The Effects of Pathogen-Associated Molecular Patterns on Peripheral Blood Monocytes in Patients with Non-alcoholic Fatty Liver Disease

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Background: Innate immune responses to gut-derived pathogen-associated molecular patterns (PAMPs) have been implicated in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). Whether NAFLD patients have increased sensitivity to PAMP exposure has yet to be reported. Methods: Peripheral blood mononuclear cell (PBMC)/monocytes were exposed to lipopolysaccharide (LPS), Pam3CSK4, or BSA conjugated palmitate in vitro. Changes in toll-like receptors (TLR), cytokines, and chemokine receptors (CR) expressions were documented by flow cytometry and/or enzyme-linked immunoabsorbent assays (ELISAs). Results: TLR2 and TLR4 expression were similar at baseline and increased to a similar extent (TLR2) or remained unchanged (TLR4) following PAMP exposure in NAFLD and healthy control (HC) monocytes. Proinflammatory IL-1β and IL-6 levels were similar at baseline but increased in a concentration-dependent manner to a greater extent in NAFLD PBMCs. CCR1 and CCR2 expressions at baseline were similar and decreased to a similar extent in NAFLD and HC monocytes. The extent of PAMP-induced proinflammatory cytokine release correlated with evidence of hepatocyte injury (CK18M30 levels). Discussion: NAFLD patients have increased proinflammatory cytokine responses following exposure to PAMPs relative to HC subjects. This response is concentration-dependent and correlates with the extent of hepatic injury. Lay summary: The precise cause of nonalcoholic fatty liver disease (NAFLD) remains to be determined. In this study, we documented that NAFLD patients have increased inflammatory responses to intestinal products that are delivered to the liver when compared to healthy controls. This finding could help to explain why some NAFLD patients have inflammation in their liver. (J Clin Exp Hepatol xxxx;xxx:xxx)

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disorder with an estimated global prevalence of 20–30%. In approximately 20% of these individuals, necroinflammatory disease of the liver or steatohepatitis (NASH) is present. NASH patients are at an increased risk of developing cirrhosis and/or hepatocellular carcinoma. Current NAFLD/NASH treatment consists of weight loss, increased physical activity, and optimal management of associated metabolic comorbidities. More targeted and effective therapy will require a complete understanding of the pathogenesis of NAFLD/NASH.

Previous studies have demonstrated that NASH patients have increased gut mucosal permeability resulting in increased entry of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides and free fatty acids into the portal circulation. Once delivered to the liver, PAMPs activate toll-like receptors (TLR) on the surface of resident liver monocytes/macrophages resulting in increased release of various proinflammatory cytokines and chemokines. These agents then contribute to hepatocyte injury and inflammatory cell infiltration, respectively. The extent of the hepatic injury can be determined by documenting serum levels of alanine aminotransferase (ALT) and/or the hepatocyte apoptosis marker CK18M30.

An important question that remains unresolved is whether NAFLD patients have increased sensitivity to PAMPs relative to healthy subjects. Also to be determined is whether the extent of the response to PAMPs in NAFLD patients correlates with the extent of hepatic injury. To address these questions we documented the expression and/or release of specific TLR, proinflammatory and anti-inflammatory cytokines, and chemokine receptors by...
peripheral blood mononuclear cells (PBMCs), and/or monocytes derived from NAFLD patients and healthy control (HC) subjects prior to and following PAMP exposure. We then correlated PBMC or monocyte responses to the extent of the hepatic injury as reflected by serum ALT and CK18M30 levels.

MATERIALS AND METHODS

Study Cohorts

NAFLD patients beyond 18 years of age and of either sex with ultrasound findings of fatty liver and the absence of alternative causes were invited to participate in the study. The ultrasound procedure was performed with a GE Healthcare LOGIQ E9 and a curvilinear probe by staff radiologists as per the standard protocol for the Section of Ultrasound, Department of Radiology, Health Sciences Centre, Winnipeg. Exclusion criteria included conditions that might alter immune responsiveness, such as acute or chronic infections, recent vaccinations, and the use of immunomodulants. HC subjects consisted of hospital staff volunteers with no ultrasound findings of fatty liver, normal serum ALT levels, and no history of liver disease, alcohol abuse, or other exclusion criteria.

Participation was voluntary and written informed consent was obtained from all study participants. The study was approved by the University of Manitoba Research Ethics Board.

Clinical Assessments

Clinical assessments documented the individual’s demographic data, medical history, and medications used. Biochemical variables, including serum glucose, creatinine, triglyceride, total cholesterol, LDL, HDL, ALT, AST, and ferritin levels, were determined by the Health Sciences Centre Clinical Chemistry Department using standard laboratory techniques. Plasma C18M30 levels were measured by an M30 Apoptosense ELISA kit (Vivalavid AB, Nacka, Sweden) as per the manufacturer’s instructions.

PAMPs

The PAMPs employed consisted of the predominantly TLR4 agonist; lipopolysaccharide (LPS) (Sigma), TLR2 agonist; Pam3CSK4 (Pam) (Invivogen) and TLR2/4 agonist; BSA conjugated palmitate (Pal) (Sigma). The final concentrations of each PAMP were calculated to approximate portal venous levels reported previously in humans.12,13

PBMC, Monocyte, TLR, and CCR Detection

PBMCs were isolated from blood after plasma collection by the Ficoll–Paque method, Ficoll Histopaque® (Sigma), as previously described.14 Freshly isolated PBMCs were cultured with either culture medium (CM) alone or in CM with final concentrations of LPS (20 ng/ml unless otherwise indicated), Pam (200 ng/ml) or Pal (200 mM). PBMCs were stained with cell viability dye (FITC, Life Technologies), CD3 (FITC, BD Biosciences), CD10 (FITC, BD Biosciences), CD56 (FITC, Biolegend), HLA-DR (PerCP, Biolegend), CD14 (APC-Cy7), CD16 (BV510, Biolegend), TLR2 (PE-Cy7, Biolegend), TLR4 (PE, Biolegend), CCR1 (APC, Biolegend), CCR2 (BV421, Biolegend) in FACS buffer (1% BSA in saline) for 0.5 h. After washing with FACS buffer, cells were fixed in 2% PFA (from 16% stock, Canemco Inc., Lakefield, Quebec) for 30 min and acquired on a flow cytometer (LSR2, BD Biosciences) within 3 h. Flow cytometry data analyses were performed by Flowjo software. Gates were adjusted following fluorescence minus one condition (FMO). Monocytes were defined as viability dye−, CD3−, CD19−, CD56−, HLA-DR+, CD14+ cells.

Cytokine Detection

Freshly isolated PBMCs (0.25 million/well) were cultured with CM alone or CM with final concentrations of LPS (0.2 ng/ml, 2.0 ng/ml, 20 ng/ml), Pam (200 ng/ml) or Pal (200 mM) in 96 well round bottom culture plates for 24 h. Culture supernatants were analyzed for IL-1β, IL-6, and IL-10 cytokines using enzyme-linked immunosorbent assays (ELISAs).14 Paired antibodies were obtained from Biolegend (clones of capture antibody and detection antibody: IL-1β, JK1B-1, JK1B-2; IL-6, MQ2-13A5, MQ2-39C3; IL-10, JES3-9D7, JES3-12G8) and standards from Peprotech.

RESULTS

Study Populations

The demographic and laboratory findings of NAFLD patients and HC subjects are provided in Table 1. The mean age of NAFLD patients was 53 years (range: 23–75), and of HC subjects 48 years (range: 33–67). Females constituted 43% of the NAFLD patients and 75% of HC subjects. NAFLD patients had significantly higher BMIs than HC subjects (33 vs. 26, respectively, P = 0.04).

Blood glucose levels and determinants of liver injury, including serum ALT, AST, and C18M30 levels, were higher in NAFLD patients than HC subjects (P < 0.0005, respectively). There were no differences in the remainder of the laboratory findings.

TLR Expression

The results of TLR2 and TLR4 expression prior to and following PAMP exposure are provided in Figure 1. Prior to PAMP exposure, the percent of monocytes expressing TLR2 was approximately twofold higher in NAFLD than HC subjects, but the difference did not achieve statistical

Table 1 Demographics and Laboratory Data of Study Groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NAFLD (n = 35)</th>
<th>Healthy Control (n = 8)</th>
<th>P value</th>
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<tr>
<td><strong>Demographics</strong></td>
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<tr>
<td>Age (years)</td>
<td>53 (23–75)</td>
<td>48 (33–67)</td>
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</tr>
<tr>
<td>Female (%)</td>
<td>43</td>
<td>75</td>
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<tr>
<td>Caucasian (%)</td>
<td>80</td>
<td>86</td>
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<tr>
<td>BMI</td>
<td>33 (26–51)</td>
<td>26 (20–30)</td>
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<td><strong>Laboratory</strong></td>
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<tr>
<td>ALT (mg/dL)</td>
<td>61 (12–209)</td>
<td>18 (13–21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AST (mg/dL)</td>
<td>38 (14–135)</td>
<td>17 (9–22)</td>
<td>&lt;0.001</td>
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<tr>
<td>CK18M30 (ug/L)</td>
<td>258 (10–894)</td>
<td>38 (10–111)</td>
<td>&lt;0.0001</td>
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<td>Glucose (mmol/L)</td>
<td>6 (3.6–11.6)</td>
<td>4.6 (3.9–6.1)</td>
<td>&lt;0.05</td>
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<tr>
<td>Creatinine (umol/L)</td>
<td>75 (43–110)</td>
<td>65 (50–93)</td>
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<tr>
<td>Triglyceride (mmol/L)</td>
<td>2.4 (1–5.7)</td>
<td>1.5 (0.6–2.2)</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>6.4 (3–13)</td>
<td>4.7 (3.7–5.9)</td>
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<tr>
<td>LDL (mmol/L)</td>
<td>1.9 (0.7–2.2)</td>
<td>1.6 (1.1–2.8)</td>
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<tr>
<td>Ferritin (ug/L)</td>
<td>265 (12–1117)</td>
<td>68 (14–118)</td>
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Concentration Dependencies

To determine whether LPS-induced cytokine responses are concentration-dependent, we exposed cultured NAFLD and HC PBMCs to three concentrations of LPS (0.2, 2.0, and 20 ng/ml). The resulting IL-1β, IL-6, and IL-10 levels were provided in Figure 4.

Following exposure to physiologic concentrations of LPS (0.2 and 2.0 ng/ml) but not 20 ng/ml (Figures 4a and 4b), IL-1β and IL-6 concentrations were significantly higher in PBMCs derived from NAFLD patients than HC subjects. IL-10 levels were similar in the two cohorts at all three LPS concentrations (Figure 4c).

Chemokine Receptor Expression

The results of CCR1 and CCR2 expression at baseline and following PAMP exposures are found in Figure 5. The percent of monocytes expressing CCR1 and CCR1 expression per monocyte prior to PAMP exposure were similar in NAFLD patients and HC subjects at baseline (Figure 5a). Following PAMP exposure, the percent of monocytes expressing CCR1 decreased to a similar extent in both monocyte study populations (LPS: 0.29 vs. 0.43, Pam: 0.36 vs. 0.49 and Pal: 0.32 vs. 0.43 fold, respectively) (Figure 5b).

The percentage of monocytes expressing CCR2 and CCR2 expression per monocyte prior to PAMP exposure were similar in monocytes derived from NAFLD patients and HC subjects (Figure 5c). Following PAMP exposure, the percentage of monocytes expressing CCR2 decreased to a similar extent in both monocyte study populations (LPS: 0.29 vs. 0.43, Pam: 0.36 vs. 0.49 and Pal: 0.32 vs. 0.43 fold, respectively) (Figure 5d).

Correlations with Liver Injury

To determine whether proinflammatory cytokine release correlates with the extent of liver injury, correlation coefficients for IL-1β and IL-6 with ALT and CK18M30 levels were calculated. As shown in Table 2, peak IL-1β levels significantly correlated with CK18M30 levels following exposure to 0.2, 2.0, and 20 ng/ml of LPS and Pam (P < 0.05, respectively) but not Pal. Similar results were obtained with IL-6. ALT levels correlated with Pal-induced IL-1β levels (P < 0.05).

Also shown in Table 2 are correlation coefficients for changes in CCR1 expression per monocyte and ALT and CK18M30 levels. Here, the extent of CCR1 downregulation associated with LPS, Pam, and Pal exposure significantly correlated with ALT and CK18M30 levels (P < 0.05, respectively).
respectively). Similar results were obtained with changes in CCR2 expression per monocyte (data not shown).

**DISCUSSION**

The results of this study indicate that following exposure to physiologic concentrations of LPS, PBMCs derived from NAFLD patients release more proinflammatory cytokines than PBMCs derived from HC subjects. These differences are not associated with differences in TLR2, TLR4, CCR1, or CCR2 expression prior to or following PAMP exposure. The extent of PAMP-induced proinflammatory cytokine release and CCR downregulation significantly correlates with CK18M30 evidence of hepatic injury.

There are a number of possible explanations as to why PBMCs derived from NAFLD patients release higher concentrations of proinflammatory cytokines following exposure to certain PAMPS than HC subjects. One possibility relates to the extent of TLR expression. TLR2 and TLR4 are the principal TLRs responsible for proinflammatory cytokine synthesis. In a NAFLD animal study, TLR2 deficiency was associated with attenuated hepatic inflammation and reduced inflammatory cell infiltration, while in other studies, TLR4 deficiency was protective against...

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**Figure 1** TLR2 and TLR4 expression depicted as percentage of monocytes staining positive for these receptors and receptor expression per monocyte (A and C). There were no differences between NAFLD patients and HC subjects at baseline (steady state) or following PAMP stimulation (B and D). Data presented as mean ± SD.
Moreover, TLR2 and TLR4 mRNA expression in adipose tissue and PBMCs tend to be elevated in obese subjects and in this study, the mean BMI in the NAFLD cohort was significantly higher than that of HC subjects. Thus, enhanced expression of either of these two receptors in NAFLD might explain the increased proinflammatory cytokine release observed. Although there was a trend toward a higher percent of NAFLD...
monocytes expressing TLR2, TLR2 expression per monocyte and PAMP-induced changes in TLR2-receptor expression were similar in the two study cohorts. In terms of TLR4 expression, there were no clear distinctions in TLR4 expression at baseline or following PAMP exposure in monocytes derived from NAFLD patients and HC subjects. Thus, significant differences in TLR2 and TLR4 expression were not identified.

Another possible explanation would have PBMCs derived from NAFLD patients containing a higher percent of monocytes, the principal cell population responsible for proinflammatory cytokine production, than those derived from HC subjects. Indeed in a Korean study of 794 subjects, increased monocyte fractions were documented in PBMCs of NAFLD patients compared to healthy controls. However, we found no such difference in monocyte fractions. Presumably, the reason for this discrepancy relates to differences in the identification of monocytes in that we employed a more stringent definition that excluded immune-activated HLA-DR positive cells in favor of the more specific CD3-, CD19-, CD56-, HLA-DR+, CD14+ monocyte markers.

IL-10 is an anti-inflammatory cytokine that downregulates proinflammatory cytokine production and blocks proinflammatory signaling. Progression of NAFLD has been associated with a relative deficiency of IL-10. Thus, the increased proinflammatory cytokine release observed in NAFLD patients would have been explained had IL-10 expression increased to a greater extent in HC subjects following PAMP exposure. However, none of the PAMPS studied disproportionally increased IL-10 expression in HC subjects.

Finally, certain cell types develop tachyphylaxis to PAMP exposure, and associated with this, attenuated proinflammatory cytokine release. Whether a similar process occurs in PBMCs derived from HC subjects but not NAFLD patients remains to be determined.

The results of LPS concentration-dependent experiments were interesting in that they demonstrated that at physiological concentrations of LPS (0.2 and 2.0 ng/ml), proinflammatory cytokine release was significantly higher in PBMCs derived from NAFLD subjects compared to HC subjects, but the difference was no longer evident at the highest LPS concentration employed (20 ng/ml). This finding suggests that once a certain concentration of LPS exposure is reached, PBMC cytokine release is maximum regardless of whether PBMCs are derived from NAFLD patients or HC subjects.

Inflammatory cell infiltration of the liver has been identified as an important feature of NAFLD disease activity.

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Figure 3 Anti-inflammatory IL-10 cytokine levels: IL-10 levels were documented by ELISA in supernatants of freshly isolated PBMCs from NAFLD patients and HC subjects cultured for 24 h with complete culture medium (A), LPS (B), Pam (C), and Pal (D). IL-10 levels were similar in supernatants derived from NAFLD and HC PBMC cultures. Data presented as mean ± SD.
and progression. Of the various chemokine receptors that regulate cell migration and tissue localization, CCR1 and CCR2 are thought to be the most important in NAFLD. Indeed, blocking CCR1 and studies in CCR1 deficient mice reveal reduced monocyte infiltration and hepatic fibrosis. Similar findings have been described with CCR2 deficiency in a murine model of NAFLD. Thus, we documented the effects of PAMP exposure on CCR1 and CCR2 expression in monocytes derived from NAFLD patients and HC subjects. CCR1 and CCR2 expression were similar in the two study populations prior to PAMP exposure and downregulated (in terms of CCR expression per monocyte) to the same extent following PAMP exposure. Although the findings were similar in NAFLD and HC monocytes, from a clinical perspective, these findings support recent attempts to attenuate hepatic inflammation in NAFLD by administering chemokine receptor antagonists. That both CCR1 and CCR2 expression per monocyte were downregulated, suggests the need to employ nonspecific or pan-CCR receptor antagonists.

Another clinically relevant question addressed in this study was whether the in vitro changes described in proinflammatory cytokine release and CCR downregulation correlate with the extent of hepatocyte injury and cellular infiltration and thereby potentially influence the progression of NAFLD to cirrhosis and/or hepatocellular carcinoma. Here, significant correlations existed between PAMP-induced proinflammatory cytokine release and CK18M30 levels (suggesting a positive correlation with hepatocyte apoptosis). Significant correlations were also documented between CCR downregulation and both ALT and CK18M30 levels (suggesting correlations with hepatocyte necrosis and apoptosis, respectively).

There are certain limitations of this study that warrant emphasis. First, liver histology was not available, and therefore the distinction between simple steatosis vs. NASH in NAFLD patients was not possible. Second, obesity is associated with increased populations of intermediate and nonclassical monocytes. Thus, the classical monocyte markers CD14+ and HLA-DR+ employed in this study may not reflect the precise monocyte profile of NAFLD subjects. Third, concentration dependency was documented for LPS but not Pam or Pal. Fourth, the effects of PAMPs other than LPS, Pam, and Pal on other TLRs, cytokines, and CRs have yet to be determined. Fifth, combinations of the various PAMPs were not tested to determine whether additive or synergistic responses occur. Sixth, given the association between increased proinflammatory cytokine release and obesity per se, it will be important to determine whether these findings also apply to lean NAFLD. Finally, the controversy as to whether PBMCs/monocytes derived from the systemic circulation reflect...
Figure 5  CCR1 and CCR2 expression depicted as percentage of monocytes staining positive for these receptors and receptor expression per monocyte (A and C). There were no differences between NAFLD patients and HC subjects at baseline (steady state) or following PAMP stimulation (B and D). Data presented as mean ± SD.
CONSENT FOR PUBLICATION
All authors have consented to the publication of the manuscript.

AVAILABILITY OF DATA AND MATERIAL
On request to the corresponding author.

CODE AVAILABILITY
Not applicable.

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REFERENCES


