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Original Article

The state of the immune system in patients with cardio rheumatic diseases and concomitant metabolic-associated liver steatosis

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Abstract

Liver steatosis, the earliest stage of metabolic-associated non-alcoholic fatty liver disease (MAFLD), is the most common form of liver damage. However, the specific immune response linked to this condition remains poorly understood. This study aimed to assess immune system parameters and their relationships in patients with cardiorheumatic diseases who also have metabolic-associated liver steatosis (MALS). A total of 53 patients were included in the study: 32.07% had ischemic heart disease, 39.62% had haemorrhagic vasculitis, and 28.31% had rheumatic fever. Various immune parameters were measured, including different lymphocyte subtypes, immunoglobulin levels, immune complexes, complement components, and phagocyte activity. In patients with MALS, immune disturbances were characterized by significant shifts in B-cell activity, elevated immune-effector indices, increased small immune complexes, and complement component levels. Low-affinity B-cells emerged as central in immune system interactions, showing significant correlations with various immune cells, such as T-cells, T-suppressors, NK cells, and IL-2 receptor-bearing T-cells, as well as with the immunoeffector index. Key immune mechanisms associated with MALS included activation of the humoral immune response, the complement system, immune complex formation, and enhanced antibacterial activity in phagocytes.

Keywords: metabolic-associated liver steatosis, T-cells, B-cells, circulating immune complexes, complement, spontaneous NBT reduction test.

Introduction

Metabolic-associated non-alcoholic fatty liver disease (MANFLD) has become the most prevalent liver disease worldwide [1, 2], which may be explained by the high prevalence of obesity, diabetes, and cardiovascular diseases, reaching epidemic ranges [3]. Liver steatosis is the earliest stage of MANFLD, affecting approximately one billion people on the planet [4]. Although complex metabolic abnormalities, changes in intestinal microbiota and immune response are well-established pathogenic factors of non-alcoholic fatty liver disease (NAFLD) [5, 6], there are few reports in the literature about the role of immune mechanisms in the case of isolated metabolic associated liver steatosis (MALS) [1]. Most of these reports consider steatosis together with steatohepatitis [5, 7], which is characterized by the activation of T-cells and B-cell responses via secretion of pro-inflammatory cytokines, Kupffer cells stimulation with subsequent stimulation of the inflammatory process [1]. Some subpopulations of T-cells (i.e., T-helpers – Th1, Th2, Th17, Th22, regulatory Treg, cytotoxic Tc) potentiate the effects of adipose tissue, change insulin sensitivity, cause necrosis of hepatocytes, and stimulate liver fibrosis [8]. The examination of 64 patients with NAFLD demonstrated an increased Th17 activity with an abnormal Th17-to-Treg ratio. The Th17-to-Treg ratio in the liver ≤1.15 was associated with activation of inflammation, oxidative stress, and fibrosis [9]. Particular attention in the pathogenesis of NAFLD is paid

to the role of natural killers (NK-cells), the number of which doubles in this condition, affecting inflammation and fibrosis through the production of interleukin (IL)-4, osteopontin, and interferon-gamma. However, this subpopulation is also not homogeneous, as pro-inflammatory and anti-inflammatory NK cells have been described [1].

In the experimental model of MALS in mice, steatosis was associated with an increased number of T cells (CD4+, CD8+, γδT cells) and B cells in the liver tissue, while the amounts of NK cells, monocytes, and Kupffer cells were decreased [7]. Although there is much evidence about the essential role of T-helper cells (CD4+) in the pathogenesis of steatohepatitis, the roles of certain subtypes of these cells remain unclear. Thus, Th1 and Th17 are considered to have pro-inflammatory and pro-fibrotic actions, whereas Th22 and Treg have both anti- and pro-fibrotic properties, and the influence of Th2 is unknown [7]. According to other data, an increased number of Th17 in the liver and a decreased Th17/Treg ratio in peripheral blood characterize the progression from MALS to steatohepatitis [10, 11].

However, the state of the cellular and humoral links of the immune system in patients with concomitant isolated liver steatosis still needs to be clearly distinguished. We hypothesize that changes in immune system activity play some role in the pathogenetic mechanisms and regulation of different processes during the beginning of the MANFLD - liver steatosis stage, before steatohepatitis appearance, which can be checked by correlative interactions between parameters. All this determines the relevance and scientific novelty of our study.

The study aims to evaluate the state of the immune system parameters and their correlations in patients with cardiorheumatic diseases and concomitant MALS.

Material and methods

Study design and patients

The results of the investigations of 53 patients (27 males, 26 females, mean age 48.74±2.09 years (y.o.), body mass index (BMI) 29.70±1.06 kg/m^2) with cardiorheumatic diseases and concomitant MALS were included into retrospective study (inpatients medical card, form 003/o). All patients were treated as inpatients according to Ukrainian Ministry of Health guidelines and amenable to the Declaration of Helsinki principles. Among patients 32.07% were with ischemic heart disease diagnosis (57.71±1.35 y.o., BMI 28.69±0.97 kg/m^2), where the immune system does not play the leading pathogenic role; 39.62% patients (49.39±2.55 y.o., BMI 31.33±1.30 kg/m^2) had haemorrhagic vasculitis with a leading role of the immune system in the pathogeneses [12], and 28.31% patients (39.11±2.37 y.o., BMI 29.09±0.92 kg/m^2) had a rheumatic fever that is an acute inflammatory disease. Liver steatosis (MALS) was diagnosed using liver ultrasound in the presence of the following signs: diffusely increased liver echogenicity, poor visualization of the walls of the portal veins, dorsal attenuation of echo-signal; the normal range of the aspartataminotransferase, alaninaminotransferase and other liver functional probes. The following conditions were exclusion criteria for the study: pregnancy, viral hepatitis, non-alcoholic or alcoholic steatohepatitis, malignant tumors, liver failure, and advanced heart or respiratory failure.

Laboratory data collection

The state of the immune system was assessed by determination of the following parameters: the absolute lymphocyte count, lymphocyte counts with the following clusters differentiation: CD3+, CD4+, CD8+, CD16+, CD19+, CD22+, CD23+, CD25+, CD71+, CD95+, which were defined using monoclonal antibodies (State Medical Institute, Belarus). Further calculation of the following two indices was performed using the formula: immunoregulatory index (IRI=CD4+/CD8+) and immunoeffector index (IEI=active T-lymphocytes/total T-lymphocytes). The content of immunoglobulins (IgM, IgG, IgA, IgE) was assessed according to C. Mancini *et al.* (1965). Circulating immune complexes (CIC) were measured by precipitation in polyethylene glycol. C3 and C4 complement components were detected by the immunoturbidimetric method. A spontaneous nitroblue tetrazolium (NBT) reduction test was performed.

Statistical analysis

Statistical analysis was performed using the program Statistica for Windows 6.0 (Statsoft, USA). Data were presented as Mean (M) ± standard deviation (SD). The Pearson correlation coefficient (r) was used to assess correlation. P-value<0.05 was taken as the threshold of statistical significance. Variability in checked immunological parameters was assessed by percentage grading system method – changes ≥20% above or below

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reference ranges were considered as deviations with the following grading: grade 1 (±1) – 20–33%, grade 2 (±2) – 34–66%, grade 3 (±3) ≥67%. Changes under 20% above or below reference ranges were considered normal variant deviations.

Results

In patients with MALS, the mean absolute number of lymphocytes, the mean relative counts of T-suppressors (CD8+), B-lymphocytes (CD19+), and B-lymphocytes with IL-2 receptors (CD25+), as well as IgG and large circulating immune complexes levels were within normal ranges (Table 1). Higher than normal levels were detected in CD22+ (B-cells), CD95+ (NK-cells), CD23+ (B-cells with receptors to Ig E), small circulating immune complexes contents, and in the spontaneous NBT reduction test and immunoeffector index meanings.

Reductions within the range of 20% were observed in the numbers of T-cells and T-helpers and in IgA levels, whereas elevations up to 20% were found for NK-cells (CD16+) number, immunoregulatory index, IgM, IgE, total circulated immune complexes and medium circulated immune complexes. Other parameters were changed more significantly. Grade 1 elevations were observed in the numbers of CD71+ lymphocytes (127.60%) and C4 level (125.00%). Grade 2 elevations were seen in B-cell count (162.10%), immunoeffector

Table 1: Immunological parameters in patients with concomitant MALS.

Parameter, units	Reference ranges of the laboratory	MALS (M±SD)	MALS(M±SD) to normal range (M±SD) Ratio (%)
Lymphocytes, absolute number x10^9/L	1.6–2.4	1.86±0.13	100.00%
CD3+ (T-cells), %	50–80	49.12±3.88	90.48%
CD4+ (T-helpers), %	33–46	33.21±3.00	91.54%
CD8+ (T-suppressors), %	17–30	19.38±1.41	100.00%
Immunoregulatory index (CD4+/CD8+)	1.4–2.0	1.94±0.32	113.00%
CD16+ (NK-cells), %	12–23	23.55±2.48	113.70%
CD22+ (B-cells), %	17–31	41.06±9.19	162.10%
CD25+ (B-cells with receptors to IL-2), %	13–24	21.81±1.48	100.00%
CD95+ (NK-cells), %	5–7	14.34±1.64	228.28%
CD19+ (B-cells), %	5–25	18.81±1.19	100.00%
CD71+ (B-cells with receptors to transferrin), %	8–15	14.73±4.41	127.60%
CD23+ (B-cells with receptors to Ig E), %	6–9	18.00±3.00	233.33%
Immunoeffector index (IEI)	0.25–0.35	0.41±0.09	142.86%
Ig M, g/L	0.5–1.9	1.46±0.55	105.89%
Ig G, g/L	8–16	12.60±2.70	100.00%
Ig A, g/L	1.4–4.2	2.20±0.88	94.28%
Ig E, IU/mL	10–150	134.44±32.57	111.34%
Circulating immune complexes, total, U	0–15	15.11±1.82	112.87%
Circulating immune complexes, large, U	60–150	91.87±27.06	100.00%
Circulating immune complexes, medium, U	200–300	259.22±61.54	106.92%
Circulating immune complexes, small, U	300–400	550.37±73.96	156.08%
Spontaneous NBT reduction test, %	≤10	17.33±0.81	181.40%
C3 component of complement, g/L	0.9–1.8	1.99±0.49	137.78%
C4 component of complement, g/L	0.1–0.4	0.46±0.04	125.00%

index (142.86%), levels small CIC (156.08%) and C3 level (137.78%). The maximum deviation (≥167%) was observed in the spontaneous NBT reduction test (181.40%), (CD95+) NK-cells (228.28%), (CD23+) B-cells with receptors to IgE (233.33%). This allows to propose the following descriptional formula for the immunological grade variations in patients with MALS: $(CD71+)^{1+}$; $(CD22+)^{2+}$; immunoeffector index^{2+}; small circulating immune complexes^{2+}; $C4^{1+}$; $C3^{2+}$; $(CD95)^{3+}$, $(CD23)^{3+}$; $(NBT\ test)^{3+}$.

In our study, significant correlations of immunological parameters also proved the activation of humoral immune system, where the most active focal point was the absolute number of B-cells with low-affinity antigen recognition receptors (CD19+) (Figure 1). The number of CD19+ cells was in direct correlations with T-cells (r=0.65), T-suppressors (r=0.67), NK-cells (r=0.74), CD25+ T-cells with IL-2 receptor (r=0.80), and an immunoeffector index (r=0.87), all $p<0.05$ (Figure 1).

Discussion

The obtained results indicate that the leading immune mechanisms at the stage of MALS are the activations of both the humoral immune response and the complement system, the formation of immune complexes, and the intensification of intracellular oxidase antibacterial system in phagocytes. Thus, we proved our scientific hypothesis about immunological changes in the steatotic first stage of fatty liver. As the main component of the humoral immune response, B-cells are involved in adaptive immunity, as they produce antibodies, present antigens, secrete cytokines, and act as mediators in oxidative stress [13]. Moreover, accumulation of B-cells in the liver of mice with experimental steatohepatitis may be caused by the gut microbiota [7, 14].

Our results confirmed the literature data about correlations of immunocompetent cells with markers of inflammation, oxidative stress, and liver fibrosis [9], which proves their regulative role. There is no doubt that dysregulation of the immune response is a critical link in the progression of metabolically associated fatty liver disease (MAFLD) [15–18]. The liver is considered to be an organ that takes an active part in immune response, particularly through the production of proteins, which participate in the active phase of inflammation, as well as complement components, cytokines, and chemokines. In addition, the liver contains a significant population of immunocompetent cells (*e.g.*, macrophages, natural killer (NK) cells, T-cells, and B-cells) [19, 20]. Experimental data demonstrated an increased number of CD4+ and CD8+ T-cells, γδT-cells and B-cells with decreased amount of NK cells, monocytes and Kupffer cells in metabolically associated hepatic steatosis [7]. Thus, hepatic steatosis, an initial stage in MAFLD, is associated with certain changes in the immune system. These changes might be responsible for the activation of inflammation and might predispose to a chronic inflammatory state.

The results of our study demonstrated that activation of humoral immune response and the complement

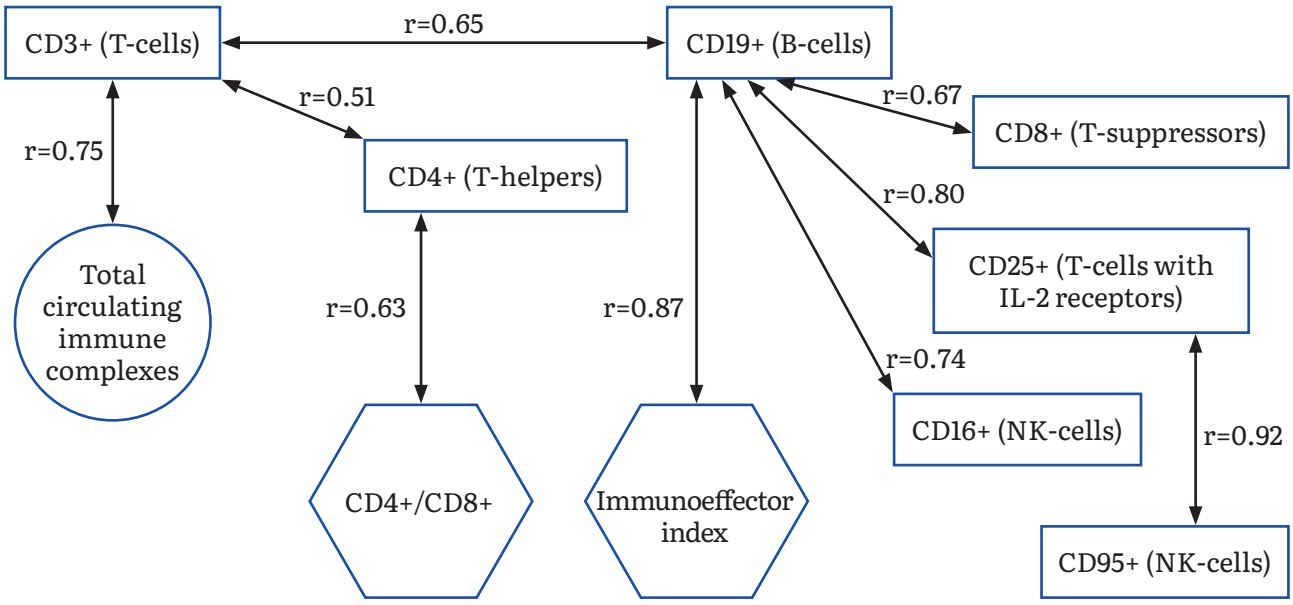

Figure 1: Pleiades of significant correlations between immunological parameters in patients with concomitant metabolic-associated liver steatosis (all $p<0.05$).

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system, immune complex formation, and intensification of antibacterial activity of phagocytes can be considered the leading immune mechanisms characteristic for metabolically associated hepatic steatosis. The activation of the complement system may become a so-called "double-edged sword" in the liver, playing the protective role and causing damage at the same time, as in the case of ischemia-reperfusion, transplant rejection-regeneration *etc*. [21]. In addition, the complement system is a regulator of B- and T-cell response, and it is also involved in essential cellular processes, primarily metabolic [22]. The association between complement system activation and an increased activity of liver enzymes has been described [23]. Therefore, the activation of the complement system revealed in our study can be either a consequence or a cause of worsening metabolic disorders in the setting of MAFLD.

We detected that CD19+ B-lymphocytes were a powerful center for focusing correlations, demonstrating multiple links with many other cellular components of the immune system in case of metabolically associated hepatic steatosis. Indeed, considerable attention is paid to the role of CD 19+ B-cells. These cells were found to be important prognostic markers of viral hepatitis C severity and response to treatment [24], complications after transplantation [25], and hepatocellular carcinoma [26]. Activation of CD19+ B-cells can be caused by interferon-lambda 4, localized mainly in the intestinal mucosal barrier [27], which links the pathogenesis of fatty liver disease with intestinal dysbiosis.

The scientific novelty of our study demonstrated that activation of humoral immune response and the complement system, immune complex formation, and intensification of antibacterial activity of phagocytes could be considered the leading immune mechanisms characteristic for metabolically associated hepatic steatosis – the beginning stage of fatty liver. The activation of the complement system may become a so-called "double-edged sword" in the liver, playing the protective role and causing damage at the same time, as in the case of ischemia-reperfusion, transplant rejection-regeneration *etc*. [21].

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Conclusions

Disorders of the immunological parameters in patients with MALS may be described as follows: $(CD71+)^{1+}$; $(CD22+)^{2+}$; immune effector index^{2+}; small circulating immune complexes^{2+}; $C4^{1+}$; $C3^{2+}$; $(CD95)^{3+}$, $(CD23)^{3+}$; $(NBT\ test)^{3+}$. The leading immune mechanisms associated with liver steatosis were the activation of the humoral immune response and the complement system, the formation of immune complexes, and the intensification of the antibacterial system of phagocytes.

B-cells with low-affinity antigen-recognition receptors (CD19+) were the most powerful focal correlation point with direct significant links with T-cells (r=0.65), T-suppressors (r=0.67), NK cells (r=0.74), T-cells with IL-2 receptors (r=0.80), and immunoeffector index (r=0.87), all $P<0.05$.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

The approval for this study was obtained from the Ethics Committee of the Danylo Halytsky Lviv National Medical University (approval ID: 275).

Consent to participate

Written informed consent was obtained from all the participants.

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