore investigated the effect of TLR9 activation by the specific agonist ODN 1826 on blood cfDNA levels and TNF-α production in mice in response to an exogenous proinflammatory stimulus, and determined whether TLR9 gene expression in immune cells can be modulated by synthetic ligands of this receptor, including blood cfDNA, whose levels increase substantially during the early phase of the inflammatory response.

The immune system controls the development and intensity of inflammatory responses, such that a shift in the helper T-cell balance toward Th1 lymphocytes, which produce proinflammatory cytokines, is a constant feature of the body's response to inflammation-inducing external stimuli [16]. Accordingly, it is reasonable to expect that, in many cases, changes in blood cfDNA levels may be significantly associated with the Th1/Th2 ratio in the body, which plays an important role in the pathogenesis of inflammatory processes in various diseases.

Materials and Methods

Study subjects

C57BL/6 and BALB/c mouse strains were selected as experimental animals because, according to published data [17], they exhibit opposite responses to inflammation. Healthy, sexually mature 6—8-week-old mice housed under conditions meeting the experimental requirements were included in the study. The animals received pelleted feed balanced for protein, fat, carbohydrate, vitamin, mineral, and trace-element requirements at appropriate levels (GOST R 50258-92). All experiments were performed under standard housing conditions, with controlled temperature and lighting. Female C57BL/6 and BALB/c mice (vivarium, Tomsk) were used in the study. The experiments were carried out in full compliance with the requirements of the International Convention for the Humane Treatment of Experimental Animals (Declaration of Helsinki). The study protocol was approved by the local ethics committee of the Research Institute of Fundamental and Clinical Immunology (approval № 92, dated 10 November 2015).

Mice of each strain were divided into four groups (6 animals each):

Group 1 — intact mice;

Group 2 — mice with an inflammatory response induced by LPS administration (control);

Group 3 — mice that, in addition to LPS, received the specific TLR9 ligand ODN 1826, containing two CpG dinucleotides;

Group 4 — mice that, in addition to LPS, received another TLR9 ligand, ODN 1982, lacking CpG dinucleotides.

All animals in the experimental groups (groups 2—4) received a single intraperitoneal injection of LPS from E. coli strain 111:B4 (Sigma, USA) at a dose of 1 μg/mouse. Animals in group 1 received an equivalent volume of PBS. Mice in groups 3 and 4 were given two subcutaneous injections of ODN 1826 or ODN 1982 as a control, at a dose of 25 μg/mouse, administered at a two-day interval before LPS injection. Four hours after LPS administration, the mice were euthanized, and blood was collected for further analysis.

Study methods

Isolation and quantification of cfDNA were performed according to the method described in [18]. Blood was collected into tubes containing 3× PBS (PBS: 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl) with 15 mM EDTA as an anticoagulant. Plasma was separated by centrifugation at 400 g for 20 min. A ninefold volume of PBS with 5 mM EDTA was added to the pelleted cells, and the supernatant was separated by centrifugation at 400 g for 20 min. To determine cell-surface-bound cfDNA, the cells were incubated for 5 min with a 0.25 % trypsin solution; the reaction was stopped by adding soybean trypsin inhibitor (Sigma, USA), and the cfDNA-containing supernatant was separated by centrifugation. DNA was isolated from the resulting fractions using BioSilica (Novosibirsk) columns according to the manufacturer's instructions. DNA content was quantified using the fluorescent dye PicoGreen (Invitrogen, USA), with concentrations calculated from a calibration curve constructed using known concentrations of standard double-stranded λ DNA.

Serum TNF-α levels in mice were determined by enzyme-linked immunosorbent assay using the Mouse TNF-alpha Quantikine ELISA kit (R&D Systems, Inc., USA).

TLR9 gene mRNA levels were determined by real-time PCR using a CFX96 Thermal Cycler (Bio-Rad, USA).

Statistical analysis

Results in the tables and figures are presented as mean values. The significance of the differences identified and the strength of associations between the measured parameters were assessed using nonparametric statistics, specifically the Mann—Whitney U test. Differences between groups were considered statistically significant at $p < 0.05$.

Results and Discussion

Assessment of the effect of synthetic TLR9 ligands on the development of the inflammatory response in C57BL/6 and BALB/c mice

The study aimed to assess cfDNA levels and TNF-α production in intact and LPS-stimulated experimental animals, and to examine the effect of the oligonucleotide ODN 1826 (a TLR9 agonist) on cfDNA levels and TNF-α synthesis in C57BL/6 and BALB/c mice.

In our earlier studies [19—21], we found that plasma cfDNA concentrations increase substantially in response to a low dose of LPS, suggesting that this parameter can serve as an indicator of the intensity of inflammatory processes in the body. This conclusion, which is consistent with published data, was based on experiments performed in other mouse strains. The results of the present study in C57BL/6 and BALB/c mice confirm that an increase in plasma cfDNA levels at the early stage of inflammatory response is observed in animals of both strains (column 1 in Tables 1 and 2). This parameter correlated well with the increase in TNF-α levels in response to the inflammatory stimulus in both C57BL/6 ($r = 0.51$, $p < 0.05$) and BALB/c ($r = 0.44$, $p < 0.05$) mice.

Table 1

The effect of TLR9 receptor ligands on LPS-stimulated inflammatory response in C57BL/6 mice

Group	Cell-free DNA, ng/mL			Ratio "cell-surface-bound cfDNA / total cfDNA"	N/L	TNF-α, pg/mL
	1	2	3			
1. Intact mice	25	255	3302	0.9	0.17	12.49
2. +LPS	249	86*	1412	0.83	0.46**	174.4**
3. +ODN 1826 + LPS	852	137	1367	0.64	0.33	476.1##
4. +ODN 1826 + LPS	436	151	827#	0.6#	0.42	217.7

Note: Designations of cfDNA fractions in the "Cell-Free DNA" column: 1 — plasma; 2 — buffer; 3 — cell-surface-bound cfDNA; N/L — neutrophil/lymphocyte ratio in the blood.

Significant difference from group 1 (control): * — $p < 0.05$; ** — $p < 0.01$.

Significant difference from group 2 (+LPS): # — $p < 0.05$; ## — $p < 0.01$.

Another significant consequence of the development of an inflammatory response in the animals is the decrease in the amount of cfDNA covalently bound to proteins on

the surface of blood cells, and the corresponding decrease in the ratio of cell-surface-bound cfDNA to total cfDNA, as shown by the results presented in Tables 1 and 2. In the BALB/c strain, these changes were statistically significant (Table 2).

Table 2

The effect of TLR9 receptor ligands on LPS-stimulated inflammatory response in BALB/c mice

Group	Cell-free DNA, ng/mL			Ratio "cell-surface-bound cfDNA / total cfDNA"	N/L	TNF-α, pg/mL
	1	2	3			
1. Intact mice	39	140	4807	0.96	0.6	21.9
2. +LPS	491**	142	2501*	0.8**	0.29	690.3**
3. +ODN 1826 + LPS	1601##	421#	3119	0.61##	0.16#	1458.1
4. +ODN 1826 + LPS	767	126	1532	0.63#	0.35	352.5

Note: Designations of cfDNA fractions in the "Cell-Free DNA" column: 1 — plasma; 2 — buffer; 3 — cell-surface-bound cfDNA; N/L — neutrophil/lymphocyte ratio in the blood.

Significant difference from group 1 (control): * — $p < 0.05$; ** — $p < 0.01$.

Significant difference from group 2 (+LPS): # — $p < 0.05$; ## — $p < 0.01$.

This study examined the effect of two TLR9 ligands on the above parameters: the oligonucleotide ODN 1826, which contains two CpG dinucleotides in its structure and is thus expected to strongly activate TLR9, and the oligonucleotide ODN 1982, which lacks CpG dinucleotides. Both compounds used were phosphorothioate derivatives of the corresponding oligonucleotides, which confers resistance to degradation by endogenous nucleases [22].

The data presented in Tables 1 and 2 show that ODN 1826, a TLR9 agonist, substantially increases the intensity of the inflammatory response to LPS, as assessed by the parameters examined in this study (plasma TNF-α and cfDNA levels, cell-surface-bound cfDNA level, and the ratio of cell-surface-bound to total cfDNA). This finding is consistent with published data and may be interpreted as reflecting the ability of activated TLR9 to influence the Th1/Th2 balance by shifting it toward a proinflammatory state. Other authors have reported that agonists of this receptor can stimulate IL-12 production by dendritic cells [23] and suppress Th2-dependent immune responses in allergy [24] and autoimmune processes [10; 25].

The CpG-free oligonucleotide (ODN 1982) did not affect indicators of the inflammatory response, such as plasma TNF-α levels and cfDNA content, in mice of either strain. However, the cell-surface-bound cfDNA fraction was significantly reduced, altering the index (i. e., the ratio of this fraction to total cfDNA). This finding may indirectly indicate that TLR9 ligands are not limited to DNA fragments rich in unmethylated CpG dinucleotides. Other potential mechanisms of activation or blockade of this receptor cannot be ruled out and clearly warrant further investigation.

The changes in inflammatory response parameters that we observed in the mouse strains studied do not support characterizing BALB/c mice as genetically predisposed to a qualitatively different response to inflammatory stimuli than that observed in C57BL/6 mice. It should be noted that, even in intact animals, TNF-α and plasma cfDNA levels are nearly twice as high in BALB/c mice as in C57BL/6 mice (Tables 1, 2),

whereas during the inflammatory response and under the action of ODN 1826, these parameters in groups 2 and 3 are 3—4-fold higher in BALB/c mice than in C57BL/6 mice. This suggests that strain differences in the response to TLR9 activation are reflected in the magnitude of the response rather than in its direction. In both strains, a significant decrease in the content of cfDNA covalently bound to proteins on the cell surface was observed, along with a corresponding decrease in the ratio of cell-surface-bound cfDNA to total cfDNA, as shown by the trypsin/total cfDNA ratio (Tables 1, 2).

Qualitatively, the changes observed in the parameters assessed were similar in both mouse strains, indicating that strain differences in the response to TLR9 activation are reflected not in opposite directions of effect, as initially assumed, but in the magnitude of the response.

This conclusion formally contradicts the well-known opposition between these strains with respect to the immune response to T-dependent antigens (BALB/c mice exhibit a stronger Th2-dependent humoral response, whereas C57BL/6 mice exhibit a stronger Th1-dependent delayed-type hypersensitivity [DTH] response), as well as the results of a recent study by a group of Chinese authors [17]. In that study, it was found that, during the development of the inflammatory process, plasma cfDNA concentrations in BALB/c mice, measured over several days after infection, were noticeably lower than in C57BL/6 mice under comparable experimental conditions. In our study, no such difference was observed; the intensity of the response to LPS in BALB/c mice was in fact somewhat higher than in C57BL/6 mice. However, there is no real contradiction between our findings and the published data. First, the cited study concerns a severe septic process resulting in the death of nearly all experimental animals, whereas in our earlier experiments, LPS-induced inflammation was of moderate intensity, with all parameters returning to baseline levels 24 h after LPS administration [20]. Second, and most importantly, the Chinese authors' study showed that the gradual increase in blood cfDNA concentration during the first day after infection was virtually identical in both mouse strains studied [17], which is fully consistent with the results of our work.

It can be assumed that the magnitude and direction of specific changes in these animals during further development of inflammation are determined by the combined action of multiple regulatory factors, one of which may be the degree of TLR9 expression in mice of different strains and its activation under various stimuli.

The results of our experiments, together with published data, suggest that bacterial DNA preparations and synthetic ligands of this receptor capable of activating TLR9 (with either agonist or antagonist properties) may prove to be effective pharmacological agents for the treatment of immunopathological processes requiring correction of the Th1/Th2 balance. Elucidating their therapeutic potential represents an important task for further research.

Assessment of TLR9 gene expression in mice of different strains under the influence of synthetic ligands of this receptor

Having examined the effects of synthetic TLR9 ligands on the development of inflammation in the experimental model, and having established that blood cfDNA and TNF-α levels in mice of both the C57BL/6 (Th1) and BALB/c (Th2) strains depend substantially on TLR9 activation, we investigated the effect of these ligands on

TLR9 gene expression (TLR9 mRNA levels) and attempted to identify a relationship between this process and cfDNA levels in the context of the Th1- and Th2-type immune responses characteristic of C57BL/6 and BALB/c mice, respectively.

The data obtained from experiments examining the potential effect of TLR9 ligands on TLR9 gene expression in splenocytes of the two opposite mouse strains are presented in Table 3.

Table 3

Changes in TLR9 mRNA levels in splenocytes of BALB/c and C57BL/6 mice under the influence of LPS and ligands of this receptor

Group	mRNA level, arb. units	
	BALB/c	C57BL/6
1. Control (n = 6)	0.96 ± 0.12	1.30 ± 0.26
2. +LPS (n = 6)	1.21 ± 0.09 (26 %↑)	1.69 ± 0.27 (30 %↑)
3. +ODN 1826 +LPS (n = 6)	1.29 ± 0.07 (34 %↑)	1.23 ± 0.19 (5 %↓)
4. +ODN 1982 + LPS (n = 5)	2.35 ± 0.54 (≥2-fold)	1.49 ± 0.28 (15 %↑)

Note: %↑↓ — relative to the control groups of the corresponding mouse strains.

The differences between the groups examined, presented in Table 3, do not support the conclusion that the synthetic ligands used substantially affect TLR9 mRNA transcription in immunocompetent cells (splenocytes), as the changes observed were not statistically significant. The same applies to the corresponding effect of cfDNA on this parameter, at least during the early phase of the inflammatory response to LPS administration, when blood cfDNA levels increase substantially.

Consequently, the observed effects of TLR9 ligands on the development of the inflammatory response cannot be explained by changes in the amount of TLR9 mRNA, but are more likely attributable to the activation of pre-existing TLR9 receptors. This does not, of course, rule out the possibility that ligands of this receptor (including blood cfDNA) may influence its expression at later time points following exposure. However, testing this hypothesis would require dedicated experiments.

Conclusion

Experiments in BALB/c and C57BL/6 mice demonstrated the feasibility of modulating the inflammatory response to LPS using synthetic TLR9 receptor ligands. Administration of the TLR9 agonist ODN 1826 increased LPS-induced inflammation intensity in mice, as characterized by higher blood TNF-α and cfDNA concentrations and a decrease in the content of cfDNA covalently bound to proteins on the cell surface. The CpG-free oligonucleotide ODN 1982 had a lesser effect on inflammatory response parameters. At the early stage of inflammation development, the response of BALB/c mice to LPS was not qualitatively different from that of C57BL/6 mice. The results of our experiments, together with published data, suggest that bacterial DNA preparations and synthetic ligands of this receptor capable of activating TLR9 (with either agonist or antagonist properties) may prove to be effective pharmacological agents for the treatment of immunopathological processes requiring correction of the Th1/Th2 balance. Investigating their therapeutic potential is a relevant and promising area for future research.

At the same time, it was found that TLR9 ligands — both synthetic ligands and plasma cfDNA, whose level increases substantially after the stimulation of an acute inflammatory response in these animals — do not produce statistically significant changes in TLR9 gene expression in splenocytes, at least during the early phase of the inflammatory response to LPS administration. However, the possibility that ligands of this receptor (including blood cfDNA) may affect TLR9 gene expression at later time points cannot be excluded.

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Authors' contribution:

O. V. Voronkova — development of the concept and design of scientific work, drafting of the manuscript text and critical revision of the manuscript;

I. D. Bespalova — conducting an examination, patient treatment, statistical analysis;

I. E. Esimova — data collection, data analysis and interpretation, statistical analysis;

I. A. Osikhov — data collection, data analysis and interpretation, drafting of the manuscript text.

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