DOI: 10.15825/1995-1191-2023-3-97-105

EQUIVALENTS OF THE NEUTROPHIL-TO-LYMPHOCYTE RATIO OF CIRCULATING POOL OF STEM AND IMMATURE HEMATOPOIETIC CELLS FOR ASSESSING LIVER TRANSPLANT STATUS

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Objective: to study the applicability of the neutrophil-to-lymphocyte ratio (NLR) for monitoring recipient status and for possible minimization of maintenance immunosuppression in the long-term period after liver transplantation (LT). **Materials and methods.** Blood samples of 19 recipients with satisfactory graft function were examined by flow cytofluorometry at various time periods after LT using hematopoietic stem cell markers CD133, their CD31 derivatives, and alpha-fetoprotein (AFP), compared with the conventional NLR. **Results.** The use of NLR equivalents with CD133 and CD31 to assess liver transplant status is due to their high representation in liver tissue. Their values change in the long-term posttransplant period (from 1.5 to 6–7 years following LT) ≈20-fold and in different directions, but only when measuring their commissural to the liver cell fractions bearing the AFP marker. **Conclusion.** In contrast to the conventional NLR, maintenance of the lowest level of CD31 AFP, an NLR "equivalent", achieved at 1.5 years after LT, can be considered a criterion for the success of immunosuppressive therapy in the long-term post-LT period. The developed technique can be used to decide on whether to reduce or discontinue medication-assisted prophylaxis of graft rejection.

Keywords: neutrophil-to-lymphocyte ratio, hematopoietic stem cells (HSCs), CD133, CD31, alpha-fetoprotein, liver transplantation.

INTRODUCTION

The impact of immunosuppressive therapy in the long-term period after liver transplantation (LT) is associated with a whole set of complications that reduce the lifespan of recipients. Among the causes of negative outcomes, the leading ones are malignancy, infections, cardiovascular and nephrological problems [1]. Therefore, it is pertinent to search for rational ways to reduce the undesirable effects of post-LT immunosuppression. Based on analysis of modern literature, minimizing immunosuppression up to complete cancellation is considered the main approach, along with intraoperative and delayed tolerance induction, individualization and rationalization of regimens in order to reduce the incidence of side effects of drugs. From the clinical perspective, immune tolerance is defined as the preservation of stable graft function in a recipient who is not taking immunosuppressants. Unfortunately, the results of experimental studies on the mechanisms of tolerance have not yet revealed reliable biomarkers of tolerance [2]. Given the complexity and inconsistency of molecular mechanisms, the only reliable way to confirm tolerance is the absence of graft rejection after deliberate cessation of immunosuppression.

In the search for a reliable control of the recipient's condition while minimizing immunosuppression, the authors paid attention to the neutrophil-to-lymphocyte ratio (NLR), which is considered a simple and universal criterion for the severity of various human pathologic conditions [3]. Increased neutrophil count is a marker of inflammation, while low lymphocytes reflect stress and hypocellularity of hematopoietic tissue [4]. NLR can be used in the selection of prospective transplant patients [5]. NLR measured at 12 months after LT predicts overall survival over the next 7–9 years and correlates closely with markers of nutritional adequacy [6]. The shortening of lymphocyte telomeres with age exceeds that of granulocytes, indicating indirectly a greater expenditure of young lymphoid cells to ensure the vital activity of the body, and, possibly, the contribution of poorly differentiated lymphoid cells in the formation of prognostic properties of the NLR indicator. In addition, some young cells are "committed" to the liver tissue by the presence of the alpha-fetoprotein (AFP) marker [7–11].

The problem of long-term survival, as well as maintenance of the functional state of a liver transplant in some recipients, may be associated with depletion of the proliferative potential of the bone marrow lympho-

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cytic sprout (the product of the number of blood stem/progenitor lymphocytes by mitotic activity), the value of which limits life expectancy during natural aging of the body [12]. However, there is no direct information in the literature about the use of the indicator to control the volume of immunosuppressive therapy in the long-term period after LT. Taking into account all the features of immature cells, the authors suggested that the NLR equivalents with such cells can be a more sensitive indicator compared to the generally accepted NLR, in particular, in solving the problems of minimizing immunosuppressive therapy in the long term.

Objective: a comparative study of conventional NLR and its equivalents with blood cells of low differentiation/maturity for more reliable monitoring of recipients' condition and making further decisions on minimizing maintenance immunosuppressive therapy in the late post-LT period.

MATERIALS AND METHODS

Patients. The results of examination of 19 liver transplant recipients in the laboratory of transplantation and stem cell research at Granov Russian Research Center of Radiology and Surgical Technologies were studied. The patients were observed from 5 days to 120 months after LT, 9 men, 10 women. The average age during the operation was 44.9 ± 9.1 years. During the entire follow-up period, clinical and biochemical blood tests, abdominal ultrasound with elastometry were performed, tacrolimus blood levels were monitored and maintained at 3–5 ng/mL. Mean NLR at 1, 3, 5 and 10 years after LT was calculated. The distribution by nosologic variants before LT is shown in Fig. 1. Graft function was considered satisfactory if there were no deviations from normal serum levels of bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase, and gamma-glutamyl transferase (GGT), if there were no circulatory disorders in the graft according to ultrasound and multi-slice spiral computed tomography (MSCT) data over time.

Materials. 7–8 mL blood samples were obtained at various times after LT and analyzed on the day of receipt, without storage. The viability of mononuclear cells (MNCs) from the entire interphase zone of the Ficoll-Paque density gradient was monitored by trypan blue exclusion test. Before cytometric phenotyping, cells were stained according to standard procedures to detect shapes in the synthetic (S) and mitotic (M) phases of the cell cycle with Hoechst 33342 reagent (bis(benzimidazole) fluorochrome; Sigma-Aldrich, St. Louis, Missouri, USA). CD133, CD31 cells, CD133AFP and CD31AFP double positive cells were stained using the standard Miltenyi Biotec protocol for CD133/2 antibodies conjugated to allophycocyanin (APC), BD Bioscience Pharmingen protocol for CD31 antibodies conjugated with fluorescein isothiocyanate (FITC), and R&D Systems protocol

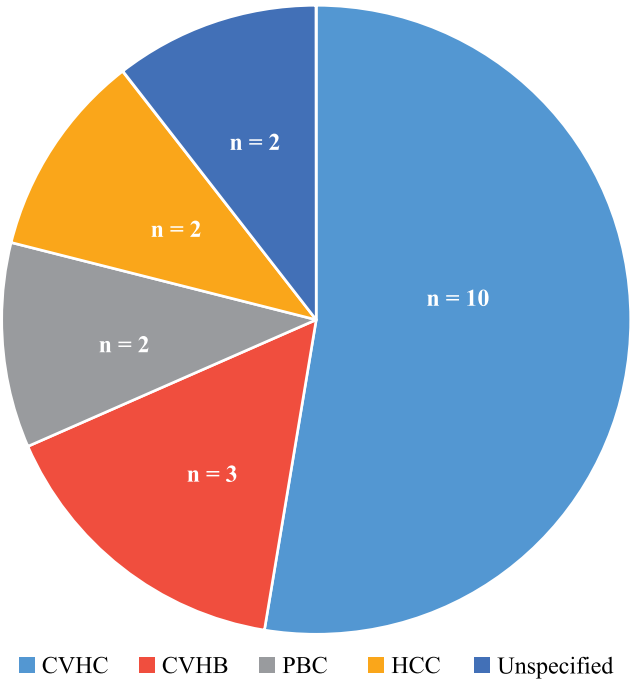

Fig. 1. Variants of liver cirrhosis before LT: CVHC, chronic viral hepatitis C; CVHB, chronic viral hepatitis B; PBC, primary biliary cholangitis; HCC, hepatocellular carcinoma; Unspecified, unspecified cirrhosis

for AFP antibodies conjugated with phycoerythrin (PE). An LSRFortessa flow cytometer (Becton-Dickinson, San Jose, CA, USA) was used.

Lymphocyte and granulocyte fractions were separated using forward scatter (FSC) and side scatter (SSC) light scatter plots, and cellular debris was excluded. Red laser (640 nm, 40 MW) was used to detect CD133+ cells, blue laser (480 nm, 50 MW) was used to detect AFP and CD31 cells, and ultraviolet (UV) laser (355 nm, 20 MW) for Hoechst 33342-labeled cells. The percentage of positive cells was calculated by subtracting the value for antibodies of the corresponding control isotype. At least 500,000 events were recorded twice to detect CD133 cells. A dot plot of Hoechst 33342 emission in blue (x-axis) and red (y-axis) wavelengths was used to separate the (G0 + G1), S and (G2 + M) phase events. Individual parameters were evaluated statistically with calculation of the mean value M, standard deviation (±σ) and standard error (±*m*). Mean values M were compared using the Student's t test and probability *p*. Parameter relationships in the graphs were analyzed by approximating the data points with regression lines that are automatically performed and described by mathematical functions in Excel, including the fit coefficient R^2.

The graphs show only those regression lines that had the maximum R^2 value, which means the marginal correspondence between the location of the points and the selected type of approximating curve / mathematical function from all those offered by the program (linear, exponential, exponential, power, logarithmic, step, po-

lynomial, parabolic). The equations for the regression lines are shown in the graphs. As a statistical measure of compliance of regression lines with the data entered into the program, we used the generally accepted criterion of reliability p (≤0.05), determined by t-test = $R/m_R = \sqrt{R^2 \times (n-2) / (1-R^2)}$ [12]. Equations for the regression lines are shown in the graphs.

RESULTS

The calculated mean NLR in recipients over a period of 3–5–10 years did not differ significantly – 2.19 ± 0.63, 2.17 ± 0.87 and 2.1 ± 0.58 according to the clinical blood test. The dynamics of neutrophil and lymphocyte content, according to flow cytometry data (see Figs. 2 and 3), is multidirectional, indicating maximum mean values of NLR up to 7 during the first 10 days after LT, followed by normalization to ≈1 by 250–500 days, and then repeated prolonged increase in NLR up to ≈4 by eight years after LT. Therefore, the entire study time was divided into relatively early and late periods. The primary decrease in NLR in the relatively early period, up to one and a half years, should be regarded as a positive effect of LT. It starts at a mean value slightly higher than the mean NLR level in those awaiting transplantation (see Fig. 3, black square in the graph), which has been determined in earlier studies. However, deviations of the mean NLR values in the early and, especially, in the late periods, were comparable to the data already known from literature, therefore, did not satisfy the objectives of the study.

Over time after LT, the percentage of lymphocytes in the synthetic phase (S phase) of the cell cycle increases, and the mitosis-to-synthesis (M/S) ratio decreases (see Fig. 4). The mean S phase in the late period (2.98 ± 0.74) is 8 times (p = 0.003) higher than that of the early period (0.35 ± 0.22). In contrast, the mean M/S ratio in the late period (0.051 ± 0.023) is 80 times (p = 0.003) smaller than the mean M/S ratio in the early period (4.25 ± 3.49), which, however, is not statistically confirmed (p = 0.21). This combination indicates a turbulent proliferation regime, classified as abnormal (synchronous). Nevertheless, the general downward trend in M/S in the combined periods (dashed line in Fig. 4) is confirmed by power law approximation: $M/S = 5.13x^{-0.649}$, $R^2 = 0.362$, $p < 0.001$. Thus, lymphocyte DNA synthesis in the long-term post-LT period increases, but this is not accompanied by increased mitotic activity, indicating turbulent lymphocytopoiesis and increased likelihood of cell apoptosis in the pre-mitotic phase of the cell cycle. In general, synthetic activity does not satisfy the task of the study, although it complements the characterization of the long-term period by a significant deficit of cell divisions in it.

The mean values of NLR equivalents in relatively early and late periods for CD133, CD133AFP, CD31, and CD31AFP subpopulations are presented in Table. Only the values of granulocyte pools (G) are shown, because the NLR is the result of arithmetic division of the percentage of the granulocyte subpopulation by the percentage of the lymphocyte subpopulation. The granulocytic constituents of NLR and its equivalents decrease by no more than 5-fold in the long-term period, except for CD133AFP. The expected decrease in the equiva-

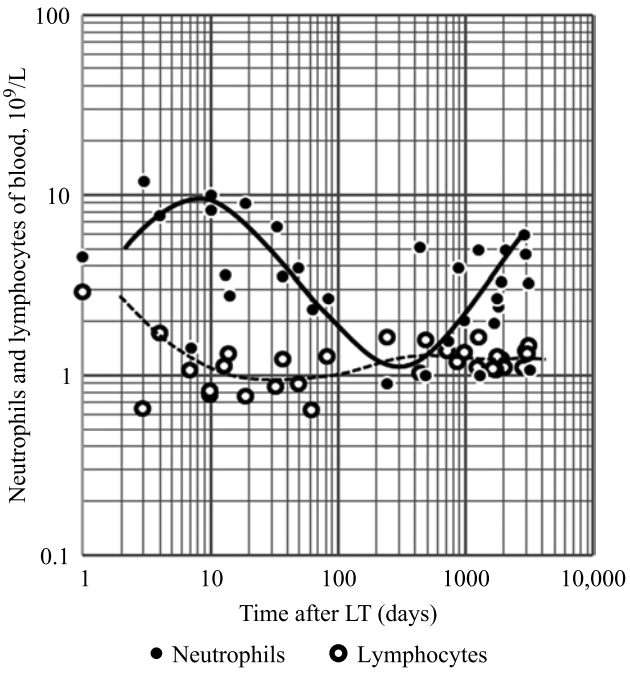

Fig. 2. Dynamics of lymphocytes and neutrophils over a long period after LT

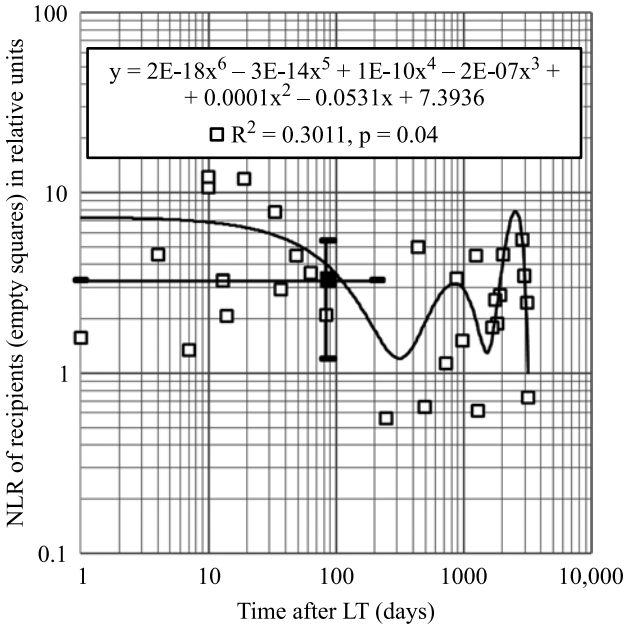

Fig. 3. Dynamics of normal NLR within the early (up to 350 days) and late (up to 3200 days) periods after LT. The approximation line shows the likely dynamics of the average indicator (p = 0.04). Black square: mean (M ± σ) for NLR of 12 patients from the LT waiting list

lents themselves does not occur, except for CD31AFP, where it is disproportionately large (≈20-fold), indicating a parity increase in the lymphocytic component of this equivalent as well. Based on period-averaged data (see Table) and comparison with conventional NLR, the CD31AFP subpopulation was not only quantitatively but also statistically preferable.

Consideration of the kinetic characteristics of CD31AFP confirms and complements this conclusion (see Fig. 5). The early decrease in NLR equivalent for liver-committed CD31AFP cells is significant (Fig. 5, center, Table), in contrast to non-committed CD31 (Fig. 5, left). The decrease in the values of the CD31AFP equivalent in the early period occurs in phase with a decrease in the conventional NLR indicator (Fig. 3), which can be interpreted as a favorable sign. There is a subsequent rise of CD31AFP in the long-term period due to a parallel rise in the granulocytic and a fall in the lymphocytic components of the equivalent (Fig. 5, right). This phenomenon may underlie late problems in recipients associated with maintenance immunosuppressive therapy. The kinetics of changes in the CD133AFP

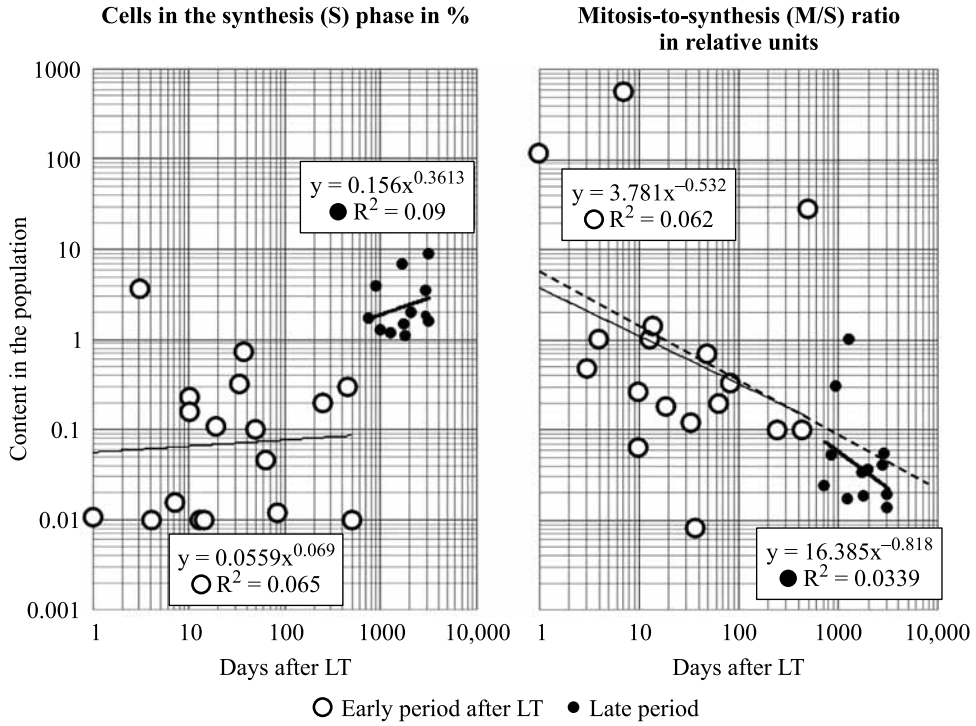

Fig. 4. Proliferative activity of lymphocytes after LT. The dotted line is the direction of change common to the two periods. P values for all equations >>0.05

Table

Mean values of "equivalents" of granulocyte/lymphocyte ratios (M ± m) for CD133, CD133AFP, CD31, CD31AFP subpopulations at relatively early (E) and late (L) periods after LT

Parameters	Normal NLR*	NLR equivalent				Mean time of early and late periods (M ± σ) in days
		CD 133	CD133AFP	CD31	CD31AFP	
Granulocytes, %	5.27 ± 0.83*	0.72 ± 0.145	0.308 ± 0.096	59.04 ± 6.36	57.33 ± 6.44	E 89 ± 153* E 29.7 ± 30
Granulocytes, %	3.1 ± 0.42*	0.257 ± 0.043	0.527 ± 0.428	12.83 ± 2.01	10.72 ± 2.1	L 1891 ± 850* L 1937 ± 905
p (between periods)	0.048*	0.008	0.62	<0.001	<0.001	<0.001* <0.001
NLR	6.8 ± 1.83*	13.85 ± 3.78	48.9 ± 22.57	10.9 ± 4.97	229.86 ± 60.65	E 89 ± 153* E 29.7 ± 30
NLR	2.6 ± 0.39*	7.97 ± 2.57	44.81 ± 11.74	2.89 ± 0.79	10.58 ± 3.44	L 1891 ± 850* L 1937 ± 905
p (between periods)	0.04*	0.21	0.53	0.13	0.003	<0.001* <0.001

* Data with normal NLR are given for comparison.

equivalent (Fig. 6, right) are inverted with respect to the CD31AFP equivalent. Its significant decrease in the long-term period (p = 0.0015) makes the CD133AFP equivalent the second contender for determining the graft condition, but only in the later stages, as its average values in two periods do not differ significantly (see Table). The decrease in CD133AFP in the long-term period is down to a significant decrease in the granulocytic component in the total CD133AFP pool and a moderate decrease in the lymphocytic component. The most probable mechanism for the inversion of kinetic trends of CD31AFP lymphocytes and CD31AFP granulocytes in the long-term period (Fig. 5, right) is that CD31 cells are the closest progeny of CD133 hematopoietic stem cells (HSC) in the series of sequential differentiation. In this case, quantitative changes in the opposite direction occur only when cells are produced in the mode of symmetric (depleted) hematopoiesis, which is confirmed by the growing deficit of mitotic activity (see Fig. 4).

So, we have identified two NLR equivalents – with CD133AFP and CD31AFP, which are promising for monitoring recipients in the long term, which significantly

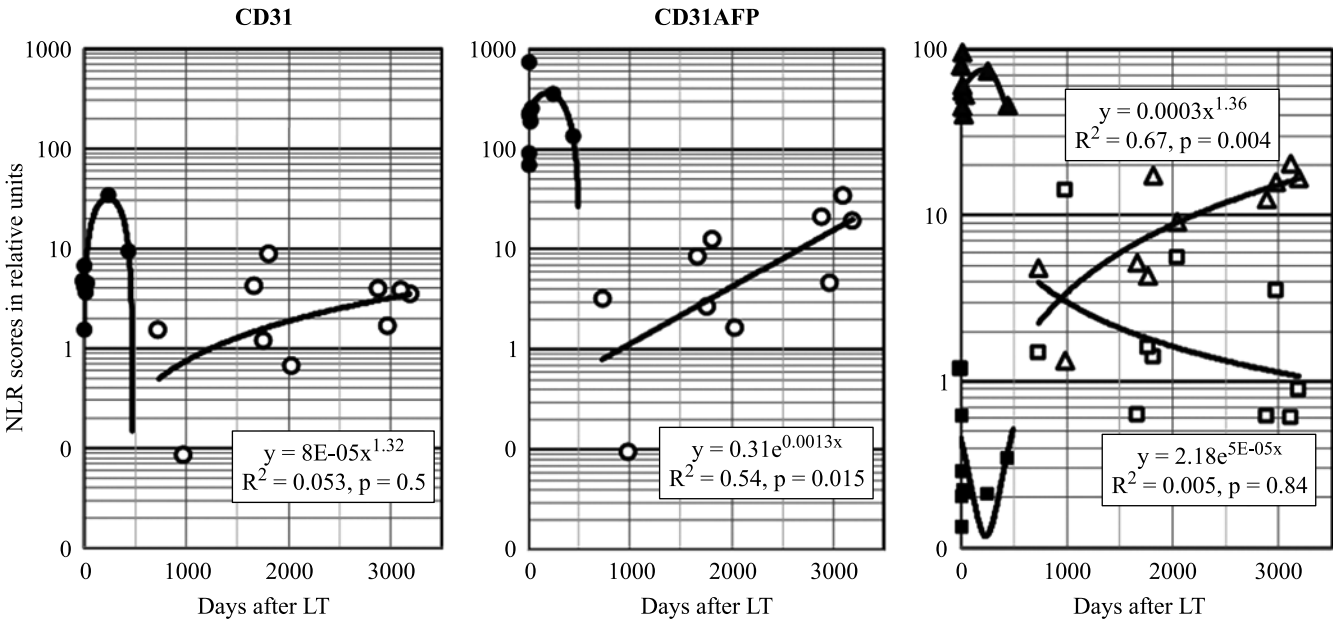

Fig. 5. Changes in NLR scores over time after LT for CD31 and CD31AFP subpopulations. Black icons represent the early period, white icons represent the late period. Solid approximation lines in Excel are given for both periods. Equations for the approximation lines are given in the boxes on the graphs for the late period only. Circles, NLR; triangles, CD31AFP in the granulocyte pool in %; squares, CD31AFP in the lymphocyte pool in %

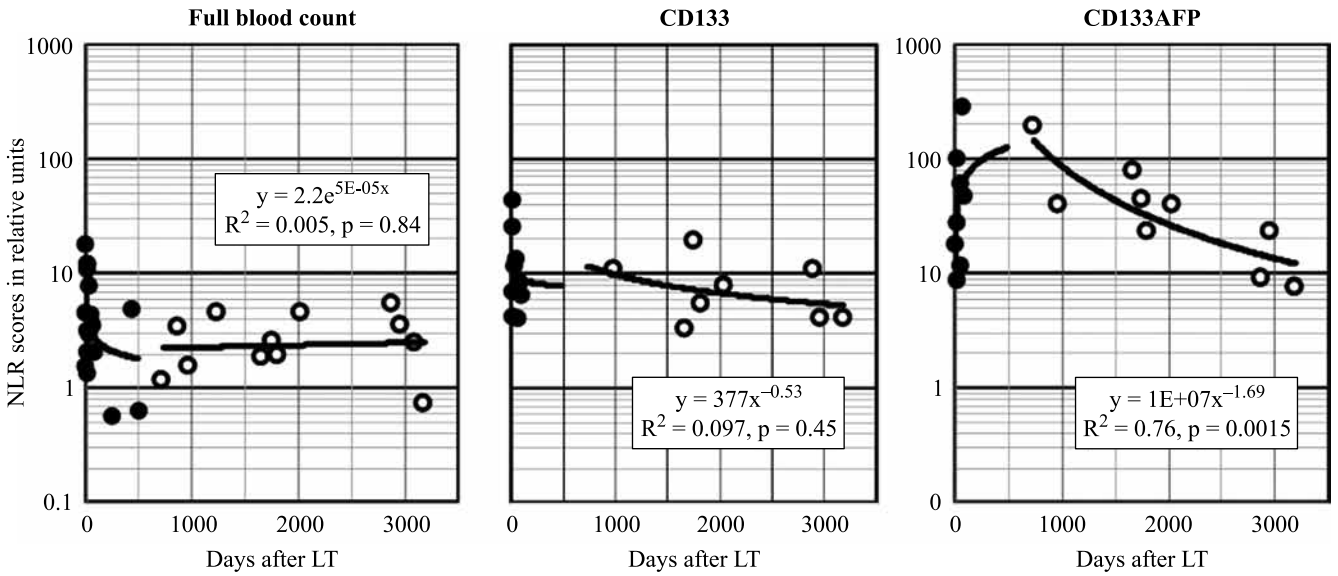

Fig. 6. Changes in NLR scores over time after LT for CD133 and CD133AFP subpopulations. Black icons represent the early period, white icons represent the late period. Equations for the approximation lines are given in the boxes on the graph

exceed the capabilities of conventional NLR. Both equivalents change smoothly and statistically significantly over the long-term period ≈20-fold, whereas the conventional NLR is virtually unchanged (Fig. 3, dashed line; Fig. 6, left) between 17 and 106 months after LT. Oppositely-directed exponential changes in the two NLR equivalents occur with a doubling period of ≈1.5 years. The prognostic capabilities of these indicators require further studies in the context of immunosuppressive therapy minimization.

DISCUSSION

Based on data obtained, the relatively early period from 0 to 1.5 years after LT seems to be optimal for identifying recipients that are most resistant to graft rejection according to the criterion of the maximum rate of decrease in the CD31AFP equivalent value. Over the late period, the NLR equivalent CD31AFP steadily increased. In parallel, there was a slow depletion of poorly differentiated, morphogenic, CD31AFP-lymphocytes committed to the liver tissue. At the same time, the opposite dynamics of lymphoid and myeloid components of CD31AFP cells may reflect the gradual depletion of lymphopoiesis with the predominance of the myeloid component over the depleting lymphoid one, like what happens in natural aging [13].

A significant reduction in the total pool of CD133AFP stem cells among 133AFP granulocytes ($p < 0.001$), along with a moderate decrease in 133AFP lymphocytes ($p = 0.06$), form a late decrease in NLR equivalent CD133AFP. Since in normal liver, there should be a constant repopulation of either pluripotent or rapidly dividing young AFP-positive cells, the identified changes may signify a progressively increasing threat to graft viability and recipient [14]. In the long-term post-LT period, one can assume the development of devascularization processes with the subsequent development of fibrosis, which requires further study.

If we allow for the influence of cells from the circulation on the migrant spectrum directly in the transplanted liver tissue, it first normalizes to optimal by 1–1.5 years after LT and then gradually depletes by 8–9 years.

Data from modern studies have proven the morphogenic properties of HSCs and their immediate undifferentiated progeny. For instance, the comparative analysis of marker composition in a normal liver showed that, like placental tissue, it is strongly polarized towards the predominance of young migrant cells compared to their content in blood [15], HSCs of double positivity for CD34 and CD133 give rise to both early endothelial precursors CD31 [16] and lymphoid stem cell lineage with terminal deoxynucleotidyl transferase marker TdT+ [15, 17–19].

Programmed death ligand-1 (PD-L1, CD274) plays an important role in processes such as tissue transplantation, pregnancy, autoimmune diseases, hepatitis, etc. [20]. Its expression on circulating CD34 HSCs closely correlates with T cell apoptosis. Apoptosis is associated with subsequent delivery of TdT into the intercellular medium and reutilization of degradation products by neighboring viable cells during regeneration [7].

Terminal-interacting protein deoxynucleotidyl transferase enhances the proliferative activity of TdT+ and vasculogenic properties of CD34 HSC [20]. Therefore, according to the authors' opinion, NLR equivalents have more informative value when evaluating recipient and graft condition in the long-term post-LT period, as well as when trying to minimize maintenance immunosuppressive therapy as a monitoring component.

CONCLUSION

Results obtained from the study suggest that maintenance of the lowest level of NLR equivalent CD31AFP, achieved by 1.5 years after LT, can be considered a criterion for the adequacy of maintenance immunosuppressive therapy in the longer period. The developed method for monitoring recipient and liver graft conditions can be used for decision making and monitoring when reducing or stopping immunosuppression.

The authors declare no conflict of interest.

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The article was submitted to the journal on 20.03.2023