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Innate Immunity Receptor TLR9 Drives Erythrocyte Cholesterol and Ferroprotin Accumulation in a Glutaminase-Independent Manner. Implications for Metabolic Associated Fatty Liver Disease

Konstantinos MIMIDIS[a], Zoi KYRIAKOU[b*], Nikolaos POLITIS[b], Panagiotis VENIAMIS[b], Dimitris VLACHOS[b], Tentes IOANNIS[b], Konstantinos ANAGNOSTOPOULOS[b*], Charalampos PAPADOPOULOS[b*]

[a]Laboratory of Study of the gastrointestinal system and the liver, Department of Medicine, Democritus University of Thrace, Alexandroupolis, Greece

[b]Laboratory of Biochemistry, Department of Medicine, Democritus University of Thrace, Alexandroupolis, Greece

[*]equal contribution as first author

ABSTRACT

Background: *During metabolic associated fatty liver disease (MAFLD), lipotoxicity induces toll-like receptor 9 (TLR9) upregulation and mitochondrial DNA-induced TLR9 activation in the liver, driving metabolic hepatic inflammation. We wondered whether there is augmented erythrocyte TLR9 in MAFLD and we explored the effect of erythrocyte TLR9 activation on cholesterol and sphingomyelin content, glutaminase activity and TLR9, ferroportin and monocyte chemoattractant protein 1 (MCP-1) levels.*

Methods: *Twenty-four patients (15 men and nine women) with MAFLD and nine healthy controls (four men and five women) were enrolled. Erythrocytes were isolated from EDTA-containing blood. Protein levels were measured in erythrocyte lysates (Triton X-100 0.1% v/v), erythrocyte membranes (isolated by hypotonic lysis) with enzyme-linked immunosorbent assays, whereas lipids and enzyme activities were measured in erythrocyte hemoglobin-free membranes by a semi-quantitative thin layer chromatography and assay kits, respectively.*

Address for correspondence:
Charalampos Papadopoulos
Mailing address: Laboratory of Biochemistry, Department of Medicine, Democritus University of Thrace, University campus, Dragana, 69100, Greece
Email: chapapa@med.duth.gr
or
Konstantinos Anagnostopoulos
Mailing address: Laboratory of Biochemistry, Department of Medicine, Democritus University of Thrace, University campus, Dragana, 69100, Greece
Email: kanagnos@med.duth.gr

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Results: *The total but not surface levels of TLR9 were increased (p=0.002) in erythrocytes of MAFLD patients. Erythrocyte TLR9 activation drove cholesterol and ferroportin-1 accumulation, but not glutaminase-1 upregulation. Toll-like receptor 9 activation did not induce a significant change on the levels of TLR9 and MCP-1.*

Conclusions: *Erythrocyte TLR9 is upregulated in MAFLD patients and drives cholesterol and ferroportin-1 accumulation in a glutaminase-independent manner. Augmented erythrocyte TLR9 could participate in metabolic inflammation during MAFLD.*

Keywords: erythrocyte, TLR9, immunometabolism, lipids, inflammation.

ABBREVIATIONS

ALT: alanine transaminase
AST: aspartate transaminase
MAFLD: metabolism associated fatty liver disease
MASH: metabolism associated steatohepatitis
MCP-1: monocyte chemoattractant protein 1
TLC: thin layer chromatography
TLR9: toll-like receptor 9

INTRODUCTION

The pandemic of obesity constantly progresses worldwide and impacts the quality of life and economy of millions of people (1). During obesity, excess fatty acids trigger a pro-inflammatory phenotype of adipose tissue and also lead to triglyceride storage in non-adipose tissues, such as skeletal muscle, pancreas and liver. During metabolic associated fatty liver disease (MAFLD), the inability of the liver to acylate fatty acids into triglycerides drives the accumulation of other toxic lipids, such as cholesterol, lysophosphatidylcholine, sphingosine-1 phosphate, ceramide and other lysophospholipids. These metabolites lead to inflammation and form an immunometabolic network known as metaflammation (2). Lipotoxicity upregulates the TLR9 (3). In the context of MAFLD, TLR9 has been found upregulated in hepatocytes, hepatic stellate cells and Kupffer cells (4). Next, lipotoxicity also drives the release of mitochondrial DNA, that activates TLR9 (5-9) and drives the development of metabolic inflammation during MAFLD. Relatively, TLR9 on endothelial cells, T cells, hepatocytes, Kupffer cells and hepatic stellate cells contribute to the development of MASH (5-10).

Importantly, erythrocytes also express TLR9 (11) and its activation leads to erythrophagocytosis and inflammation (12). In fact, CpG DNA induced inflammation requires the existence of erythrocyte TLR9, while erythrophagocytosis is a major pathogenic mechanism in MAFLD (12). For these reasons, we explored if there are increased TLR9 levels in erythrocytes of MAFLD patients. We also explored the surface TLR9 levels on erythrocytes. In addition, we examined if TLR9 activation induces increased total TLR9 levels.

Recently, we reported elevated cholesterol levels in MAFLD (13). Since erythrocyte TLR9 activation leads to altered structure and crenation (12), we wondered if TLR9 activation drived cholesterol accumulation. Since the levels of erythrocyte cholesterol are regulated by glutamine (14) and sphingomyelin metabolism (15-17), we also quantified the levels of sphingomyelin and glutaminase activity after erythrocyte TLR9 activation.

We previously reported increased MCP-1 release from erythrocytes of MAFLD patients (18). Because erythrocyte TLR9 activation leads to altered protein distribution (12), we wondered whether this is accompanied by alterations on the levels of erythrocyte chemokine. Finally, because membrane lipids affect the ferroportin levels (19), we also quantified its levels after TLR9 activation. ❑

MATERIALS AND METHODS

Subjects

Twenty-four patients (15 men and nine women) with MAFLD and nine healthy controls (four men and five women) were enrolled. All healthy controls were non-obese (BMI<25) and free of chronic diseases, with no signs of infection in the previous two months and normal AST and ALT levels. They were selected from the outpatient liver clinic of the First Department of Internal Medicine, School of Medicine, Democritus Uni-

Parameter	Healthy controls (n=9)	MASH patients (n=24)	t(df), p value
Age (years)	38±15.4	58±11.1	t(11.9)=-3.57, p=0.0039
BMI[a]	24.8±3.79	33.4±4.22	t(16.85)=-5.46, p<0.0001
AST (U/L)	21±8.7	47±19.3	t(26.97)=-4.91, p=0.0001
ALT (U/L)	21±6.5	80±66.8	t(19.78)=-3.91, p=0.0008
Triglycerides (mg/dL)	152±17.1	224±160	t(15.61)=-1.71, p=0.1062
Cholesterol (mg/dL)	155±17.1	197±46.8	t(22.26)=-3.30, p=0.0032
HDL cholesterol (mg/dL)	65±14.8	45±7.3	T(10.10)=3.71, p=0.0040
LDL cholesterol (mg/dL)	65±14.8	110±42.6	t(20.40)=-3.83, p=0.0010
Platelets (thousands/μL)	232±50.4	223±63.4	t(19.57)=0040, p=0.6926
FIB-4[b]	0.75±0.364	1.90±1.279	t(20.34)=-3.45, p=0.0025
AST/ALT	0.75±0.364	0.91±0.514	t(21.01)=-1.01, p=0.325

MASH: metabolism associated steatohepatitis; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; FIB-4: fibrosis-4; AST: aspartate transaminase; ALT: alanine transaminase

TABLE 1. Anthropometric and clinical characteristics of MAFLD patients and healthy subjects.

versity of Thrace, Alexandroupolis, Greece. Hepatic steatosis was assessed by conventional ultrasound performed by a skilled radiologist according to the European guidelines for adults at risk for MAFLD. All patients had elevated AST and ALT levels at baseline. Viral hepatitis (A, B, C, D, E) was ruled out by ELISA and/or polymerase chain reaction test. Alcoholic and drug induced hepatitis was excluded by a questionnaire, and autoimmune hepatitis by antibody detection. Liver fibrosis stage was also evaluated by non-invasive serum markers (Fibroscan, FIB-4, AST/ALT ratio). Table 1 shows their anthropometric and clinical characteristics. The Scientific Council of the University Hospital of Alexandroupolis and the Ethics Committee approved the study after obtaining each participant's informed consent.

Results are reported as mean ± standard deviation (SD). The p-value is for the difference between means.

Erythrocyte isolation

From every patient or healthy volunteer three ml of blood in tubes containing 5.4 mg ethylenediaminetetraacetic acid (EDTA) were isolated. To isolate erythrocytes, the tubes containing blood were centrifuged at 200×g for 10 minutes at 4°C in order to remove the buffy coat and plasma. Then, 1 mL of erythrocyte pellet was washed with cold saline solution and centrifuged at 200×g for 10 minutes at 4°C. The last step was repeated four times, until a clear erythrocyte population was achieved.

Erythrocyte membrane isolation

After erythrocyte isolation, 500 μL of packed erythrocytes were incubated for 30 minutes at 4°C with continuous shaking, diluted with cold hemolysis solution [Tris 1 mM–NaCl 10 mM-EDTA 1 mM, pH 7.2, at a final concentration of 1:10 (vol/vol)]. This was followed by centrifugation at 10,000×g, at 4°C for 15 minutes. The pellet, corresponding to erythrocyte membranes, was collected. The last step was repeated as many times as needed, until the removal of hemoglobin-free erythrocyte membranes. Samples were kept at −80°C until analysis.

Lipid extraction

For lipid extraction, we use a modified Folch method (20). Briefly, 500 μL of 2:1 chloroform/methanol was added to 100 μL of erythrocyte membrane. After a vortexing for 30 seconds and a centrifugation for 10 minutes at 5000×g, the organic phase was collected. The previous step was repeated for the aqueous phase. The two organic phases were washed with water to remove any remainders of polar molecules by centrifugation for 10 minutes at 5000×g and then evaporated at 70°C.

Lipid separation and visualization

Thin-layer chromatographic (TLC) analysis of lipids was performed on chromatographic plates

(TLC Silica gel 60 F254; Merck KGaA 64071, Darmstadt, Germany) according to our previously published protocol (21).

Cholesterol and sphingomyelin quantitation
The characteristics of the method (specificity, linearity, accuracy, precision, limit of detection, limit of quantification and sensitivity) can be found in our previous study (21).

Erythrocyte TLR9 activation
Erythrocytes from three healthy volunteers were incubated in PBS in final concentration of 40% vol/vol, with or without CpG DNA (100 ng/mL). After incubation, conditioned media were isolated with centrifugation at 200 g for 10 minutes.

Lysis of erythrocytes
One milliliter of packed erythrocytes was lysed with Triton X-100 at a final concentration of 0.1% vol/vol (13).

Determination of TLR9, ferroportin-1 and MCP-1 protein levels
The levels of TLR9 (OriGene, USA), ferroportin-1 (assay genie) and monocyte chemoattractant protein 1 (OriGene, USA) were determined by ELISA in erythrocyte lysates, conditioned media or plasma according to the manufacturer's instructions.

Determination of surface TLR9 levels
Surface TLR9 was measured on erythrocyte membranes after permeabilization with Triton x 100 (1% v/v).

Determination of erythrocyte glutaminase-1 activity
The activity of erythrocyte glutaminase-1 was determined in erythrocyte membrane preparations according to the manufacturer's instructions (Abbexa. UK, respectively).

Statistical analysis
Results were expressed as mean ± SD and median, unless otherwise stated. Statistical analysis was performed with the R programming language v. 4.0 (22) Welch's independent samples t-test (23) was used for comparison of means between groups. This test is an adaptation of Student's t-test, and does not assume equal variances and/or sample sizes, being more reliable when these conditions occur. Glass's Δ was used to quantitate the effect size (24) since it is more appropriate when the standard deviations between groups are significantly different. According to the above, effect sizes with values $\Delta<0.2$ are considered very small, small for $0.2\leq\Delta<0.41$, moderate for $0.41\leq\Delta<0.63$ and large for $\Delta\geq0.63$.

The results are reported as t(degrees of freedom)= test statistic, the p-value, followed by Glass's Δ [along with is 95% confidence interval (CI)], the statistical power 1-β for a significance level $\alpha=0.05$ and the 95% CI for the difference between means of the healthy and MAFLD groups, as shown in Table 2.

Linear regression was performed using the base R lm command. All plots were created using the ggplot package. In all the boxplots, the box extends from the lower quartile to the upper quartile – that is the range corresponds to the inter-quartile range (IQR). The vertical lines extend to $\pm1.5\times$IQR. Individual points shown correspond to extreme values, outside the $\pm1.5\times$IQR.

For the measurements regarding the activation of TLR9, the sample size was small (three paired samples, that is three samples before and three samples after activation). Because of the small sample size, the traditional frequentist t-test gives unreliable results or no results at all. For this reason, a Bayesian approach for statistical analysis was employed. Bayesian analysis is much more suited to provide meaningful results for small datasets, since it does not assume large sample sizes. Thus, smaller datasets can be analyzed while retaining statistical power and precision (25).

The brms package was used for the analysis. The brms model used was:
value ~ group + (0 + group | sample) + (1| sample2).
This model tests for the effect of group (that is before and after activation) on the values of each sample: value ~ group + (0 + group | sample). The next part of the model: (1 | sample2) duplicates the sample variable. This essentially tells the model to estimate a separate intercept for each sample. This allows brms to estimate the correlation between the "Before" and "After" measurements within each sample. The priors used were wide, non-informative. Specifically, for the effect of the intervention a normal distribution prior was used with a mean of 0 and an SD of 100. For the SD of the errors of the model, as well as the random effects) a Student t-distri-

bution prior was used with three degrees of freedom, a location of 0 and a scale of 100. For the correlation between the random intercepts and slopes, an LKJ prior was used Eta of 2. For the Hamiltonian Monte Carlo sampling algorithm, an adaptDelta value near to 1 (0.99) was used, meaning smaller steps, leading to more accurate sampling. A maxTreeDepth value of 15 was used (compared to the default of 10). This allows the the No-U-Turn Sampler (NUTS) algorithm to improve exploration of the posterior, especially for complex models.

Results were reported as the probability P of the difference between the means after activation being greater that the means before activation, P(After>Before). This is more intuitive than the more convoluted meaning of the frequently used p value, which is the probability of observing the data, assuming the null hypothesis (no difference between means) is correct. Probabilities above 90% were considered statistically significant. ❑

RESULTS

Erythrocyte TLR9 levels

Erythrocytes express TLR9 (11). This receptor binds and carries mitochondrial DNA to macrophages after erythrophagocytosis, leading to inflammation (12). In addition, TLR9 ligation can lead to CD47 loss (12). For these reasons, we quantified TLR9 on erythrocytes. In MAFLD patients TLR9 levels were statistically significantly greater (mean=3.6 ng/mL) than in healthy subjects (mean=1.0 ng/mL), with p=0.002 and a large effect size (Table 2). Surface TLR9 levels were unaltered.

Erythrocyte TLR9 activation

Previous studies have shown that erythrocyte TLR9 activation triggers crenation, resistance to hemoysis, altered protein distribution and surface TLR9 upregulation (12). We show here that activation of erythrocyte TLR9 led to cholesterol and ferroportin-1 accumulation, but not to alterations on the levels of TLR9 and MCP-1 and glutaminase-1 activation (Table 3). ❑

DISCUSSION

Metabolic associated fatty liver disease constitutes a metabolic hepatic disease, where metabolic perturbations precede inflammatory mechanisms (26). Otogawa *et al* (27) and Park *et al* (28) have reported that hepatic erythrophagocytosis resulted in inflammation during MAFLD. We have also reported extended erythrocyte changes during MAFLD (13, 18, 21). In this study, we tried to elaborate on the role of erythrocytes in the metabolic inflammation of MAFLD.

Lipotoxicity induces TLR9 upregulation (3, 4) and activation in the liver through hepatocyte mitochondrial DNA release (4, 6). This step actu-

TABLE 2. Test for difference between means using Welch's t-test

Parameter	Unit	Healthy	MAFLD[i]	t (df)[a]	p value	Δ[b]	Δ 95% CI[c]	1-β[d]	Mean difference 95% CI[e]
RBC[f] TLR9[(h)]	ng/mL packed RBC	N=6 mean=0.97 SD=1.325, median=0.16	N=24 mean=3.62 SD=1.681 median=3.71	-4.13 (9.50)	0.0022	-1.52	-2.36–-0.67	0.90	-4.08–-1.21
sTLR9[(g)]	ng/mL packed RBC	N=9 mean=3.34 SD=1.631 median=3.29	N=9 mean=2.72 SD=1.110 median=2.83	0.95 (14.09)	0.593	0.51	-0.58–1.57	0.17	-0.79–2.03

[(a)]t-statistic (degrees of freedom); [(b)]Glass's Δ for effect size; [(c)]95% confidence interval (CI) for Δ; [(d)]statistical power; [(e)]95% CI for the difference of means between healthy and MAFLD groups; [(f)]red blood cells; [(g)] surface toll-like receptor 9; [(h)]toll-like receptor 9; [(i)]MAFLD: metabolic dysfunction-associated fatty liver disease

TABLE 3. Bayesian test for difference before and after TLR9 activation

Parameter	Before activation mean±SD	After activation mean±SD	P (after > before)
SM (μg/mL)	16.6±10.74	25.1±18.90	84%
Cholesterol (μg/mL)	19.0±8.89	30.5±7.92	91%
MCP-1 (pg/mL)	79±21.3	70±31.8	38%
FPN (ng/mL)	36±17.5	70±12.1	92%
TLR9 (pg/mL)	2416±1005	2260±510	45%
Glutaminase (U/L)	2.6±0.22	2.8±0.62	57%

SM: sphingomyelin; MCP-1: monocyte chemoattractant protein 1; FPN: ferroportin; TLR9: toll-like receptor 9

ally connects dysmetabolism with metabolic inflammation in the liver during MAFLD (4). Similarly, reduced TLR9 levels on T-lymphocytes protect from the transition from hepatic steatosis to metabolic inflammation (10). Of note, a previous study reported that, in general, mitochondrial DNA-induced inflammation requires erythrocyte TLR9 and erythrophagocytosis (12).

We show that erythrocytes of MASH patients contain increased TLR9 levels and follow the trend of upregulated TLR9 in the liver of MASH animals and patients (3, 4). In this perspective, erythrocytes join hepatocytes (6), liver macrophages (6), endothelial cells (29), T-cells (10) and hepatic stellate cells (9) in sensing metabolism – induced liver damage through TLR9. Erythrocytes with high TLR9 levels could bind the increased cell-free mitochondrial DNA (37, 41), and deliver to the liver through their phagocytosis resulting in increased delivery of mitochondrial DNA to macrophages (30). Importantly, mitochondrial DNA delivery through erythro- phagocytosis could drive the development of a pro-inflammatory erythrophagocyte subset (31).

Increased circulating mitochondrial DNA has been shown to be followed by augmented surface TLR9 levels on erythrocytes (12). Since we observed increased total erythrocyte TLR9 levels, we wondered whether there were also increased surface TLR9 levels. We noticed that surface TLR9 were not statistically significantly different. Hence, TLR9 upregulation probably concerns TLR9 on intracellular vesicles of erythrocytes. This seems to be in contrast to the reported effect of augmented circulating mitochondrial DNA during sepsis on erythrocyte surface TLR9 levels (12). We speculate that in MAFLD the levels of circulating mitochondrial DNA are not high enough to induce such detrimental changes.

We wondered if TLR9 activation explains the increase of erythrocyte TLR9 levels. We showed that, following TLR9 activation, the levels of TLR9 were not altered. Hence, the increase in the surface TLR9 levels that has previously been reported (12) was possibly due to translocation of TLR9 from the endocytic compartment to the plasma membrane. Erythrocyte cholesterol could be part of an extended membrane reorganization giving access to TLR9 to the plasma membrane.

Erythrocyte TLR9 activation leads to erythrocyte crenation and altered structure (12). These changes have previously been reported to be mediated by cholesterol accumulation (32). We previously reported erythrocyte cholesterol accumulation in MAFLD (13) and hypothesized that TLR9 activation would lead to altered membrane cholesterol content. We show that erythrocyte TLR9 activation leads to cholesterol accumulation. Cholesterol levels on erythrocytes are necessary for the tight linkage of the membrane to the cytoskeleton (32). In fact, it has been shown that increasing erythrocyte cholesterol made the cell more resistant to hemolysis due to the tighter linkage of BAND3 (32). Relatively, TLR9 activation has been shown to make erythrocytes resistant to hemolysis (12). Hence, cholesterol accumulation due to TLR9 activation could be partly responsible for the TLR9-induced physicochemical alterations of erythrocytes. We hypothesize that augmented erythrocyte cholesterol along with augmented erythrophagocytosis could lead to increased cholesterol levels in the liver.

We wondered what was driving cholesterol accumulation after TLR9 activation. Since erythrocytes are not able to synthesize cholesterol de novo, the only way to induce cholesterol accumulation is through reduced exchangeability. A previous study found that sphingomyelin impaired the efflux of cholesterol from red blood cells (16) and protected cholesterol from oxidation (17). We show that erythrocyte TLR9 activation does not lead to sphingomyelin accumulation. An effect of erythrocyte sphingomyelin on cholesterol content has also been reported previously for atherosclerosis (33, 34). In the past we found reduced erythrocyte sphingomyelin in erythrocytes of MAFLD patients (21), possibly due to increased serum sphingomyelinase action.

Apart from sphingomyelin metabolism, glutamine metabolism also impacts the erythrocyte cholesterol levels, possibly through protection from oxidation and release through vesiculation (14). We show that the TLR9-induced cholesterol accumulation is independent of glutaminase-1 activity.

In a previous study we found that erythrocytes of MASH patients release increased content of the chemokine MCP-1(18). We wondered whether TLR9-induced altered protein distribution (12) also mediated chemokine release. We showed that erythrocyte TLR9 activation did not

drive MCP-1 release. Since BAND3 exists in various complexes in the erythrocyte, it is possible that the junctional compartment where DARC is found along with BAND3 and other membrane proteins (but not TLR9 and CD47)(35) is not strongly affected by the altered distribution of BAND3.

Cholesterol affects the distribution of ferroportin (19). We sought to investigate whether TLR9-induced cholesterol accumulation was followed by ferroportin accumulation. We show that TLR9 activation leads to increased ferroportin-1 levels on eryrhrocytes. We speculate that this difference is due to decreased degradation. This result could unravel a novel mechanism for inflammation related increase in serum iron. ❑

CONCLUSION

To conclude, we report here augmented erythrocyte TLR9 levels. Based on previous findings of increased circulating mitochondrial DNA in MAFLD patients (6), we explored the effect erythrocyte TLR9 activation. We show that TLR9 leads to cholesterol and ferroportin-1 accumulation, possibly in a sphingomyelin- and glutaminase-1-independent manner. Our results shed light on the role of erythrocytes in MAFLD (36) and physiology (37). ❑

Authors' contribution: CP designed the research/study; ZK, KM, NP, PV, DM and CP performed the research/study; CP collected data; CP and KA analyzed data; CP wrote the manuscript; and CP, KA and IT supervised the study.

Conflicts of interest: none declared.

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