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# PROLIFERATIVE ACTIVITY OF STROMAL CELL ELEMENTS AT FIBROUS-CAVERNOUS PULMONARY TUBERCULOSIS DEPENDING ON MACROPHAGEAL IMMUNOPHENOTYPE

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***Abstract***

*Aim of this study was to evaluate the proliferative activity of stromal cellular elements at fibrous-cavernous pulmonary tuberculosis depending on macrophageal immunophenotype.*

*Biopsy samples from patients with fibrous-cavernous tuberculosis divided into group with active bacillation (n=52) and with clinical abacillation (n=31) were studied. To evaluate the immunophenotype of the macrophageal pool the immunohistochemical study was performed using CD68 and VEGF-A markers. The proliferative activity of pulmonary stroma cellular elements was evaluated using Ki-67 marker.*

*The performed immunohistochemical evaluation has revealed the presence of two immunophenotypes of macrophages: VEGF-A$^+$ and VEGF-A$^-$, characterized by various localization and quantitative characteristics depending on bacillation activity. Active bacillation was accompanied with predominance of highly active CD68$^+$/VEGF-A$^-$ macrophages (M1), intensification of exudative reactions and phagocytosis, as well as formation of perifocal areas of serous / caseous pneumonia, while the clinical abacillation group was characterized by the increased number of CD68$^+$/VEGF-A$^+$ cells without signs of proliferative activity and stabilization of alterative and exudative processes.*

*Thus, in fibrous-cavernous tuberculosis it is present two immunophenotypically different subpopulations of macrophages CD68$^+$/VEGF$^-$ and CD68$^+$/VEGF$^+$ with an inductive effect on the severity of immunological reactions, as well as remodeling of the pulmonary parenchyma in the form of pathological angiogenesis and pneumofibrosis are recorded.*

*As the severity of alterative-exudative processes increases and/or the proximity to the site of specific inflammation, an increase in the pool of VEGF$^-$ macrophages characterized by high and moderate phagocytosis activity is noted.*

***Keywords:*** *fibrous-cavernous tuberculosis, immunohistochemistry, macrophages, angiogenesis*

## *Introduction*

Tuberculosis is one of the leading problems of the global medical community, possessing a leading position within morbidity and mortality. A set of measures developed as part of World Health Organization's End Tuberculosis Strategy (May, 2014) is aimed primarily at improving the diagnostic measures and early detection of specific inflammatory process, development of effective vaccines and prevention of the global epidemic, particularly in association with HIV infection [*Fox G et al., 2016; WHO, 2017*]. However, the therapeutic management strategy of patients with TB remains virtually unchanged for decades and includes a toxic chemotherapy. Either in case of treatment failure or drug resistance, formation or progression of the destruc-

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tive forms is seen, with irreversible fibrotic transformation of the lung parenchyma, leading to palliative surgery [*Cliff J.M. et al., 2015*].

One of the basic pathogenetic mechanisms of fibrosis is proliferative activity and differentiation of the fibroblasts, followed by amplification of their synthetic activity [*Golubinskaya E.P. et al., 2017*]. However, the mechanisms of their activation and increase in reactivity of the extracellular matrix are still understudied. The potential inducers of inadequate collagen synthesis are cells of histiocytic origin, in particular macrophages [*Upadhyay S et al., 2017; Yakar H et al., 2017*]. The in vitro experimental studies have shown the polarization of histiocytes pool into two major subpopulations: M1 and M2 cells [*Gordon S et al., 2014; Martinez F et al., 2014*].

M1 cells are classically activated proinflammatory macrophages producing the intermediate compounds of nitrogen and oxygen, characterized by the subsequent activation of Th1-mediated immune response and pronounced phagocytic function [*Huygen K, 2014; Jasenosky L et al., 2015; Bhattacharya D et al., 2017*].

M2 alternatively activated cells exhibit an anti-inflammatory activity by means of target-specific remodeling of the surrounding stroma by producing vascular endothelial growth factor (VEGF), fibroblast growth factor and metalloproteinases 9 and 13, providing fibroblast chemotaxis and initiation of their synthetic activity [*Italiani P et al., 2014; Tan S, Krasnow M, 2016; Golubinskaya E et al., 2017*].

The studies of population heterogeneity of macrophages and their potentiating effect on the morphogenesis of tuberculosis under real-life conditions in humans are occasional, fragmented and highly controversial [*Kim P.S. et al., 2012*]. However, we believe, such studies are of crucial significance, because the key role of macrophages in the pathogenesis of immune response and formation of the subsequent specific pathological manifestations is well known. As well, they can be potential targets for targeted therapies as an alternative method for the therapeutic correction of the infectious process and prophylaxis of the pulmonary fibrosis and progressive respiratory insufficiency, accordingly.

Thus, the aim of this study was to evaluate the proliferative activity of stromal cellular elements at fibrous-cavernous pulmonary tuberculosis depending on macrophageal immunophenotype.

### *MATERIAL AND METHODS*

The fragments of the cavern wall, pericavernous zone and intact pulmonary tissue of patients with verified diagnosis of fibrous-cavernous tuberculosis (FCT) (n=83) obtained during surgery or autopsy were used as a study material. This material was divided into 2 groups: FCT-MBT+ group with active bacillation (n=52) and FCT-MBT- group with clinical abacillation (n=31). The fragments of the lungs of patients who died from the pathologies not related to respiratory diseases (n=30) were used as a control group.

Patient age (from 18 to 65 years old), confirmed absence of viral hepatitis B, C and HIV, as well as confirmed absence of chronic diseases exacerbation of other organs and systems were taken into account during the sampling.

For routine histological examination using hematoxylin and eosin staining fragments of lung tissue were fixed in 10% neutral formalin with subsequent paraffin embedding and serial sectioning of 4 to 5 $\mu m$ thick [*Yanin V.L. et al., 2015*].

To evaluate the immunophenotype of the macrophageal pool the immunohistochemical (IHC) study was performed using CD68 (clone KP1) and VEGF-A (VG1 clone) markers. The proliferative activity of pulmonary stroma cellular elements was evaluated using Ki-67 marker (clone MIB-1). System of visualization EnVision™ FLEX+, Mouse, HighpH (Link), CodeK8012 with DAKO autostainer [*Dabbs D.J., 2006*].

Quantitative evaluation of positive cells was performed in 10 fields of vision at 200-fold magnification taking into account the zonal distribution (pyogenic layer, specific granulation tissue, fibrous layer, pericavernous zone, draining bronchus, and intact pulmonary tissue) and the patterns of interaction with other structural elements of the lung tissue. The obtained results were reported in absolute number of immunopositive cells per area of the field of view. Semi-quantitative evaluation of the level of IHC expression was performed in the pyogenic layer of the fibrous cavern as follow: «+++» high level, «++» moderate level, «+» low level, and «–» no expression.

Viewing and making photographs of slides was performed using a light microscope "Olympus CX-41", OLYMPUS DIGITAL CAMERA C5050

ZOOM. The morphometric data processing was performed using the licensed software ImageJ.

Statistical data analysis was performed using Statistica for Microsoft Windows software package, version 10.0 (StatSoft Inc., USA). Data were reported as M±SD, where M – arithmetic mean and SD – standard deviation. Statistical analysis included making of variation series of quantitative data, determination of distribution normality using the Kolmogorov-Smirnov test, calculating an arithmetic mean, standard deviation, mean error, coefficient of variation and percentage deviation compared to the control. The significance of compared values differences was determined using the non-parametric Mann-Whitney U-test at a significance level α=5%. To assess a statistical interrelationship Pearson's correlation coefficient was calculated [*Satake E.B., 2015*].

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### Results

Evaluation of Ki-67 expression in FCT regardless of bacillation activity has allowed to identify statistically significant increase in the proliferative activity of cells of mesenchymal and epithelial origin compared to the control group ($p<0.05$). Additionally, uneven expression in regard to cavernous pulmonary destruction focus and heterogeneity of cells with readiness to entry into mitosis cycle was observed (Fig. 1).

The pyogenic layer, presented in all FCT-MBT+ cases as a caseous necrosis, was characterized by the absence of the proliferative activity of any cellular elements, as well as diffuse positive expression of VEGF-A and CD68.

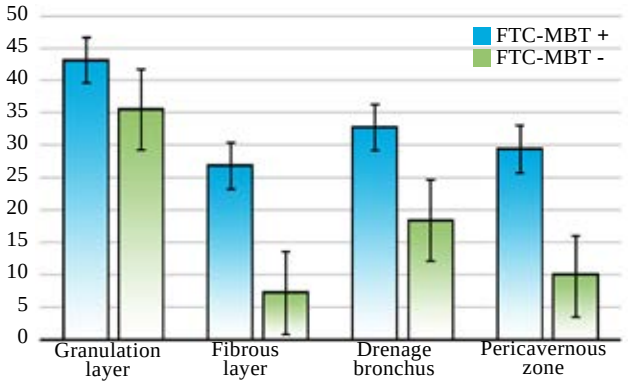


***Figure 1.*** *Graph of Ki-67 expression level distribution in lung tissue at FCT as a function of bacillation activity.*

IHC study in the specific granulation tissue has revealed that number of $CD68^+$ cells was 143.45 ± 5.27 (FCT-MBT+) and 117.32±3.03 (FCT-MBT-), and VEGF-A positive macrophages – 33.50±1.13 and 43.12±1.24 among them, respectively. The Ki-67 proliferative activity index also showed a peak value of 43.2±2.4 and 35.5±3.2 with no statistically significant differences between groups, but with a significant increase compared to the control group (Table).

Assessment of Ki-67 expression in the macrophageal population has allowed determining the positive nuclear staining in $CD68^+/VEGF^-$ cells characterized by the high phagocytic activity. However, the giant multinucleated Pirogov-Lang-

***Table***

Assessment of CD68, VEGF-A and Ki-67 marker expression levels in lung tissue at FCT according to bacillation activity.

| Zone | | Pyogenic layer | Granulation tissue | Fibrous layer | Draining bronchus | Pericavernous zone |
|---|---|---|---|---|---|---|
| MBT+ | CD68 | +++ | 143.45±5.27* | 122.30±6.11* | 103.00±4.31* | 86.74±3.28* |
| | VEGF-A | +++ | 33.50±1.13* | 38.01±1.27* | 35.54±2.07* | 42.12±1.12* |
| | Ki-67 | - | 43.20±2.40* | 26.80± 1.9 * | 32.80±2.50* | 29.41±1.08* |
| MBT- | CD68 | +/- | 117.32±3.72*# | 75.81±2.11*# | 84.69±4.52*# | 64.92±3.51*# |
| | VEGF-A | +/- | 43.12± 1.24 * | 29.41±0.92*# | 84.69±3.87*# | 47.24± 2.01 * |
| | Ki-67 | - | 35.5±3.20 * | 7.20 ± 2.10*# | 18.3 ± 1.90* # | 9.70 ± 1.01* # |
| Control | CD68 | 23.70± 0.03 | 23.70± 0.03 | 23.70± 0.03 | 23.7± 0.03 | 23.7± 0.03 |
| | VEGF-A | 10.02± 0.21 | 10.02± 0.21 | 10.02± 0.21 | 10.02± 0.21 | 10.02± 0.21 |
| | Ki-67 | 3.70± 0.04 | 3.7± 0.04 | 3.70± 0.04 | 3.70± 0.04 | 3.70± 0.04 |

***Notes:*** * - $p \leq 0.05$ compared to the control group; # - $p \leq 0.05$ compared to MBT+

hans cells were characterized by the negative reaction in all the cases. Furthermore, it was found that regardless of bacillation activity the cells in an active phase of the cell division cycle, apart from macrophages, are lymphocytes, fibroblasts of the immature connective tissue and vascular endothelial cells of newly formed vessels.

The fibrous layer of cavern in patients with FCT-MBT- was characterized by the decreased inflammatory infiltration: number of $CD68^+$ cells was 75.81±2.11, of which VEGF-$A^+$ macrophages – 29.41±0.92. The number of $Ki67^+$ lymphocytes, occasional fibroblasts and endothelial cells of differentiated vessels with positive nuclear reaction was 7.2±2.1. The number of macrophages with cytoplasmic macrosialin expression at bacteriologically confirmed FCT-MBT+ was also reduced with a parallel increase in the number of VEGF-$A^+$ cells. The predominant mitotically active cell population was $CD68^+$/VEGF-$A^-$ macrophages. Lymphocytes with positive Ki67 expression diffusely infiltrated the entire fibrous wall of the cavern, but the most pronounced accumulations were visualized in the germinal centers of lymphoid aggregates on the border with pericavernous area. Fibroblasts also actively proliferated. A comparative intergroup analysis of the number of Ki-67 positive cells showed a statistically significant increase in proliferative activity in the group with active bacillation ($p<0.05$) compared both to the control group (by 60%) and FCT-MBT- (by 30%).

Draining bronchus was characterized by the intrapopulation macrophageal ratio of 1:1 in the group with clinical abacillation, and Ki-67 was 18.3±1.9 positive cells with nuclear expression due to epithelial mucosa cells and active fibroblasts that synthesize thin and disorganized collagen fibers (Fig. 2).

IHC analysis of this zone in patients with FCT-MBT+ showed that the expression level of studied markers corresponds to the specific granulation zone with the prevalence of mitotically active $CD68^+$/VEGF-$A^-$ macrophages, lymphocytes and fibroblasts.

The pericavernous zone in FCT outside the foci of specific inflammation was characterized by alternating portions of alveolar collapse and alveolar emphysematous dilation with uneven Ki-67 expression, which intensity was directly correlated with the severity of exudative reactions (29.41±1.08) (Fig. 3). Thus, increase in the inflammatory processes as serous and/or caseous pneumonia was accompanied with intensification of proliferative activity of macrophages, lymphocytes and fibroblasts, suggesting the inductive effect of the inflammatory cells on collagen formation, i.e. initiation of the proliferation phase mechanisms.

At MBT- group a statistically significant decrease in the number of cells with mitotic activity (9.70±1.01) was observed due to the stabilization of sclerotic processes and predominance of inactive fibrocytes in mature fibrous tissue.

Regardless of bacillation activity, the dilated emphysematous areas were characterized by the minimal number of Ki-$67^+$ cells. These occasional positive cells were alveolar macrophages, located freely in the lumen of the dilated alveoli. Also, few positive alveolocytes fixed in the alveolar niche and active fibroblasts in thinned interalveolar septa were seen.

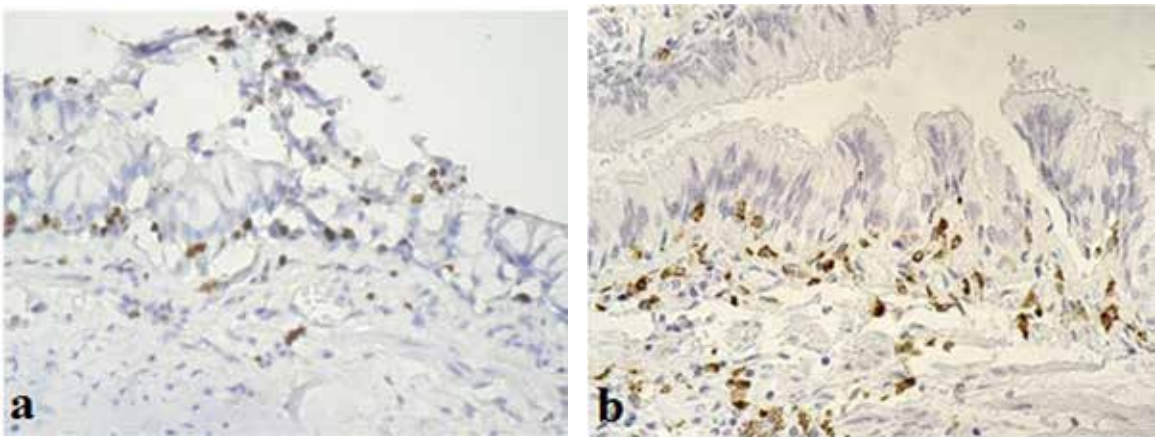


***FIGURE 2.*** *FCT-MBT-. IHC reaction in the wall of the draining bronchus:* ***a*** *– nuclear expression of Ki-67 marker, x400;* ***b*** *– cytoplasmic expression of CD68 marker, x400.*

***Discussion and conclusion***

Raising number of patients with progressive destructive forms of secondary tuberculosis and resistance to standard chemotherapy makes it relevant to search for potential cellular targets for the targeted intervention [*Golubinskaya E et al., 2018; Golubinskaya E, Kramar V, 2018*]. For that matter, we believe the most promising is antigen-presenting cells, such as macrophages. This is a key cell population, supporting the morphogenesis of tuberculosis granulomatous inflammation due to inefficient phagocytosis and intracellular persistence of M. tuberculosis [*Chistiakov D et al., 2014; Kim H et al., 2015; Aryanpur M et al., 2016*]. Experimental data on the heterogeneity of the macrophageal population suggest their inductive effect not only on the severity of immune reactions, but also on remodeling of the surrounding lung parenchyma [*Gordon S. et al., 2014; Italiani P. et al., 2014; Chávez-Galán L. et al., 2015*].

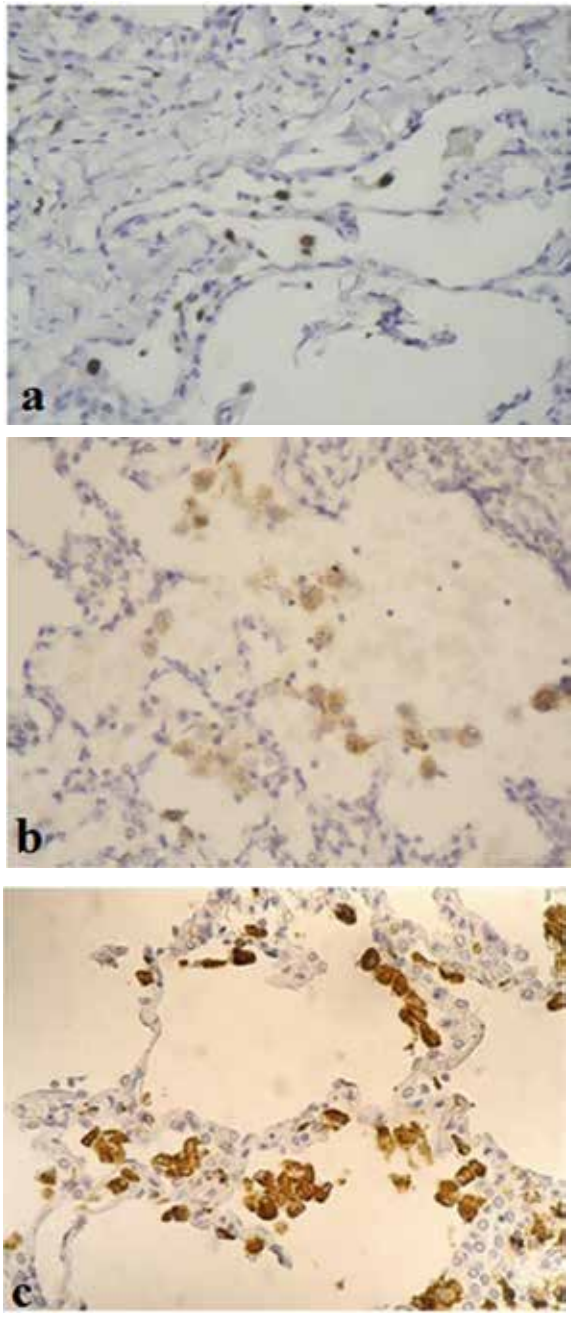


***Figure 3.*** *FCT-MBT+. IHC reaction at pericavernous zone:*
***a*** *– nuclear expression of Ki-67 marker, x400;*
***b*** *– cytoplasmic expression of VEGF-A marker, x400;*
***c*** *– cytoplasmic expression of CD68 marker, x400.*

Our study based on an assessment of vascular growth factor expression in patients with fibrous-cavernous tuberculosis has revealed the presence of two immunophenotypes of macrophages: VEGF-A$^+$ and VEGF-A$^-$, characterized by various localization and quantitative characteristics depending on bacillation activity. Furthermore, their potentiating effect on the proliferative activity of pulmonary stromal cellular elements, namely fibroblasts, endothelial cells and lymphocytes, was shown by means of correlation analysis.

The predominant Ki-67$^+$ cell population in biopsy samples of lung tissue in patients with active bacillation was highly active CD68$^+$/VEGF-A$^-$ macrophages that diffusely infiltrate specific granulation tissue, fibrous layer, pericavernous zone and draining bronchus. It is associated with intensification of exudative reactions and phagocytosis, as well as formation of perifocal areas of serous/caseous pneumonia. Correlation analysis has revealed a strong direct correlation ($R = 0.821$, $p<0.05$) between the number of CD68$^+$ macrophages and bacillation activity with increased proliferative activity of inflammatory cells, namely lymphocytes (Figure 4). Parallel to this, there was a proportional increase in the population of actively proliferating fibroblasts and endothelial cells, which indirectly indicates the hyperactivation of their synthetic activity, collagen-producing function and angiogenesis of imperfect vessels, leading to irreversible remodeling of the pulmonary parenchyma such as fibrous transformation and critical increase in respiratory failure.

IHC analysis of the lung fragments of patients with FCT-MBT- indicates on the stabilization of alterative and exudative processes by increasing the fibrous transformation of cavern wall in the form of lack of caseous necrosis zone, reduction in

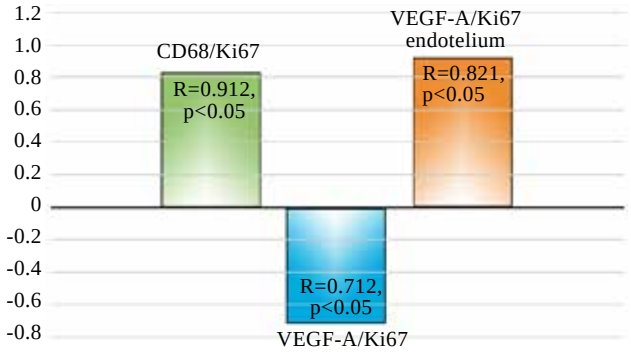


***FIGURE 4.*** *Graph of correlation coefficient of the proliferative activity of stromal cell elements as a function of the immunophenotype of macrophages in the lung tissue at FCT.*

area of the specific granulation tissue and increase in pneumosclerosis at pericavernous zone and surrounding intact pulmonary tissue. Macrophageal pool undergo the substantial intrapopulation reorganization due to increased number of $CD68^+$/$VEGF\text{-}A^+$ cells without signs of proliferative activity. At the same time, a direct correlation was revealed between the number of macrophages with VEGF-A immunophenotype ($R=0.912$, $p<0.05$) with the mitotic activity of endothelial cells of both newly formed and differentiated vessels. As to the potentiating effect of macrophages with VEGF-A positive immunophenotype, a direct correlation with the increase in the number of fibrocytes ($R=0.654$, $p<0.05$), which are elongated spindle-shaped cells with a small nucleus and which are involved into the final stage of the connective tissue formation, as well as inverse correlation with the bacillation into the environment ($R = -0.712$, $p<0.05$) was shown.

Thus, in fibrous-cavernous tuberculosis it is present two immunophenotypically different subpopulations of macrophages $CD68^+/VEGF^-$ and $CD68^+/VEGF^+$ with an inductive effect on the severity of immunological reactions, as well as remodeling of the pulmonary parenchyma in the form of pathological angiogenesis and pneumofibrosis are recorded.

As the severity of alterative-exudative processes increases and/or the proximity to the site of specific inflammation, an increase in the pool of $VEGF^-$ macrophages characterized by high and moderate phagocytosis activity is noted.

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