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JLR RESEARCH ARTICLE

Associations between serum cholesterol and immunophenotypical characteristics of circulatory B cells and Tregs

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Abstract **Blood lipids play a major role in the manifestation of cardiovascular diseases. Recent research suggested that there are connections between cholesterol levels and immunological alterations. We investigated whether there is an association between serum cholesterol levels (total, HDL, and LDL) and immune cells (B cell and regulatory T cells [Tregs]). The analysis was based on data from 231 participants of the MEGA study in Augsburg, Germany, recruited between 2018 and 2021. Most participants were examined two different times within a period of 9 months. At every visit, fasting venous blood samples were taken. Immune cells were analyzed immediately afterward using flow cytometry. Using multivariable-adjusted linear regression models, the associations between blood cholesterol concentrations and the relative quantity of several B-cell and Treg subsets were analyzed. We found that particularly HDL cholesterol concentrations were significantly associated to some immune cell subpopulations: HDL cholesterol showed significant positive associations with the relative frequency of CD25++ Tregs (as proportion of all CD4+CD25++ T cells) and conventional Tregs (defined as the proportion of CD25+CD127− cells on all CD45RA−CD4+ T cells). Regarding B cells, HDL cholesterol values were inversely associated with the cell surface expression of IgD and with naïve B cells (CD27−IgD+ B cells). In conclusion, HDL cholesterol levels were associated with modifications in the composition of B-cell and Treg subsets demonstrating an important interconnection between lipid metabolism and immune system. Knowledge about this association might be crucial for a deeper and more comprehensive understanding of the pathophysiology of atherosclerosis.**

Supplementary key words cholesterol • HDL • flow cytometry • regulatory T cells • B cells

Blood cholesterol levels are strongly associated with major cardiovascular diseases, such as myocardial infarction, pulmonary embolism, or stroke (1, 2). LDL transports cholesterol from the liver to the tissues and is considered to play an important role in the pathophysiological process in atherosclerosis (3). HDL on the other hand transports excess cholesterol from peripheral tissues back to the liver; thus, high HDL cholesterol levels were shown to be protective for cardiovascular events (4, 5). A lot of research has been conducted investigating the causes, underlying pathophysiology, and consequences of alterations in blood cholesterol levels. Interestingly, recent work indicated an important interconnection between serum cholesterol concentrations and immunological characteristics (5–7). However, knowledge on potential associations between plasma cholesterol levels and adaptive immune systems in particular remains incomplete. Such interactions might play a major role in hypercholesterolemia and cardiovascular diseases in general. Therefore, the aim of the present study was to investigate associations between serum blood cholesterol levels (total, HDL, and LDL) and phenotypic characteristics of B cells and regulatory T cells (Tregs) in a study cohort including men and women from the general population.

MATERIALS AND METHODS

Study population and data collection

This analysis was based on data from the MEGA study (German acronym for metabolic health study), which was conducted between 2018 and 2021 in Augsburg, Germany. Participants between 25 and 65 years were recruited and examined up to four times within a period of 9 months (baseline visit, follow-up visits after 1 and 6 months, and a final visit after 9 months). The main objective of the study was to investigate immunological particularities that are associated with body composition/anthropometric measures, diet and general lifestyle (e.g., physical activity, etc.). For that reasons, a best as possible population-based sample of participants was recruited including normal-weight individuals as well as individuals with higher BMI. The recruitment of participants was done in various ways, which were posters and flyers (e.g.,

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at doctor's offices), announcements in newspapers and on social media, direct recruitment of participants in public places (e.g., shopping mall, university hospital), and in later stages by word of mouth. The examination of the participants included, amongst others, a venous blood sample, anthropometric parameters and bioelectrical impedance analysis, liver elastography, pulse wave velocity measurements, oral glucose tolerance test, resting metabolic rate measurements and an extensive face-to-face interview about comorbidities, lifestyle (e.g., physical activity), medications, and other issues. Every participant of the study received the same examination (no grouping or similar was conducted).

A total of 232 participants were examined at least once. One patient had missing values for cholesterol levels and was therefore excluded beforehand. For 200 participants, additional data of the final visit approximately 9 months after the first visit were available. For the T cell panel, measures had to start with a certain delay, which means, it was not established well enough yet at the time the examination of the participants started. Therefore, only flow cytometry results for 215 participants at the baseline visit (and 200 for the final visit) were available.

The examinations included, among other assessments, a collection of venous blood samples, blood pressure measurements, and an extensive survey addressing comorbidities and existing diseases, risk factors, and mental health. Next to other topics, the following information was obtained within the scope of the face-to-face interviews: total alcohol consumption (beverages per day), smoking status (current smoker, never smoker, and previous smoker), moderate and intensive physical activity (average hours per day), vegetarian diet (no, vegetarian, and vegan), education (no professional education, professional education, and academic education), diabetes mellitus (yes and no), arterial hypertension (yes and no [self-reported]), hypothyroidism (yes and no), and statin medication use (yes and no; dosage per day). Anthropometric measurements were performed during the examination (e.g., weight, height, BMI).

For this study, only patients without fever (>38.5°C in the last 24 h) and without antibiotics or immunosuppressant use in the last 3 months were included. All examinations were performed in a 12 h overnight fasting state.

All study participants gave written informed consent. Methods of data and biosample collection have been approved by the ethics committee of the Ludwig-Maximilians Universität München, and the study was performed in accordance with the Declaration of Helsinki. The study was registered at "Deutsches Register Klinischer Studien" (DRKS) with the project number DRKS00015784.

Serum cholesterol concentrations and covariables

Cholesterol levels were determined at baseline and at the final visit, so for most participants, there were two measurements within a period of approximately 9 months. The following serum cholesterol levels were measured: total cholesterol (mg/dl, test principle: enzymatic colorimetric method, assay CHOL2 [analyzer: cobas c], Roche Diagnostics GmbH, Mannheim, Germany), HDL cholesterol (mg/dl, test principle: homogeneous enzymatic color test; assay HDLC4 [analyzer: cobas c]), and LDL cholesterol (mg/dl, test principle: homogeneous enzymatic color test [not by Friedewald approximation], assay LDLC3 [analyzer: cobas c]). For the blood collection, serum tubes were used, and the measurements were performed by the central laboratory of the University Hospital Augsburg immediately after collection.

Flow cytometry

For the fluorescence-based flow cytometry (Cytoflex LX flow cytometer, 6 lasers, Fa. Beckman Coulter), venous EDTA blood samples were used. First, erythrocytes were lysed using VersaLyse Lysing Solution from Fa. Beckman Coulter. In several subsequent washing steps, the immune cells were isolated. In order to avoid nonspecific antibody binding, the leucocytes were treated with an FC receptor block. After that, antibody staining was performed with fluorescence-labeled liquid antibodies. The first panel (Treg panel) included the following antibodies: anti-CD4, anti-CD25, anti-CD127 and anti-CD45RA. For the second panel (B cells), the following antibodies were used: anti-CD19, anti-CD21, anti-CD24, anti-CD27, anti-CD38, anti-IgM and anti-IgD. The best concentration of antibodies was predetermined by titration. Then, the antibody-coupled immune cells were fixed using IO-Test 3 Fixative Solution from Beckman Coulter. Finally, the cells were analyzed using flow cytometry. The subset analysis (gating) was performed using Kaluza software (Fa. Beckman Coulter); the gating strategy applied is displayed in **Figure 1** and **Figure 2**. A publication by Streitz *et al.* (8) served as a template for the gating strategy. In supplemental Tables S1 and S2 of the Supplemental data, the exact definition of each subpopulation according to their cell surface marker expression is displayed. For every cell subset, the relative frequency of the respective cells (as a share of the total cell count of the parent gate) was used for the statistical analysis.

Statistical analysis

In general, categorical variables were analyzed using Chi-square tests, and the results were presented as absolute frequencies with percentages. For normally distributed continuous variables, Student's *t*-tests were used. For continuous variables that were not normally distributed, we used nonparametric tests. The results were presented as mean and SD or median and interquartile range.

Even though there were repeated measurements for most participants (baseline and follow-up visit), the statistical analyses are characterized by a purely cross-sectional approach. So the intention of the repeated measurements was not to identify any longitudinal changes in time, neither in the dependent variables nor in the independent variables, but it was rather to reduce the influence of short-term immune cell fluctuations (e.g., caused by infection).

Linear models

Conventional linear regression models were chosen to assess the associations between the cholesterol parameters (exposures) and the frequencies of different B cell and T cell subsets (outcomes). Therefore, we standardized the obtained values for the different B cell and T cell subsets as well as the values of cholesterol measurements (the variables were centered and then divided by the SD so that the transformed variable had an expectancy value of 0 and a statistical variance of 1). The standardization of the exposure and outcome variables ensures comparability of the effect sizes. In order to avoid excessive impact of single outliers, we removed observations with a deviation from the mean of greater than three SDs.

All models were adjusted to account for potential confounding. First, as for most participants, there were two measurements, one at baseline and one about 9 months later, all models were adjusted for the corresponding visit (baseline visit and final follow-up visit). According to the

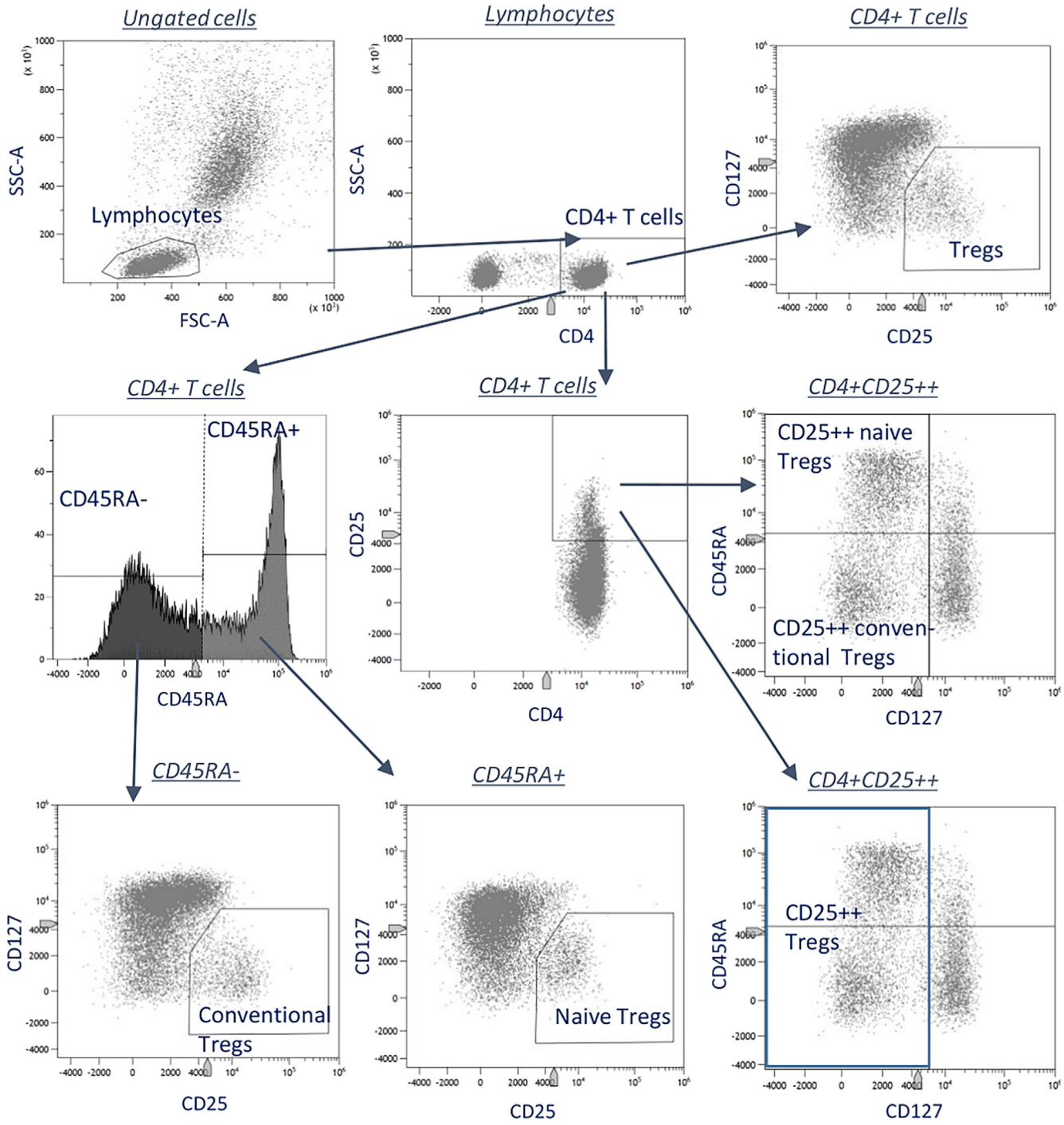

Fig. 1. Gating strategy for the Treg panel. In the first step, T-helper cells were identified by their expression of CD4. Then, different CD4+ T-cell subsets were analyzed using the following antibodies: anti-CD25, anti-CD127, and anti-CD45RA. The identified cell subsets were quantified by calculating their proportion of the parent gate cells.

literature review, the following potential confounder variables were in addition included into the models: sex, age, corresponding visit (baseline and follow-up), education, diabetes mellitus, smoking status, alcohol consumption, BMI, vegetarian diet, moderate and intensive physical activity, and statin medication. Normal distribution of the regression residuals was checked graphically and considered to be fulfilled.

For every cell line (B cells and CD4+ T-cells), the obtained *P* values were adjusted to control the false discovery rate (FDR) regarding multiple testing issues (CIs displayed in Figure 3 and Figure 4 were not FDR adjusted). The displayed effect estimates (β-coefficient and 95% CI) must be interpreted as the expected change in standardized outcome associated with one SD increase in the exposure variable.

Since loss of follow-up was low and missing observations were very scarce, we performed a complete-case analysis (only participants without missing data on relevant variables were considered; patients with only one examination [baseline visit] were included into the analysis).

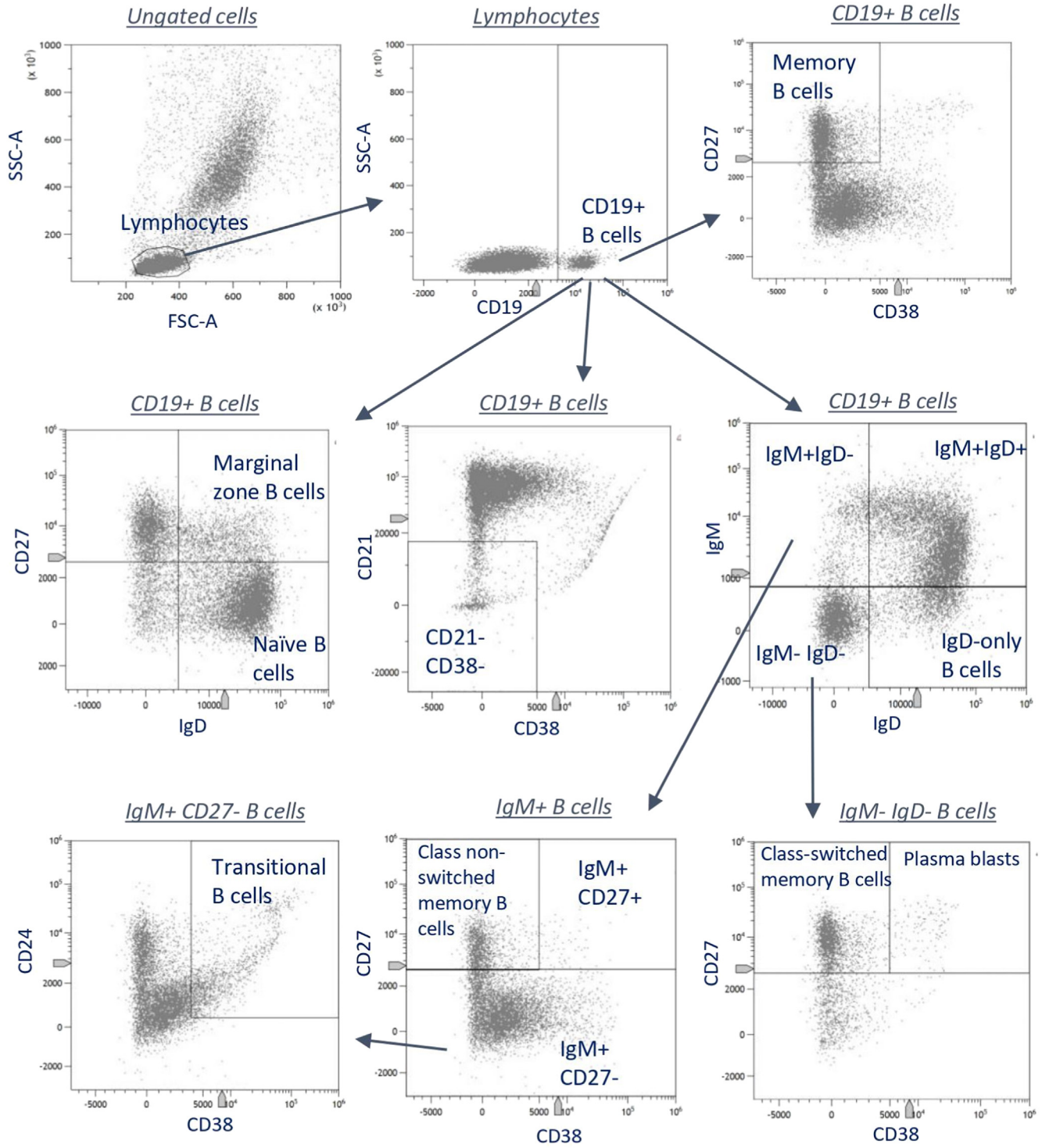

Fig. 2. Gating strategy for the B cell panel. After selecting the B cells using anti-CD19 antibodies, the B cells were further analyzed using anti-IgD, anti-IgM, anti-CD38, anti-CD27 and anti-CD24 antibodies. The identified cell subsets were quantified by calculating their proportion of the parent gate cells.

All statistical analyses were performed using R, version 4.2.1.

RESULTS

Table 1 displays the baseline characteristics for the total sample and stratified for hypercholesterolemia (total cholesterol levels > 200 mg/dl).

The results of the linear regression models are shown in Figs. 3 and 4. In the following, we give a brief description of the main results with significant *P* values after FDR adjustment. Generally, the associations between immune cell subsets and cholesterol levels were stronger for HDL cholesterol than for total cholesterol and LDL cholesterol levels.

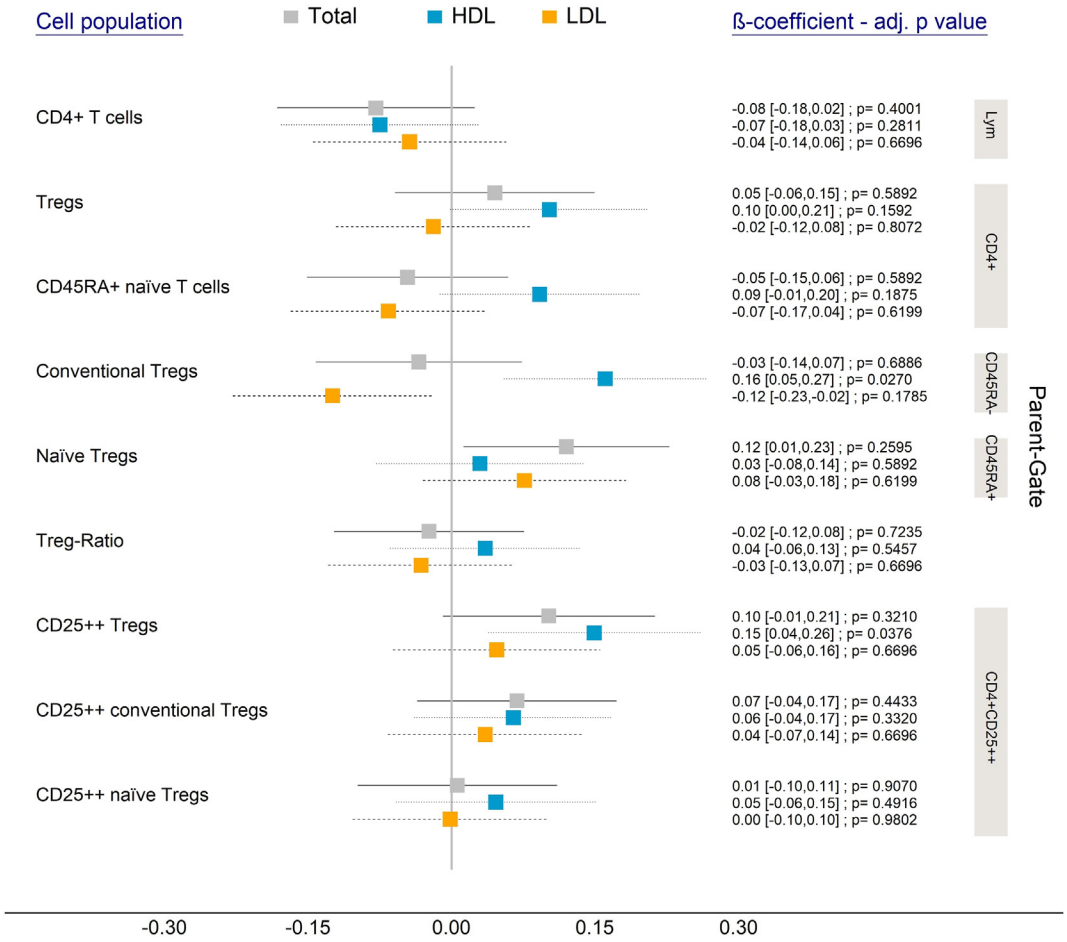

Fig. 3. Association between total serum cholesterol levels, serum HDL cholesterol and serum LDL cholesterol levels, and Treg subsets. The interconnections were examined by calculation of linear regression models adjusted for sex, age, corresponding visit (baseline and follow-up), professional education, diabetes mellitus, smoking status, alcohol consumption, BMI, vegetarian diet, moderate and intensive physical activity, and statin medication. Both the exposure variables (total cholesterol, HDL and LDL) and the outcome variables (specific cell subsets as proportion of parent gate) were standardized. The regression models were based on 415 observations (215 participants at the baseline visit and 200 at the 9-month follow-up visit). The figure displays the estimated β-coefficients with 95% CI.

Tregs and cholesterol

HDL cholesterol levels were positively associated with the number of CD25++ Tregs (as proportion of all CD4+CD25+ T cells) (Fig. 3). In addition, HDL cholesterol was positively related to the relative frequency of conventional Tregs (defined as share of CD25++ CD127− cells in CD4+CD45RA− T cells). Further to be noted is the, in comparison to HDL cholesterol, inverted association of LDL cholesterol with the conventional Treg subset (only significant before FDR adjustment, Fig. 3).

B cells and cholesterol

HDL cholesterol levels were inversely associated with the cell surface expression of IgD, an effect that was more pronounced in IgM− B cells than in IgM+ B cells (only nonsignificant associations for the latter) (Fig. 4). In line with these results, HDL cholesterol showed an inverse association with naïve B cells (characterized as IgD+CD27− B cells).

Supplementary results

In the Supplemental data (supplemental Figs. S1 and S2), the associations between immune cells (B cells and Tregs) and non-HDL cholesterol (defined as total cholesterol minus HDL cholesterol) are displayed. In general, the associations were comparable to those of LDL and immune cells.

In addition, the results of the regression models including only baseline visits (supplemental Figs. S3 and S5) and only follow-up visits (supplemental Figs. S4 and S6) are provided in the Supplemental data. The associations at those two time points were similar to each other and overall comparable to the main results including both visits at once.

In supplemental Table S3 (Tregs) and supplemental Table S4 (B cells), the correlation (Spearman) between baseline and follow-up visits for each cell population is displayed.

Finally, the median percentages of different T cell and B cell subpopulations on total T cells and B cells,

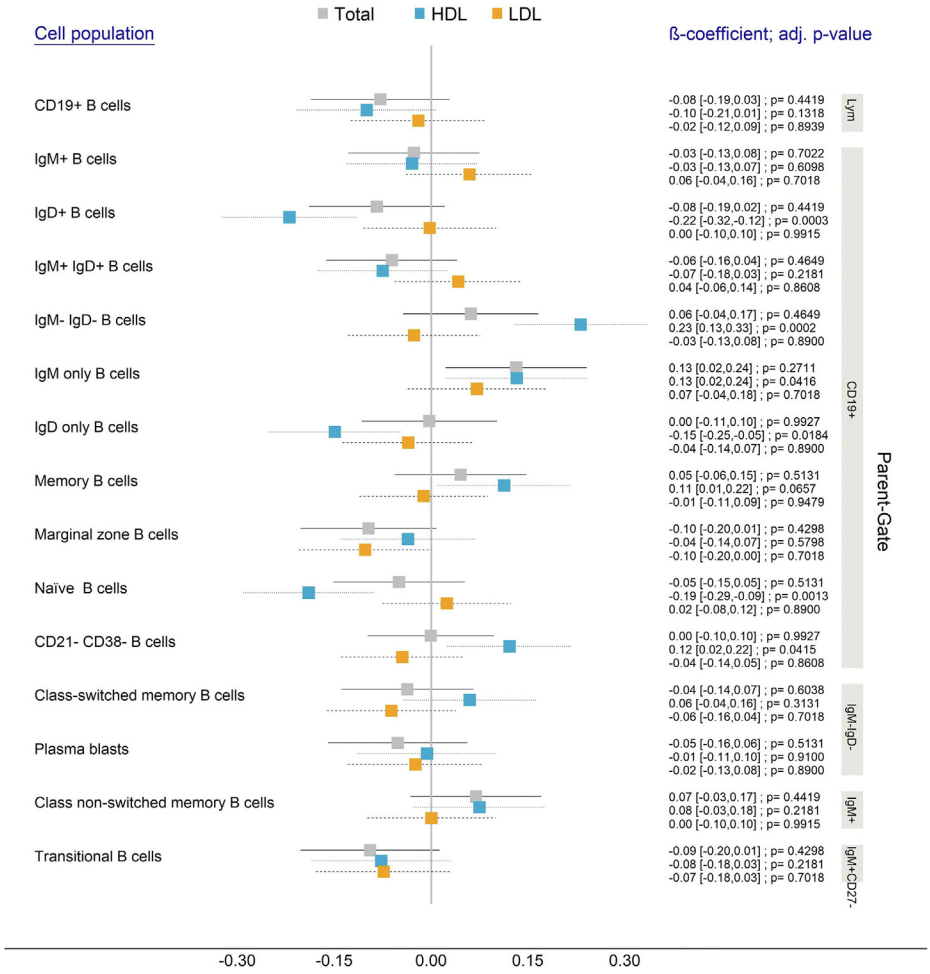

Fig. 4. Association between total serum cholesterol levels, serum cholesterol, and serum LDL cholesterol levels and B cells. The associations were examined by linear regression models adjusted for sex, age, corresponding visit (baseline and follow-up), professional education, diabetes mellitus, smoking status, alcohol consumption, BMI, vegetarian diet, moderate and intensive physical activity, and statin medication. Both the exposure variables (total cholesterol, HDL and LDL) and the outcome variables (specific B cell subsets as proportion of parent gate) were standardized. The regression models were based on 431 observations (231 participants at the baseline visit and 200 at the 9-month follow-up visit). The figure displays the estimated β-coefficients with 95% CI.

respectively, are displayed in supplemental Tables S5 and S6.

DISCUSSION

In this study, we investigated the associations between serum cholesterol levels (total, HDL and LDL) and immunological modifications in the composition of B cell and Treg subsets. Phenotypic characteristics of circulating B cells and Treg cells were examined using flow cytometry. In the Treg panel, a significant positive association between HDL cholesterol and Treg subsets was observed. In the B cell panel, a strong inverse association between HDL cholesterol and IgD expression of the B cells was found including an inverse association with naïve B cells.

In general, we obtained several statistically significant hits for HDL cholesterol and immune cell subsets but none for LDL and total cholesterol (after FDR adjustment). This is a plausible result, since interconnections between HDL and immunological processes in general are well described (5). Nevertheless, the majority of associations (in terms of point estimators/β-coefficients) pointed in the same direction for total cholesterol levels, HDL cholesterol and LDL cholesterol. One major exception from this was the conventional Treg subset: while HDL cholesterol levels were significantly positively associated with a higher frequency of conventional Tregs, high LDL levels went along with a reduced number of conventional Tregs (nonsignificant after correction for multiple testing).

Tregs and HDL cholesterol

In this study, HDL cholesterol showed a significantly positive association with the relative proportions of the conventional Tregs and the CD25++Tregs. In addition to that, HDL cholesterol was also nonsignificantly positively associated with the general Treg subset

TABLE 1. Baseline characteristics for the total sample and stratified by hypercholesterolemia (defined as total cholesterol levels >200 mg/dl)

Variable	Total sample (n = 231)	No hypercholesterolemia (n = 145)	Hypercholesterolemia (n = 86)	*P* value
Age (years; mean, SD)	46.2 (12)	51.6 (9.8)	43.1 (12.1)	<0.001
Sex: Females	158 (68.4)	59 (68.6)	99 (68.3)	1.000
BMI (kg/m^2; mean, SD)	28.7 (7.6)	28.8 (7.4)	28.6 (7.7)	0.896
Hypertension (yes)	62 (26.8)	23 (26.7)	39 (26.9)	0.403
Diabetes mellitus (yes)	10 (4.3)	4 (4.7)	6 (4.1)	0.974
Total alcohol consumption (beverages per day[a]; median, IQR)	0.15 (0.00–0.54)	0.15 (0.11–0.53)	0.15(0.00–0.53)	0.298
Physical activity (hours per day; median, IQR)				
Medium intensity	0.0 (0.0–3.0)	1.3 (0.0–5.0)	0.0 (0.0–3.0)	0.002
High intensity	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.852
Smoking status				0.578
Current smoker	34 (14.7)	11 (12.8)	23 (15.9)	
Never smoker	115 (49.8)	41 (47.7)	74 (51)	—
Previous smoker	82 (35.5)	34 (39.5)	48 (33.1)	—
Diet				0.138
Mixed	208 (90)	80 (93)	128 (88.3)	
Vegetarian	12 (5.2)	5 (5.8)	7 (4.8)	—
Vegan	11 (4.8)	1 (1.2)	10 (6.9)	—
Statin medication (yes)	12 (5.2)	2 (2.3)	10 (6.9)	0.228

[a]One beverage represents approximately 12–14 grams of alcohol.

(CD25+CD127− cells as proportion of the total CD4+ T cell population). Tregs are modulatory immune cells that regulate and suppress overall immune response and inflammation, which makes them essential for maintaining immune homeostasis. All Treg subsets are characterized by a limited expression of CD127 and by a high or very high expression of CD25, which is a high-affinity interleukin-2 receptor (9, 10). Tregs that express high amounts of CD25 (in this study, represented by the CD25++Tregs) are presumed to have particularly strong immune regulatory effects (11). CD127, an interleukin-7 receptor subunit on the other hand, is proposed to be inversely correlated with the suppressive function of human Treg cells (12). So the observations of the present study suggest that high HDL cholesterol plasma levels are associated with a higher immune regulatory capacity by the virtue of the Treg populations. A number of prior studies have shown that alterations in the Treg population are connected to the development of several diseases, for example, the development of autoimmune diseases (9, 13).

Moreover, previous scientific results also strongly indicated an involvement of Tregs in many cardiovascular diseases and thereby implicate cardioprotective effects of Treg cells by suppressing chronic vascular inflammation and atherogenesis (14–19). Dolati *et al.* (20) for example reported significantly reduced frequencies of Tregs in elderly patients with ischemic stroke compared with controls. Likewise, patients with acute coronary syndrome showed strongly decreased numbers of Tregs (21). In acute coronary syndrome, Tregs are protective for myocardial cells and positively modulate cardiac tissue repair (22). Furthermore, it is suggested that aging not only goes along with a higher incidence of cardiovascular diseases but also impairs the immunomodulatory functionality of Tregs (23).

In the present study, we demonstrated that HDL cholesterol is positively associated with the relative frequency of two Treg subsets. Even though we cannot prove any causal relationship, the scientific literature suggests some potential pathophysiological mechanisms: as described, HDL removes cholesterol from the cell membrane and transports it from peripheral tissue to the liver (4, 5). Membrane cholesterol accumulation stabilizes the cell membrane and severely influences the cell function and its ability to react to exogenous stimuli (24). In this way, high HDL might modulate general immune response and T cell activation in particular (5). The results of a study in mice indeed indicated a cause-effect relationship: the injection of apolipoprotein A-I, a protein that is part of HDL particles and essential for its function, caused an increased effectiveness of Treg response (25). It must be noted though, that in our study we investigated the associations between Tregs and HDL cholesterol and not any specific HDL proteins like apolipoprotein A-I, so it is unclear to which extent the above is applicable also to HDL cholesterol.

B cells and HDL cholesterol

We observed that HDL cholesterol was not only associated with Treg populations but showed also significant associations with B cells: First, it was strongly negatively associated with membrane-bound IgD. This association was found to be more pronounced in IgM−B cells than in B cells expressing cell surface IgM. In particular, the quantity of the IgM−IgD− subset increased with serum levels of HDL cholesterol. Second, we also found a significantly inverse association between HDL cholesterol and naïve B cells (characterized as IgD+CD27− B cells), which confirmed the overall negative association between HDL cholesterol and the

cell surface expression of IgD in B cells. Naïve B cells are mature but antigen naïve B cells prior to activation and massive antibody production; they are usually characterized by a coexpression of IgD and IgM (26).

In a review article, Yvan-Charvet *et al.* (6) demonstrated important interactions between B cells and atherosclerosis. B2 cells are characterized by a T-cell-dependent secretion of proatherogenic IgG antibodies and consequently possess pathogenic properties (6). B1 cells on the other hand produce and secrete antiatherogenic IgM antibodies. It has been shown that these cells have protective effects on the development of atherosclerosis because of their IgM secretion (6). Even though we did analyze membrane-bound antibody expression and not antibody secretion in the present study, our results are well in line with these considerations as we found an inverse association between antiatherogenic HDL cholesterol and potentially proatherogenic IgD-expressing B cells.

The relationship between HDL cholesterol and B cells described in this study might represent an important link between the adaptive immune system, cholesterol levels and arteriosclerosis and thus helps to expand the understanding of these highly complex connections.

Strengths and limitations

This study is characterized by some particular strengths. We examined a relatively large number of individuals, and for most participants, we have two measurements at baseline and after 9 months with a low drop-out rate. The repeated measurements reduce the susceptibility to short-term immune cell fluctuations caused by infection or illness. Measurements were highly standardized and conducted by qualified and certified staff. The flow cytometry measurements were performed immediately after blood sampling. Next to the laboratory measurements, a variety of additional data was collected that allowed to consider many potential confounders in the linear regression models.

Nevertheless, there are some limitations to consider. To mention is the limited quantitative comparability of flow cytometry measurements among different laboratories. Even though the procedure of measurement was highly standardized in this study, other laboratories might slightly differ in methodological details. One example for this is the selection of used staining antibodies and the applied gating strategy including the classification of specific subpopulations. Moreover, we did not use negative controls for the flow cytometry measurements, which might have affected the obtained results. Another limitation of this study is the exclusive investigation of B cells and Tregs (both cells of the adaptive immune system) but not any other types of T cells. There might also be important interconnections with the innate immune system, which we were not able to examine within this study. Since the overwhelming majority of our study sample consisted of a typical European population, the results cannot be generalized to other ethnicities. Finally, we might have neglected relevant confounding covariables and, as argued previously, we cannot make reliable statements about the causality of the observed associations.

CONCLUSION

This study elucidates important interconnections between plasma HDL cholesterol levels and phenotypic characteristics of cells of the adaptive immune system. On the one hand, HDL cholesterol levels were positively associated with the conventional Treg subsets and the CD25++ Treg subset. On the other hand, HDL cholesterol was negatively associated with expression of membrane-bound IgD in B cells and naïve B cells. Serum HDL cholesterol levels as well as the mentioned modifications in immune cell subsets are known to be involved in the pathophysiology of arteriosclerosis; but the understanding of their interconnection remains incomplete. With our results, we demonstrated an important relationship between blood cholesterol levels and the adaptive immune system, which helps to gain a deeper understanding of the underlying pathophysiological mechanisms of arteriosclerosis.

Data availability

The datasets generated during and/or analyzed in the current study are not publicly available because of data protection aspects but are available in an anonymized form from the corresponding author on reasonable request.

Supplemental data

This article contains supplemental data.

Ethics approval and consent to participate

The study was registered at the DRKS under the number DRKS00015784 and was approved by the Ethics Committee of the Ludwig-Maximilians-Universität München (project number: 18-637). All study participants gave written informed consent.

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Author contributions

C. M. and J. L. conceptualization; D. F. and T. S. formal analysis; C. M. and J. L. data curation; T. S. writing–original draft; C. M. supervision.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

DRKS, Deutsches Register Klinischer Studien; FDR, false discovery rate; Treg, regulatory T cell.

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