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Expression of TLR2 and TLR4 Receptors on Lymphoid Cells as a Marker of Infectious Lesions of Periodontal Tissues, Associated with the Dominance of *Porphyromonas gingivalis* and *Filifactor alocis* in Periodontal Biofilm

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Abstract

A research was carried out of the Toll-like receptors TLR2 and TLR4 expression on non-stimulated peripheral blood cells (neutrophils, monocytes and lymphocytes) and gingival fluid cells in people with healthy periodontal disease and in patients with chronic periodontitis associated with osteopenia and osteoporosis. *P. gingivalis* and *F. alocis*, asaccharolytic anaerobic bacteria, are emerging markers of periodontitis. It was established that the expression of TLR2 and TLR4 on peripheral blood leukocytes in different people, despite of their study group, varied significantly. Multidirectional expression of TLR2 and TLR4 on peripheral blood cells was found (both upward and downward) in patients with chronic periodontitis associated with *P. gingivalis*, *F. alocis*.

Keywords: *TLR2; TLR4; Receptors; P. gingivalis; F. alocis; Periodontitis; Osteoporosis; Osteopenia*

Abbreviations

TLR: Toll-Like Receptors; CP: Chronic Periodontitis in the Acute Phase; PCR: Multiplex Polymerase Chain Reaction; RPC: Research and Production Company; FITC: Fluorescein Isothiocyanate

Introduction

Chronic periodontitis begins with the sequential colonization of a wide range of periodontal pathogenic bacteria that induce an inflammatory response with the involvement of innate and adaptive immunity factors, which affect the periodontal biofilm [1-5]. Microbial recognition is mediated by Toll-like receptors that interact with conserved pathogen-associated molecular patterns. It has been established that, recognizing specific ligands, TLR-2 and TLR-4 interact with the majority of representatives of periodontal pathogenic bacterial species [6-8].

Among the factors of innate immunity, more attention is paid in recent years, to Toll-like receptors (TLR). The universality of the mechanisms, due to both: wide representation of these markers on various cells of the body and wide range of ligands for them, determines the inclusion of TLR in the pathogenetic links of the development of many diseases [8-10]. Currently, various disorders have been established in the innate immunity mechanisms system associated with TLR, in particular, violations of TLR expression and ligand recognition, signal transduction, production of effector molecules, as well as polymorphism of TLR genes. The manifestation of these disorders in the development of atherosclerosis, infectious, autoimmune, allergic and some other diseases has been shown [9-12]. Thus, it was noted that defects in molecules involved in signal transduction from TLR underlie increased susceptibility to infectious diseases [8].

P. gingivalis and *F. alocis*, asaccharolytic anaerobic bacteria, are emerging markers of periodontitis. Severe periodontitis causes destruction of the alveolar bone that supports teeth and can even lead to tooth loss. It is assumed that these bacteria - derived extracellular vesicles contain various effectors molecules and have immunostimulatory activity on osteogenesis, what was shown using mouse model of bone-derived mesenchymal stromal cells. As a result, it was found that these effectors molecules dramatically inhibited bone mineralization similar to whole bacteria and reduced the expression levels of osteogenic marker genes [13,14].

The importance of these mechanisms for wide clinical practice determines the need to introduce adequate and reliable methods for assessing the components of the TLR system, which can be reproduced in the clinical laboratory of medical institutions.

Aim of the Study

The aim of this work was to study the expression of innate immunity markers TLR2 and TLR4 on lymphoid cells of the periodontal (gingival fluid) and peripheral blood by immunofluorescence microscopy in almost healthy patients and patients with chronic periodontitis.

Materials and Methods

The study involved 150 patients - 88 (59%) women and 62 (41%) men aged 18 to 73 years with chronic periodontitis in the acute phase (CP) and 32 people without signs of chronic periodontal inflammation (conditionally healthy people of the same age category). Patients with CP were divided into 2 groups of periodontal criteria accepted in the Russian Federation: 1st group - CP of moderate severity, 2nd group - severe CP. Patients of the latter group had X-ray signs of severe osteoporosis of the jaw bones.

Multiplex polymerase chain reaction (PCR) was conducted to confirm the diagnosis of periodontitis using "MultiDent-5" reagent kit to detect *P. gingivalis*, *P. intermedia, T. forsythia, T. denticola, A. actinomycetemcomitans*, and individual - for *F. alocis* in periodontal pocket (RPC "GenLab", Russia).

To assess cells carrying CD282 and CD284 markers, a test material was used in the form of gingival fluid lavage from the periodontal pocket or from the periodontal sulcus with Hanks solution.

The isolated cells were stained with antibodies to CD282 (corresponds to the TLR2 receptor) or CD284 (corresponds to the TLR4 receptor) markers labeled with FITC and fixed with paraformaldehyde. The finished preparations were examined with ECLIPSE 50i microscope (Nicon, Japan) at a magnification × 1000. Phenotype of peripheral blood leukocytes was determined using the flow laser cytofluorimetry method [15]. To do this, 100 µl of blood was mixed with a solution of antibodies labeled with fluorescent multicolor dyes against TLR2 (TLR2 IgG1 - FITC), CD14 - PE, CD45 - PerCP, CD19 - APC, CD3 - AF700 or TLR4 (TLR4 IgG1 - FITC) and CD14 - PE, CD45 - PerCP, CD19 - APC, CD3 - AF700. Negative control tubes were supplemented with mouse antibodies labeled with FITC (IgG1 - FITC), as well as with antibodies labeled with PE, PerCP, APC, and AlexaFluor700. Incubation lasted for 30 minutes in the dark at ± 4°C.

To remove erythrocytes, a FACS Lyse lysis buffer (BD Bioscience, USA) was used in a volume of 2.0 ml for 10 minutes at room temperature in the dark. Then centrifugation was carried out at 500g for 5 minutes. The supernatant was removed. 2.0 ml of phosphate-buffered saline were added to the precipitated cells. The cell suspension was washed twice by centrifugation, after which the leukocytes were resuspended, and 0.5 ml of FACS CellFix (BD Bioscience, USA) was added. The samples obtained were examined on a FACS Canto 2 flow cytometer, determining the expression of TLR2 and TLR4 on the main subpopulations of T-lymphocytes (CD3+ lymphocytes), B lymphocytes (CD19+ lymphocytes), monocytes (CD14+ monocytes) and granulocytes (CD45dim neutrophils).

The likelihood of differences in the studied indicators was assessed using the Student's t-test. All calculations were performed using the Statistica 7.0 software package.

Results and Discussion

The results of the periodontal pocket molecular biological research conducted by PCR showed that DNA of *P. gingivalis* with chronic periodontitis was detected in 94 (62,7%) patients out of 150, and *F. alocis* - in 97 (64,7%), while representatives of other periodontopathogenic species were less common than in 50% of patients. Thus, *P. intermedia* was identified at 72 (48,0%) people, *T. forsythia* - at 73 (48,6%), *T. denticola* - at 52 (34,6%), *A. actinomycetemcomitans* - in 69 (46,0%). In 11 patients (7,3%), representatives of these periodontal pathogenic species were not found. In the control group with intact periodontium (35 patients) incidence of periodontal kinds did not exceed 8,6% (*P. intermedia*) - 11,4% (*F. alocis*, *T. Forsythia*). Representatives of *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola* were not found in patients with intact periodontium (Table 1).

Index	**Patient's groups**		χ^2	p
	Periodontitis (n = 150)	**Healthy (n = 35)**		
A. actinomycetemcomitans	69 (46,0)	0 (0)	25.677	p < 0,001
F. alocis	97 (64,7)	4 (11,4)	32.447	p < 0,001
P. gingivalis	94 (62,7)	0 (0)	44.590	p < 0,001
P. intermedia	72 (48,0)	3 (8,6)	18.302	p < 0,001
T. forsythia	75 (50,0)	4 (11,4)	17.256	p < 0,001
T. denticola	52 (34,6)	0 (0)	16.877	p < 0,001
No germs	11 (7,3)	29 (82,9)	95.515	p < 0,001

Table 1: *Frequency of periodontal pathogenic bacterial species detection in biofilm material in patients with chronic heart disease and intact periodontium (%).*

Note: **Statistically significant difference compared with the control group at p < 0.001.*

When conducting a study using immunoluminescence microscopy, for the first time in domestic practice, it was shown that the cells of the gingival fluid or periodontal pocket exudate, most of which were leukocytes, expressed the innate immunity receptors TLR2 and TLR4 with different intensities.

According to the data obtained in the control group, the detection rate of cells expressing these markers was 12,3 ± 3,2%, and in the group of patients with CP in the acute phase - 4.1 ± 2.1% (p < 0.05), i.e. turned out to be statistically significantly lower (3 times). Thus,

figure 1 shows 2 cells expressing CD282 (TLR2) labeled with FITC in the field of a fluorescent microscope view. Brightly colored granule clusters are visible. Figure 2 shows 4 cells in phase contrast, isolated from the exudate of the periodontal pocket. Figure 3 shows the same 4 cells at a luminescence microscopy with dim diffuse staining of CD284 FITC (TLR4).

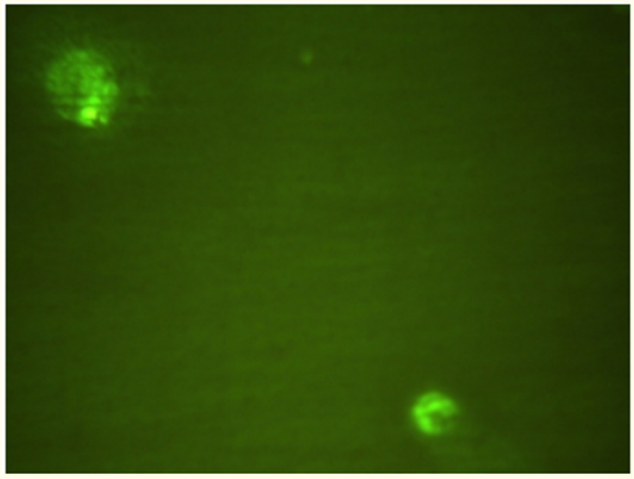

Figure 1: *Gingival fluid cells expressing CD282 - FITC. Magnification × 1000.*

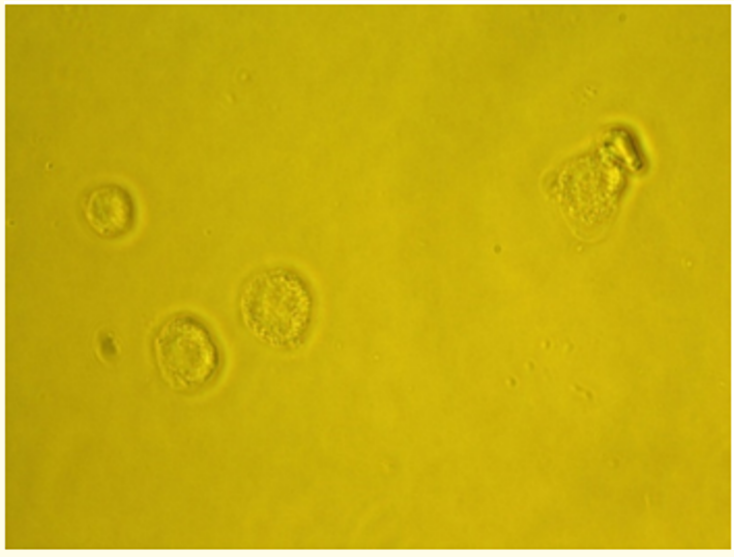

Figure 2: *Gingival fluid cells. Phase contrast. Magnification × 1000.*

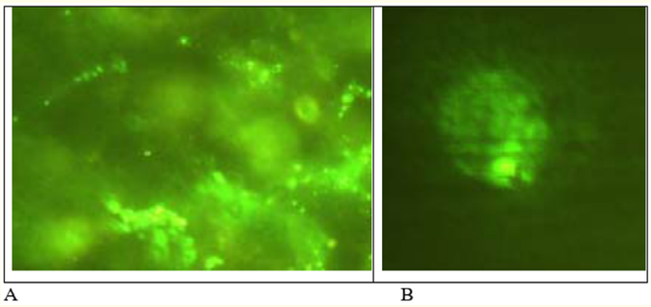

Figure 3: *Gingival fluid cells expressing CD284 - FITC (the same field of view): A. Magnification × 1000; B. Magnification × 6000.*

Thus, as a result of this pilot study, the expression of innate immunity receptors TLR2 and TLR4 in the gingival fluid and exudate of the periodontal pocket in periodontal pathology (CP) was identified and visualized.

Unfortunately, the content of cellular elements in the gingival fluid is less than required for the study of the immunophenotype of cells on a flow cytometer - 3,5 - 9,4 × 10^6 cells per ml [15]. When cells are isolated from the gingival fluid, its lavage with isotonic sodium chloride solution is usually used, which makes it possible to obtain 5 - 10 × 10^3 cells in 1 ml, i.e. about 2 times less than required. Therefore, to obtain additional data, studies were carried out on peripheral blood cells.

In the first series of experiments, the expression of TLR2 and TLR4 was determined on leukocytes isolated from the peripheral blood of patients (and conventionally healthy people) by sedimentation of erythrocytes on 2% gelatin in phosphate-buffered saline, according to standard methods used to determine the phenotype of immunocompetent cells [15].

The obtained data confirms that the expression of TLR2 and TLR4 on peripheral blood leukocytes in different people, regardless of the group to which they belonged (CP patients, control group with intact periodontium), significantly varied from complete absence to 100% expression on blood monocytes of some of people. Figure 4 and 5 show examples of markers CD14/CD282 and CD3/CD284 determination on neutrophils, lymphocytes and monocytes of peripheral blood.

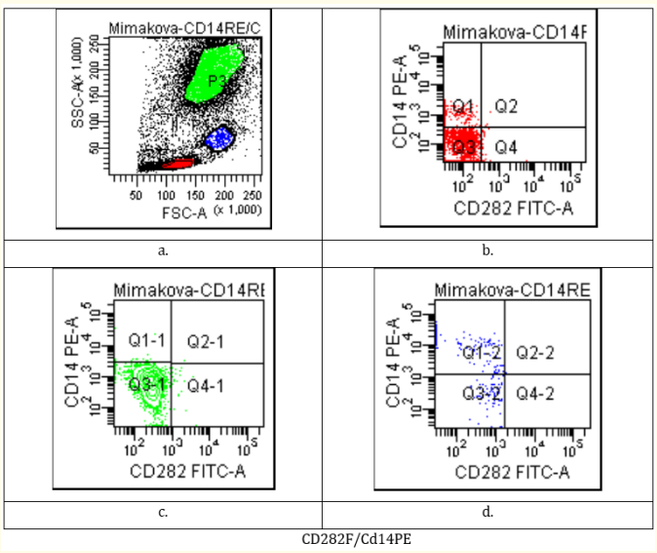

Figure 4: *Expression of TLR2 on peripheral blood leukocytes: a - graph of anterior and lateral light scattering: neutrophils - area colored green, lymphocytes - red areas, monocytes - blue; b-d - two-dimensional dot plot - expression of CD14, CD282 and CD14/CD282 on cells of different populations of leukocytes.*

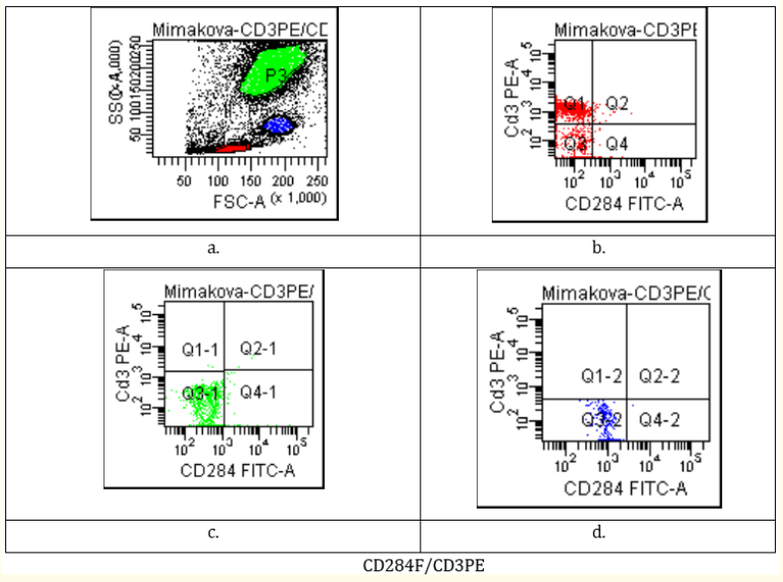

Figure 5: *Expression of TLR4 on peripheral blood leukocytes: a - graph of anterior and lateral light scattering: neutrophils - area colored green, lymphocytes - red areas, monocytes - blue; b-d - two-dimensional dot plot - expression of CD14, CD284 and CD14/CD284 on cells of different populations of leukocytes.*

It turned out that the expression of TLR2 on monocytes (double staining for CD14/CD282) and TLR4 on lymphocytes (double staining for CD3/CD284) was very low and did not exceed 1%.

In the second series of experiments, whole blood cells were simultaneously stained with several variants of antibodies against certain populations of leukocytes marked with different fluorescent labels. In this modification subpopulations immunophenotyping cells are not lost what usually occurs in the process of allocating leukapack from peripheral blood. Nevertheless, the proportion of cells expressing CD282 (TLR2) was also low.

It turned out that the highest level of CD282 (TLR2) markers expression was observed on lymphocytes of people with healthy periodontium (1.06 ± 0.45%), i.e. by 56,24 ± 24,12 million cells/liter (Table 2). Relative and absolute lymphocytes content expressing CD282 in group I was 3 times lower than of the control group (0,35 ± 0,01% and 18,82 ± 0,93 million cells/liter). Relative content of lymphocytes expressing CD282 in group II was 4 times lower than that of the control group (0,26 ± 0,05%), and absolute - in 5,7 times (9,91 ± 1,91 million cells/l).

Group	Unit	Expression of CD 282 (TLR 2)		
		Neutrophils	Monocytes	Lymphocytes
Control	%	0,34 ± 0,04	0,65 ± 0,11	1,06 ± 0,45
	abs., mln/l	18,15 ± 2,16	34,45 ± 5,71	56,24 ± 24,12
I (Moderate CP)	%	0,12 ± 0,01 *	0,91 ± 0,43 *	0,35 ± 0,01 *
	abs., mln/l	6,13 ± 0,93 *	43,72 ± 5,34 *	18,82 ± 0,93 *
II (Severe CP)	%	0,08 ± 0,01 *	0,065 ± 0,028	0,26 ± 0,05 *
	abs., mln/l	2,67 ± 3,05 *	2,48 ± 1,12 *	9,91 ± 1,91 *

Table 2: *Expression of CD282 (TLR2) on peripheral blood leukocytes*

Note: **Statistically significant difference compared with the control group at p < 0.05.*

Expression of CD282 on monocytes of peripheral blood in group II patients (0,91 ± 0,43%, 43,72 ± 5,34 million cells/l) was 1,4 times higher than in the control group (0,65 ± 0,11%, 34,45 ± 5,71 million cells/l). However, relative number of monocytes expressing TLR2 in group II, was 10 times lower than in the control group, and the absolute content - 13,9 times (2,48 ± 1,12 million cells/l).

CD282 expression on the neutrophils was even lower. Thus, in representatives of the control group, these receptors were detected in 0,34 ± 0,04% (18,15 ± 2,16 million cells/l) of neutrophils. In group I patients - relative and absolute content was 2,8 (0,12 ± 0,01%) and 3 times (6,13 ± 0,93 million cells/l) less, in group II - 4,3 (0,08 ± 0,01%) and 6,8 times (2,67 ± 3,05 million cells/l) less than in the control group.

Expression of CD284 (TLR4) on peripheral blood leukocytes had the same character, though on lymphocytes of group I patients the amount of these receptors was 2,8 (0,73 ± 0,13%) and 2,9 times (39,06 ± 2,79 million cells/l) more than in the control group (0,26 ± 0,12%, 13,52 ± 6,50 million cells/l). In group II relative content of CD284+ lymphocytes was higher than in people with healthy periodontium, only 1,2 times higher (0,31 ± 0,11%), and the absolute quantity - 1,14 times lower (11,81 ± 4,19 million cells/l). The highest expression of CD284 on monocytes was also in group I patients with CP of medium severity: 2,17 ± 0,18% (117,02 ± 3,8 million cells/l) - 15 times more than in the control group (0,145 ± 0,003%, 7,63 ± 1,51 million cells/l). The lowest expression in group II patients with severe stage of CP and signs of jaw bones osteoporosis - relative amount of (0,085 ± 0,038%) was 1,7 times lower, and the absolute amount (3,39 ± 2,67 million cells/l) - 2,3 times lower than in the control group with intact periodontium (Table 3).

Group	Unit	Expression of CD 28 4 (TLR 4)		
		Neutrophils	Monocytes	Lymphocytes
Control	%	0,35 ± 0,05	0,145 ± 0,003	0,26 ± 0,12
	abs., mln/l	18,25 ± 3,11	7,63 ± 1,51	13,52 ± 6,50
I (Moderate CP)	%	0,275 ± 0,083*	2,17 ± 0,18*	0,73 ± 0,13*
	abs., mln/l	14,79 ± 1,78	117,02 ± 3,8*	39,06 ± 2,79*
II (Severe CP)	%	0,21 ± 0,07*	0,09 ± 0,04*	0,31 ± 0,11
	abs., mln/l	8,05 ± 2,67*	3,39 ± 2,67*	11,81 ± 4,19*

Table 3: *Expression of CD 284 (TLR -4) on peripheral blood leukocytes.*

Note: **Statistically significant difference compared with the control group at p < 0.05.*

The highest expression of CD284 on neutrophils was in the blood of the control group patients with intact periodontium (0,35 ± 0,05%, 18,25 ± 3,11 million cells/l); in group I patients - relative amount (0,275 ± 0,083%) was 1,3 times lower, absolute (14,79 ± 1,78 million cells/l) - 1,2 times lower, in group II patients the relative amount (0,21 ± 0.07%) was 1,7 times lower, absolute (8,05 ± 2,67 million cells/l) - 2,3 times lower than in the control group (Table 2). It should be noted that relative number of neutrophils expressing TLR2 in group I patients was 1,5 times higher than in group II, the number of monocytes - 15 times higher and lymphocytes - 1,3 times higher. The difference in absolute content was even greater - 2,3, 17,6 and 1,9 times, respectively. The relative number of neutrophils expressing TLR4, in group I patients was 1,3 times higher than in patients of group II, of monocytes - 25,5 times higher and lymphocytes - 2,3 times higher. The difference in the absolute content was also higher - 1,84, 34,5 and 3,3 times respectively.

Thus, as a result of the studies carried out, it was found that the main populations of peripheral blood leukocytes (neutrophils, monocytes and lymphocytes) express TLR2 and TLR4 on their surface, which are detected using monoclonal antibodies to CD282 and CD284. In people with healthy periodontal disease, the highest expression of TLR2 is observed on lymphocytes, less on monocytes, and the lowest

on peripheral blood neutrophils. On the contrary, the largest number of neutrophils expressing TLR4 was detected in healthy people, in a smaller number - lymphocytes and even less - monocytes.

At the same time, in patients with chronic periodontitis, the expression of TLR2 was higher on monocytes, but lower on neutrophils and lymphocytes compared to the control group; TLR4 expression was higher on monocytes and lymphocytes, but lower on neutrophils.

Discussion

In our opinion, the nature of periodontal microbiota and severity of destruction or osteoporosis of alveolar bone also deserves attention. The studied parameters testify the dominance among the local population of three representatives of periodontal microbiota - *P. gingivalis*, *F. alocis* and *T. forsythia*, which were detected in 62,7%, 64,7% and 50% of patients with chronic periodontitis, respectively. It is known that *P. gingivalis* and *T. forsythia* are responsible for the destruction of the alveolar bone [17-20].

Regarding the new periodontal species - *F. alocis* such data was obtained for the first time. It is possible that these processes can also be associated with the expression of the innate immunity receptors TLR2 and TLR4 [21-24].

The osteogenic differentiation of TLR2-deficient BMS cells was not inhibited by *F. alocis* extracellular vesicles, suggesting that their inhibitory effect on osteogenesis is dependent on TLR2 signaling. In addition, *F. alocis* extracellular vesicles effectively activated TLR2 downstream signaling of the MAPK and NF-κB pathways. It is assumed that *F. alocis* extracellular vesicles regulated RANKL and OPG gene expression, increasing the RANKL/OPG ratio in BMS cells in a TLR2-dependent manner. Thus study suggests that *F. alocis*-derived extracellular vesicles interfere with bone metabolism via TLR2 activation, providing insight into the pathogenesis of bone loss associated with periodontitis [13,14].

In early studies it was found that *F. alocis* has different mechanisms of action on white blood cells, however, the link with osteoporosis or osteopenia has not been considered in detail [23-25]. Our results showed that TLR2 expression in all studied leukocyte populations in patients with CGP associated with osteoporosis was 4 - 14 times lower than in healthy people and 1,3 - 15 times lower than in patients with periodontitis without osteoporosis. The expression of TLR4 in these patients was also 1,2 - 2,3 times lower than in healthy people and 1,3 - 25,5 times lower than the expression of Toll-like receptors on the leukocytes of patients with periodontitis associated with osteopenia.

Conclusion

- The level of expression of Toll-like receptors TLR2 and TLR4 does not fundamentally differ on unstimulated peripheral blood cells (neutrophils, monocytes and lymphocytes) and gingival fluid in people with healthy periodontal disease, but varies with the development of chronic periodontitis.
- Multidirectional expression of TLR2 and TLR4 receptors was revealed on peripheral blood cells, both upward and downward, in patients with moderate to severe chronic periodontitis stage in the exacerbation phase associated with periodontal microbiota, especially *P. gingivalis, F. alocis, T. forsythia*.
- Significantly lower levels of expression of TLR2 and TLR4 markers on peripheral blood cells were observed in severe chronic periodontitis, which was accompanied by signs of osteoporosis of the jaw bones. This fact allows to suggests that changes in the innate immunity system, manifested at the level of expression of Toll-like receptors are associated with systemic loss of bone mineral density and progression of chronic periodontitis.

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