The Influence of Gut Derived Antigens and Hepatic Steatosis-Associated Fatty Acids on Chemotaxis, Innate Immunity and Pro-Inflammatory Cytokine Expression in Patients with Non-Alcoholic Fatty Liver Disease.

by

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ABSTRACT

Background: Emerging data suggests that enhanced chemotaxis, innate immunity and pro-inflammatory mediators, namely cytokines and oxylipins are critical in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Data also suggests that gut-derived antigens and hepatic steatosis-associated fatty acids are potent regulators of these activities. To date, there have been no studies documenting the extent of correlation between these factors and the extent of hepatic inflammatory activity in NAFLD. **Objectives:** The objectives of this study were: 1) To compare plasma oxylipin levels in 43 different NAFLD patients and healthy controls (HCs). 2) To compare basal and stimulated levels of chemokine receptor (CCR), Toll-like receptor (TLR),cytokines IL-1β, IL-6, TNF-α and IL-10 in peripheral blood mononuclear cells (PBMCs) and monocytes derived from NAFLD patients and HCs, and 3) To determine whether the differences observed in NAFLD patients correlate with hepatic apoptotic activity as reflected by levels of the hepatic apoptotic marker plasma ck18. **Methods:** 35 adult NAFLD patients and 8 HCs participated in the study. Plasma oxylipin levels were determined by mass spectrometry. PBMCs and monocytes were isolated and analyzed *in vitro* prior to and following exposure to varying concentrations of the gut-derived antigens, lipopolysaccharide (LPS) and Pam3CSK4 (PAM) or the hepatic steatosis-associated fatty acid, palmitate (PAL) for 12-24 hours. CCR1, CCR2, TLR2, TLR4, IL-1β, IL-6, IL-10 and TNF-α surface or secreted protein levels were measured by flow cytometry and ELISA respectively. **Results:** Plasma oxylipins, 20-HETE and 8-HETrE

were elevated in NAFLD patients with 5-HETE, 8-HETE, 15-HETE, 20-HETE, 11,12-DiHETrE and 14,15-DiHETrE correlating positively with ck18 levels. At baseline, CCR, TLR and cytokine levels were similar in NAFLD patients and HCs. In NAFLD patients, the levels of CCR, TLR and cytokines did not correlate with plasma ck18 levels. Following stimulation with LPS, Pam3CSK or palmitate, CCR1 and CCR2 levels decreased in both NAFLD patients and HCs but to a lesser extent in HCs. The extent of the CCR1 but not CCR2 decrease inversely correlated with plasma ck18 levels. TLR2 levels significantly increased following stimulation while TLR4 expression was found to decrease following LPS, but not Pam3CSK4 or palmitate stimulation. The changes in toll-like receptors were similar in NAFLD patients and HCs and did not correlate with plasma ck18 levels in NAFLD patients. The levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α increased following stimulation in NAFLD patients and HCs. Importantly, gut derived antigen-induced IL-1β and IL-6 levels were increased in NAFLD patients compared with HCs and positively correlated with ck18 levels. **Conclusions:** The results of this study suggest that specific oxylipin levels differ between NAFLD subjects compared to HCs. In addition, NAFLD was associated with an increased PBMC sensitivity to gut antigens reflected by increased stimulated IL-1β and IL-6 production. The increased PBMC sensitivity in simple steatosis is likely prior to the inflammation in the liver which might contribute to NASH development. The increased PBMC sensitivity correlated with hepatic apoptosis levels which indicated that PBMC may play a role in hepatic inflammation and cell death. We also found CCR1 expression was decreased after stimulation, possibly reflecting

reduced monocytes trafficking from the site of inflammation which suggests hepatic apoptosis is associated with an increased ability of monocytes to stay in the liver with gut derived stimuli.

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LIST OF ABBREVIATIONS

AA Arachidonic acid
AASLD American Association for the Study of Liver Diseases
AFLD Alcoholic fatty liver disease
ALA α-Linolenic acid
ALT Alanine aminotransferase
AST Aspartate aminotransferase
BMI Body mass index
BSA Bovine serum albumin
CCR C-C chemokine receptor
CCL2 C-C motif ligand
CM Culture medium
COX Cyclooxygenases
cPLA2 cytosolic phospholipase A2
CXCR CXC chemokine receptor
CX3CR CX3C chemokine receptor
CVD cardio vascular diseases
CYP Cytochrome P450
DAMPs Damage-associated molecular patterns
DCs Dendritic cells
DHA Docosahexaenoic acid
DGLA Dihomo-γ-linolenic acid
ELISA Enzyme-linked immunosorbent assay
EPA Eicosapentaenoic acid
FACS Fluorescence-activated cell sorting
FFAs Free fatty acids
FMO fluorochrome minus one
GLA γ-Linolenic acid
HC healthy controls
HCC hepatocellular carcinoma
HDL high-density lipoprotein
HLA-DR human leukocyte antigen DR
HPLC-MS/MS High performance liquid chromatography-tandem mass spectrometry
HSC Hepatic stellate cells
IL-1β Interleukin one beta
IL-10 Interleukin ten
IL-6 Interleukin six
IR insulin resistence
KCs Kupffer cells
LA Linoleic acid
LDL low-density lipoprotein
LOX Lipoxygenases

LPS Lipopolysaccharide
MHC Major histocompatibility complex
MFI Median fluorescence intensity
NAFLD non-alcoholic fatty liver disease
NASH non-alcoholic steatohepatitis
Nf-kB Nuclear factor-κB
NHANES National health and nutrition exami-nation survey
PRRs Pattern recognition receptors
PAM Pam3CSK4
PAMPs Pathogen-associated molecular patterns
PAL palmitate
PBMCs Peripheral blood mononuclear cells
PBS Phosphate buffer saline
PGE2 Prostaglandin E2
PNPLA3 Patatin-like phospholipase domain-containing protein 3
PRRs Pattern recognition receptors
PUFA polyunsaturated fatty acid
ROS Reactive oxygen species
SS Simple steatosis
Tetranor 12-HETE Tetranor12-hydroxy-eicosatetraenoic acid
TNF-α Tumor necrosis factor-alpha
TLR Toll-like receptor
TM6SF2 Transmembrane 6 superfamily 2 human gene
TXB2 Thromboxane B2
10-HDoHE10-hydroxy-docosahexaenoic acid
11,12-DiHETrE 11,12-dihydroxyeicosatrienoic acid
11,12-EpETrE 11,12-epoxy-eicosatrienoic acid
11-HDoHE 11-hydroxy-docosahexaenoic acid
11-HETE 11-hydroxy-eicosatetraenoic acid
12,13-diHOME 12,13-hydroxy-octadecadienoic acid
12,13-EpODE 12,13-epoxyoctadecadienoic acid
12,13-EpOME 12,13-epoxyoctadecenoic acid
12-HEPE 12-hydroxy-eicosapentaenoic acid
12-HETE 12-hydroxy-eicosatetraenoic acid
13-HDoHE 13-hydroxy-docosahexaenoic acid
13-HODE 13-hydroxy-octadecadienoic acid
13-HOTrE 13-hydroxy-octadecatrienoic acid
13-oxoODE 13-oxo-octadecadienoic acid
14,15-DiHETrE 14,15-dihydroxyeicosatrienoic acid
14,15-EpETrE 14,15-epoxy-eicosatrienoic acid
14-HDoHE 14-hydroxy-docosahexaenoic acid
15-HEPE 15-hydroxy-eicosapentaenoic acid
15-HETE 15-hydroxy-eicosatetraenoic acid
15-HETrE 15-hydroxy-eicosatrienoic acid

15-oxoETE 15-5-oxo-eicosatetraenoic acid
16-HDoHE 16-hydroxy-docosahexaenoic acid
16-HETE 16-hydroxy-eicosatetraenoic acid
17-HDoHE 17-hydroxy-docosahexaenoic acid
17-HETE 17-hydroxy-eicosatetraenoic acid
18-HEPE18-hydroxy-eicosapentaenoic acid
18-HETE 18-hydroxy-eicosatetraenoic acid
19,20-DiHDoPE 19,20-dihydroxydocosapentaenoic acid
20-HDoHE 20-hydroxy-docosahexaenoic acid
20-HETE 20-hydroxy-eicosatetraenoic acid
4-HDoHE 4-hydroxy-docosahexaenoic acid
5,6-DiHETrE 5,6-dihydroxyeicosatrienoic acid
5,6-EpETrE 5,6-epoxy-eicosatrienoic acid
5-HETE 5-hydroxy-eicosatetraenoic acid
5-oxoETE 5-5-oxo-eicosatetraenoic acid
7-HDoHE 7-hydroxy-docosahexaenoic acid
8,9-DiHETrE 8,9-dihydroxyeicosatrienoic acid
8-HDoHE 8-hydroxy-docosahexaenoic acid
8-HETE 8-hydroxy-eicosatetraenoic acid
8-HETrE 8-hydroxy-eicosatrienoic acid
9-oxo-OTrE 9-oxooctadecatrienoic
9,10-diHOME 9,10-hydroxy-octadecadienoic acid
9,10,13-triHOME 9,10,13-trihydroxy-octadecenoic acid
9,12,13triHOME 9,12,13-trihydroxy-octadecenoic acid
9-HETE 9-hydroxy-eicosatetraenoic acid
9-HODE 9-hydroxy-octadecadienoic acid
9-HOTrE 9-hydroxy-octadecatrienoic acid
9-oxoODE 9-oxo-octadecadienoic acid

1. Introduction

1.1 Non-alcoholic fatty liver disease (NAFLD)

1.1.1 Definition of NAFLD

NAFLD is the most common liver disease worldwide, affecting 30% of people in the Western world. NAFLD encompasses a wide spectrum of disorders that ranges from simple steatosis (SS), to nonalcoholic steatohepatitis (NASH), to steatofibrosis which can lead to cirrhosis and hepatocellular carcinoma (HCC). According to the guidelines of the American Association for the Study of Liver Diseases (AASLD), NAFLD is defined as detection of primary hepatic steatosis (via microscopy of a liver biopsy) where over 5% to 10% of hepatocytes have overloaded lipid accumulation, excluding subjects with excessive alcohol consumption or drug exposure [1]. Simple steatosis is characterized by a lack of inflammation or hepatocyte damage termed 'ballooning'. NASH is characterized by steatosis, ballooning, inflammation and possibly fibrosis that can induce end-stage liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). The majority of patients with simple steatosis have benign and non-progressive clinical features. Their risk of progression into NASH is 10-20%. The risk of direct progression with no NASH intermediate stage to cirrhosis and liver failure is very low [2]. However, patients with NASH are at high risk to develop cirrhosis and end-stage liver disease. There is no effective pharmacological treatment for NAFLD, likely due to a lack of understanding of the biology of NAFLD-progression. Thus, it is critical to understand the mechanisms behind NAFLD-progression, especially the role of immune cells and immune-mediated inflammation.

1.1.2 Epidemiology of NAFLD

The US National Health and Nutrition Examination Survey has reported that the percentage of NAFLD cases constituting chronic liver disease rose from 47% in 1988 to 75% in 2008 [3]. It is estimated that up to 1 billion people have NAFLD worldwide [4] and the prevalence of NAFLD is still rising. Globally, the estimated prevalence has reached more than 30% for adults and 10% for children. The estimated prevalence of NAFLD is around 24% in African Americans [5] 20–30% in Europeans and people from the Middle East [6, 7]. In Australia, the prevalence has been reported to be 20-30% [4]. NAFLD is no longer only a "western disease". Increased prosperity and lifestyles consisting of excessive calorie intake and lack of exercise have raised the prevalence of this metabolic syndrome worldwide. According to recent studies from Asia, the prevalence of NAFLD in Japan, China and Korea are estimated to be 9–30% [8, 9], 15–30% [10, 11] and 16%-18% [8, 12] respectively. In India, the prevalence of NAFLD ranges from 16% to 32% in urban populations [13] and 9% [8] in rural areas where traditional lifestyles and diets are still dominant. The prevalence of NAFLD in Hispanic populations is as high as 40% [14]. The rising prevalence of NAFLD likely reflects the impact of sedentary lifestyle and excess nutrition in emerging and growing economies.

1.1.3 Diagnosis of NAFLD

The early stage of NAFLD is usually asymptomatic or only presents with minor symptoms, such as abdominal discomfort [15]. More often the disease tends to be detected by elevated liver alanine aminotransferase **(**ALT) and aspartate aminotransferase (AST) values in patients undergoing routine blood testing or by abnormal ultrasound scores performed for other conditions. When elevated liver enzymes are detected, alcohol consumption will then be evaluated on NAFLD patients to exclude the possibility of alcoholic fatty liver disease

(AFLD). Sufficient alcohol consumption to diagnose AFLD is defined as a minimum of 2 years drinking greater than 21 drinks per week for men and more than 14 drinks per week for women [16]. Additionally, to diagnose NAFLD, drugs associated with fatty infiltration of the liver such as hormones, methotrexate, valproic acid etc. also need to be considered. Finally, autoimmune disorders, Wilson's disease and other causes of liver steatosis should also be screened and excluded by related tests [17].

Serum levels of AST and ALT are the most common laboratory parameters used to detect liver disease. AST is found in hepatocytes as well as in other organs and tissues such as the heart, kidney, brain and skeletal muscles. As a result, AST is not a specific marker for liver diseases. Different from AST, ALT is found almost exclusively in the cytosol of hepatocytes, at a concentration of approximately 3000 times more than that of serum [18]. Upon liver injury, release of ALT from hepatocytes increases the ALT level in the serum. Due to its technical feasibility, low cost and association with NAFLD disease, ALT level is widely used in clinics as a surrogate marker of NAFLD [18]; however, release of ALT from other tissues, for example kidney and heart as well as natural variations in ALT levels makes it difficult to diagnose liver disease accurately using only ALT. In addition, ALT is found elevated in only 50% of patients that are confirmed with simple steatosis and in 80% of patients with NASH [19].

Fat accumulation in the liver of NAFLD patient can be detected by ultrasonography, computed tomography and magnetic resonance imaging. Ultrasonography represents the most widely used diagnostic tool for fatty liver in clinical practice. It is relatively inexpensive and has good sensitivity to detect moderate or severe steatosis (>15%) but poor sensitivity to detect minor steatosis (<15%). However, it may have intra-observer and

inter-observer interpretation variability [20, 21]. Additionally, ultrasound is not able to distinguish simple steatosis from NASH [20]. Another method is computed tomography which is more quantitative and objective than ultrasound, but is also only able to assess moderate or severe steatosis [22]. It is not routinely used for fatty liver diagnosis due to the harmful radiation exposure to patients. Magnetic resonance imaging is a new approach that is able to not only detect, but also quantify, mild steatosis with a high sensitivity and specificity [23]; however, it too cannot distinguish between simple steatosis and NASH. Additionally, the relatively high-cost equipment of magnetic resonance imaging limits its application in NAFLD diagnosis. Currently, a liver biopsy is considered the gold standard to evaluate NAFLD. The liver biopsy provides important information including the degree of fat infiltration, inflammation as well as fibrosis. A liver biopsy is the removal of a small piece of the liver tissue from the patient; however, the procedure is invasive and introduces the possibility of developing serious complications. Furthermore, differences in location of the biopsy (i.e. where in the liver the sample is taken from) as well as variation in the interpretation of the result can impede proper diagnosis [24].

Due to the limitations of existing diagnostic methods, investigators have attempted to identify non-invasive biomarkers that can accurately reflect NAFLD disease severity and distinguish simple steatosis from NASH. Among them, the detection of the ck18 fragment, M30, is a promising method since apoptosis is a crucial cell death mechanism in NAFLD patients [25] and ck18 is a major intermediate filament protein found in hepatocytes. During hepatocyte apoptosis, activated caspases, mainly caspase3 cleave ck18 in the cytosol and release it into the plasma, which correlates with hepatocyte death [26]. Ck18 can be detected by enzyme-linked immunosorbent assay (ELISA). More than 10 groups

have demonstrated the independent function of Ck18 M30 to distinguish between simple steatosis and NASH patients with the confirmation of histopathology liver biopsies. Cut off values for a diagnosis of NASH ranged from 121.6 to 380.2 U/L with a sensitivity as high as 0.95 and specificity of 0.97 [27].

In summary, NAFLD patients were recruited based on their elevated ALT levels. ck18 was used to distinguish simple steatosis and NASH in this study.

1.1.4 Risk factors for NAFLD

The metabolic syndrome is the primary risk factor for NAFLD. Metabolic syndrome is a group of medical conditions including insulin resistance, obesity, dyslipidemia and hypertension, to name a few. Insulin resistance (IR) is a known contributor to the pathogenesis of NAFLD. In a study examining liver biopsies, two thirds of NAFLD patients with IR had significant higher mean steatosis grades than patients without IR [28]. Diabetes or even a family history of diabetes is also associated with an increased risk of NAFLD [29, 30]. In a large study of 3166 type 2 diabetes (T2D) patients, the prevalence of NAFLD was 69.5%, which is significantly higher than the global prevalence of NAFLD [31]. Obesity is another risk factor for NAFLD; both a high body mass index (BMI) and abdominal obesity are associated with an increased risk of NAFLD. In a study of patients that underwent bariatric surgery (BMI>40 kg/m^2), the prevalence of NAFLD and NASH was found to be 91% and 37%, respectively. Abnormal lipid metabolism also appears to have a substantial influence on NAFLD development. A Chinese study grouped 60,000 subjects undergoing ultrasound into 4 groups based on their LDL-c level and found the prevalence of NAFLD was higher in these cohorts with high LDL-c levels [32]. In addition to a higher prevalence of NAFLD patients with the metabolic syndrome are at higher risk

of NASH, fibrosis and liver failure. Other factors such as older age, male gender, Hispanic background, patatin-like phospholipase domain-containing protein 3 (PNPLA3) or transmembrane 6 superfamily 2 human gene (TM6SF2) allele carriers, psychosocial stress and smoking have also been reported to be associated with a higher risk of NAFLD [33-36].

1.1.5 Complications of NAFLD and NAFLD-related death

The interaction between the metabolic syndrome and NAFLD is bi-directional. Increased levels of lipid in the liver decrease hepatic insulin sensitivities, which then leads to hyperglycemia. Hyperglycemia stimulates insulin secretion and resulting in hyperinsulinemia. Hyperinsulinemia increases the risk of progressing to T2D, cardiovascular disease, extrahepatic malignancies and the metabolic syndrome [37]. Evidence has shown that high fat diet induced hepatic insulin resistance develops earlier than systemic insulin resistance [38]. Indeed, NAFLD is regarded as the hepatic component of the metabolic syndrome [39].

NAFLD is the most common cause of chronic liver disease such as liver fibrosis and cirrhosis. In a 13 year prospective study, survival was significantly lower in NAFLD patients than reference subjects without NAFLD (78% vs 84%) [40]. Approximately 30-40% of NASH patient progress to fibrosis and cirrhosis and thereby, an increased risk of hepatocellular carcinoma (HCC) [15]. HCC is also found in NAFLD patients without cirrhosis [41]. It is reported that NASH is the second leading disease responsible for liver transplantation in the United states [42].

1.2 Liver anatomy and cell composition

The liver is one of the most essential organs in mammals. In the human body, it is in the

upper right portion of abdomen beneath the diaphragm under the rib cage. It is irregularly shaped; and is thicker on the right and narrower on the left. By weight, the liver is the largest organ in the human body, weighing 1200-1600g in a healthy adult. The liver has many vital functions, including metabolism of protein, fat and carbohydrates, bile secretion, and glycogen storage. It is also responsible for detoxification, hematopoiesis and coagulation.

The liver contains 14% of the blood volume in the human body. A volume of 800-1200ml of blood flows through liver per minute [43]. The blood coming to the liver is supplied by two blood vessels, hepatic artery and portal vein. Approximately [44]. Blood from the hepatic artery originates from the aorta and providing oxygen to the liver. Blood coming from the portal vein is rich in digested food, bacterial products and toxins, as it traffics blood originating from the large and small intestines, the stomach and the spleen to the liver. Blood flowing through these two vessels mixes when they reach the lobule (Figure 1). Lobules are basic functional units of the liver. Each lobule is constituted by a sinusoid, a bile duct and millions of liver cells. The sinusoid is present as small capillaries, each of which has a branch of the hepatic portal vein and a branch of the hepatic artery. Blood from these two branches flow through the sinusoids reach to the central vein. The bile duct transports bile, which helps emulsify the lipids and aid food digestion in the small intestine. Liver cells consist of 80% parenchymal cells and 20% non-parenchymal cell [45]. Hepatocytes are the principal cell population of parenchymal cells which are responsible for liver metabolism, biosynthesis, biliary secretion, and detoxification. The non-parenchymal cells primarily include liver sinusoidal endothelial cells, and a diverse variety of immune cells. The innate immune cells that resident in the liver include KCs, dendritic

cells (DC) and natural killer (NK) cells [46]. Once the liver is injured, other immune cells such as neutrophils, monocytes and lymphocytes infiltrate into the liver. Sinusoidal endothelial cells and hepatic stellate cells [47] may also contribute to immune responses.It is not surprising that the liver contains many immune cells due to its unique anatomical location and particular functions.

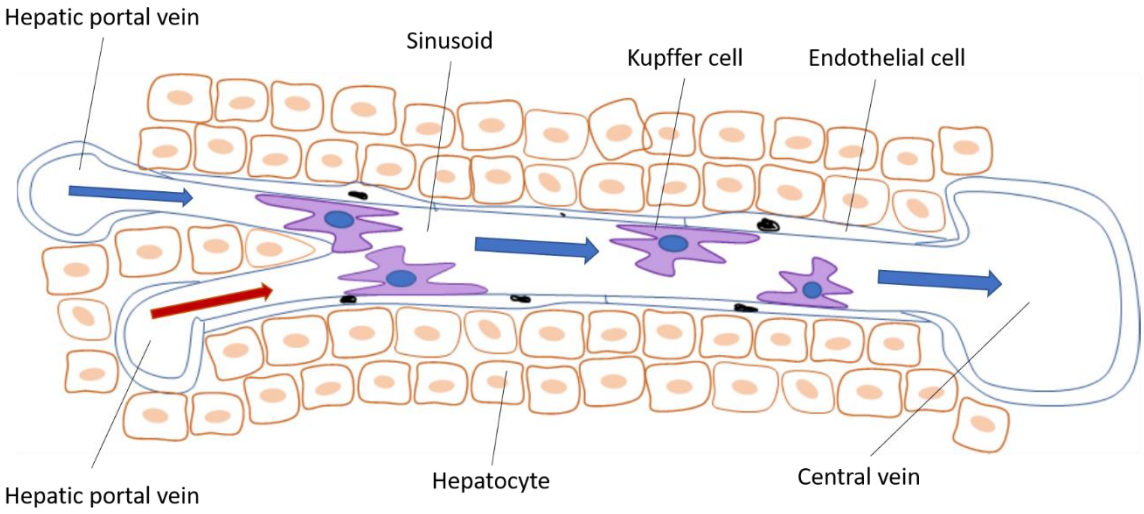


Figure 1. Liver anatomy and cell composition

The liver consists of many functional units called lobules. Each lobule contains blood vessels, bile duct and millions of liver cells. A sinusoid is a blood vessel derived from branches of the hepatic portal vein and a hepatic artery. Blood in the sinusoids flow to the central vein. Bile ducts transports bile, which helps emulsify the lipids and aids food digestion in the small intestine. Liver cells consist of 80% parenchymal cells, hepatocytes, and 20% non-parenchymal cell. The Non-parenchymal cells include liver sinusoidal endothelial cells, hepatic stellate cells and a diverse variety of immune cells of which Kupffer cells (KCs) is the predominant cell type. KCs are located throughout the liver sinusoids and are exposed to the blood where they capture and clear pathogens.

KCs are liver resident macrophages [47] accounting for 15% of liver cells, which represent the largest tissue specific macrophage population in the human body. There are two subsets of KCs in the liver [48], one subset originates from embryonic progenitor cells. These cells are located more in the portal area where they first meet the exogenous materials and serve to function in immune surveillance [45, 49]. They clear the blood by phagocytosis of low levels of microbes or microbial-derived products, apoptotic cells and debris [50]. In this basal position, due to the down regulation of major histocompatibility complex (MHC) expression, KCs are not being activated [51, 52]. Stimulations such as infection, inflammation and additional TLR ligands could activate KCs by upregulating MHC expression. The other subset of KCs are differentiated from bone marrow derived circulating monocytes. These monocytes infiltrate into liver during liver injury [52]. They produce pro-inflammatory cytokines, reactive oxygen species (ROS) and activate components to promote inflammation and initiate fibrosis.

1.3 Mechanisms of NAFLD development

1.3.1 "Two-hit" and "multiple-hit" hypotheses

Despite the high prevalence of NAFLD and underlying complications, NAFLD pathogenesis is poorly understood; however, some hypotheses have been postulated. According to the "two-hit" hypothesis [53], peripheral insulin-resistance is thought to be the "first hit" that results in hepatic steatosis [54]. A number of other processes that associate with liver inflammation such as activation of liver resident immune cells (Kupffer cells), increased production of hepatic chemokines and cytokines and increased immune cell infiltration, are thought to be the "second hit" that leads to transition of simple steatosis to NASH and fibrosis. However, recent studies have proposed a "multiple-hit" hypothesis

which suggests that steatosis and inflammation happen simultaneously [55]. It has been proposed that pro-inflammatory cytokines such as IL-6 and TNF-α that promote inflammation can also contribute to hepatic steatosis [56]. Additionally, fatty acids from high-fat diet that induce steatosis and IR contribute to hepatic inflammation via binding to TLR4. Finally, obesity and high fat diet are associated with increased gut permeability which could lead to microbial translocation and directly initiate and enhance liver inflammation [57]. Thus, there are multiple hits that may contribute to the pathogenesis of NASH parallelly which include the interactions between the host metabolic system, immune system, gut microbiota and environmental factors [2, 58].

1.3.2 Simple steatosis is associated with increased circulating FFAs and insulin resistance

Circulating free fatty acids (FFAs) are the source of fat in the liver. They are stored in adipocytes as the energy resource. In healthy individuals, degradation or hydrolysis of the stored fatty acid is carefully regulated. However, in obese individuals, excess fat exceeds their lipid storage capacity and metabolism. This causes adipocyte hypertrophy, hyperplasia and cell death. Dead adipocytes release free fatty acids into the blood resulting in elevated levels of circulating free fatty acids. Insulin resistance is another cause of increased FFAs in the blood due to the decreased inhibition of lipolysis. Finally, FFA are carried to the liver through blood vessels.

Insulin resistance contributes to hepatic fat accumulation. Insulin resistance occurs when cells fail to respond normally to insulin. For example, insulin resistance in skeletal muscle leads to inadequate glucose uptake from the bloodstream, leading to post-prandial hyperglycemia.

In adipose tissue, insulin signaling promotes the uptake and storage of fatty acids and inhibits lipolysis. In the liver, insulin signaling is important in suppressing endogenous glucose production when dietary carbohydrate intake is high. In the healthy state, insulin is produced by the pancreatic beta cell when blood glucose levels increase after digestion of carbohydrates from food. Insulin resistance pushes the beta cells to increase the production of insulin to compensate, which can lead to hyperinsulinemia. Hyperinsulinemia is thought to contribute to hepatic fat accumulation due to the predominant role of liver in lipid and glucose metabolism in the human body. The overload of fatty acid infiltration to the liver as well as IR lead to increased *de novo* hepatocyte lipid generations and decreased hepatocyte lipid metabolism [59].

In summary both enhanced release of fatty acid from adipose tissue and/or systemic or adipose tissue insulin resistance leads to systemic fatty acid flux and liver steatosis.

1.3.3 NAFLD is associated with increased Pathogen-associated molecular patterns (PAMPs) caused by increased intestinal permeability

Emerging studies have investigated the role of intestinal permeability and its association with NAFLD progression. In patients with NASH, intestinal permeability was found to be strongly associated with liver injury [60]. In addition, a study has found there is a significant higher presence of increased intestinal permeability in NAFLD patients compared with healthy controls [57]. Another study has shown that obesity may impair intestinal barrier function that results in increased intestinal permeability related to a loss of integrity of epithelial tight junctions [61]. The alteration of tight junctions has been shown to be directly induced by a high fat or high sucrose diet in murine models [62]. The intestinal barrier segregates exogenous substances from the host. It is responsible for the

selection of nutrients, water and defense against bacterial products such as lipopolysaccharide (LPS) to the portal circulation [63].

Increased gut permeability leads to releasing of PAMPs in the blood. PAMPs refer to a variety of bacterial products such as bacterial RNA or DNA, and cell wall components such as lipopolysaccharides and lipoproteins [64]. In the context of NAFLD, PAMPs reaching the liver are not liver specific pathogens but microbial components from blood delivered to the liver (sterile inflammation) [65]. By binding to Toll-like receptors, the DAMP and PAMP activate pro-inflammatory signaling pathways [66, 67]. In the liver, this process happens mainly in KCs. These pathways then activate the transcription of many pro-inflammatory genes including cytokines, chemokines and other effectors of the innate immune response [67], result in mixed inflammatory cells such as neutrophils and lymphocytes infiltration [68].

1.3.4 NAFLD is associated with an increased immune activation

Innate immunity is that component of the immune system which respond to antigens in a non-specific way. Innate immune responses play an important role in the onset and progression of inflammation in NAFLD. Under normal circumstances, liver resident immune cells, mainly KCs assist liver detoxify by phagocyting gut-derived bacterial products such as LPS, lipoproteins and DNA. This process is immune tolerant and not harmful to the liver.

However, in NAFLD, increased PAMPs caused by changes in gut microbiome and intestinal wall permeability are transported via the portal venous system into the liver where they activate TLR2 and TLR4 on the surface of resident macrophages/Kupffer cells. Activation of Kupffer cells in NAFLD triggers liver inflammation. However, the degree of

inflammation is affected by many factors including the cell activation sensitivity to PAMPs and the level of chemotaxis. The sensitivity of cell activation can be affected by the level of Toll like receptor a cell is expressed and reflected by the cytokine production levels. Besides, chemotaxis can be influenced by chemokine receptor expression levels on one cell.

Toll-like receptors (TLRs)

TLRs can recognize DAMPs and PAMPs and are expressed by monocytes/macrophages [69]. The most studied TLRs in NAFLD, and particularly in NASH, are TLR2 and TLR4. These receptors recognize a variety of PAMPs from bacteria, viruses, fungi and parasites. TLR2 recognizes the bacteria lipoproteins; peptidoglycan, and lipoteichoic acid. In a NAFLD animal study, TLR2 deficiency was associated with reduced inflammatory cell infiltration and ameliorated hepatic inflammation [70-72]. TLR4 recognizes LPS, viral proteins and other components of microbes. Continuous subcutaneous injection of low dose LPS in WT mice resulted in liver insulin resistance and steatosis [73] which highlight the LPS-TLR4 response in NAFLD onset and progression. TLR4 deficiency in animal models also displayed a protection role against steatohepatitis [74, 75]. Both TLR2 and TLR4 can recognize certain fatty acid like palmitic acidand trigger inflammatory signalling pathways. Palmitic acid is a common fatty acid that can be found in meats, dairy products and plant oil. By binding to TLRs, PAMPs and DAMPs activate pro-inflammatory signaling pathways [69]. These pathways then activate the transcription of many pro-inflammatory genes that encode pro-inflammatory molecules including cytokines, chemokines, oxylipins and other effectors of the innate immune response. In the liver, this process occurs mainly in KCs. In the context of liver inflammation, KCs are recruited from

blood.

Chemokine receptors

Monocyte migration to the site of inflammation is driven by the interaction of chemokine and chemokine receptors as well as adhesion molecules [76]. In NAFLD, recruited monocytes can further contribute to liver inflammation. Monocyte expresses chemokine receptors migrate from blood to the inflamed tissue following the gradient of chemokines. Production of chemokines could be initiated by hepatocyte damage by fat infiltrate or activated KCs. CCL2 (MCP-1), CCL3 (MIP-1α) and CCL4 (MIP-1β) are chemokines that directly target monocytes to promote cell migration [77]. Chemokine receptors are a group of G protein-coupled receptors. Monocyte chemokine receptors include CXC chemokine receptor CXCR1, CXCR2, CXCR4, C-C chemokine receptor CCR1, CCR2, CCR4, CCR5, CCR8 and CX3C chemokine receptor CX3CR1. In animal studies, CCR1 and CCR2 expression are shown to be associated with liver diseases. CCR1 expression was upregulated in KCs of fibrotic mice [78]. Further, blocking CCR1 or using CCR1 deficient mice showed reduced fibrosis and macrophage infiltration [79]. Knocking out CCR2 decreased monocyte liver infiltration and reduced liver inflammation and fibrosis in a NAFLD murine model [80, 81]. Moreover, the pharmacological CCR2/5 antagonist Cenicrivirocis, has been demonstrated to be anti-inflammatory in animal models and is in phase 2b clinical trials for reducing hepatic inflammation and fibrogenesis in NAFLD treatment [82].

Cytokines

There is increased immune activation in NAFLD reflected by elevated cytokine levels in NAFLD livers. These cytokines include IL-β, IL-6 and tumor necrosis factor alpha (TNF-

α) [83-85]. IL-1β can promote hepatic lipid accumulation. Ex-vivo exposure to IL-1β in a choline-deficient, l-amino acid-defined diet induced NAFLD mice model increased hepatocyte apoptosis and necrosis. Additionally, IL-1β was shown to promote the activation of hepatic stellate cells, the fibrosis producing cells in the liver. Knock out of IL-1β receptor had a protective effect against hepatic steatosis, apoptosis and fibrosis [86].

IL-6 is responsible for stimulating neutrophil cytokine production, B cell proliferation, and the inhibition of regulatory T cells. In addition, IL-6 also stimulate hepatocytes to produce acute phase reactants. Evidence has shown that IL-6 levels detected from liver biopsies were elevated in NASH patient livers compared to simple steatosis patients. As well, IL-6 expression in the liver positively correlates with the stage of liver fibrosis [85].

TNF-α is able to influence insulin resistance and hepatic lipid accumulation [87]. TNF-α induces cell apoptosis and insulin resistance [88], and is a potent pro-inflammatory cytokine. Release of TNF-α promotes inflammation and hepatic dysfunction. Kudo et al. reported TNF-α contributes to reactive oxygen species (ROS) accumulation in the liver which then promotes hepatocyte cell death. In turn, by pretreatment with H_2O_2, promotes TNF-α inducible e hepatocyte apoptosis. Contrary to pro-inflammatory cytokines, IL-10 is an anti-inflammatory cytokine and can down regulate pro-inflammatory cytokines and blocks pro-inflammatory signaling. IL-10 contributes to improve insulin sensitivity by balancing the pro-inflammatory cytokines and reducing inflammation. Then, IL-10 play a protective role in NAFLD and progression of NAFLD may associated with a relative deficiency of IL-10 [89]. Overall, an imbalance of pro- and anti-inflammatory cytokine production resulting in enhanced pro-inflammatory and a relative decrease in anti-inflammatory cytokines may contribute to IR, fat accumulation and inflammation in

NAFLD development.

1.3.5 The role of monocyte in NAFLD progression is not clear

Monocytes are bone marrow-derived leukocytes that constitute 2-10% of the total white blood cell population. Monocytes circulate in the blood providing a resource for tissue macrophage replacement. Blood monocytes and tissue macrophages are from the same cell lineage but in two different stages of development. Older models suggest that monocytes continually replenish tissue resident macrophage. However, this does not appear to be the same for all tissues. In the liver, monocytes are recruited from blood as replenishment during liver injury [90]. If not being recruited to tissues, monocytes in the circulation can live 1-2 days [91]. Monocytes that enter the liver can differentiate into macrophages and survive for a longer period. Monocytes do not proliferate, but remain in a steady state. The number of monocytes in the blood is normally within a set range, however, an increase of circulating monocyte numbers is found in NAFLD [92]. There are limited studies describing monocyte function and phenotype in NAFLD.

Monocytes, similar with macrophages, express Toll-like receptors on their cell surface or intracellularly that recognize DAMP and PAMP. The TLR family include 9 members TLR1-9. Similar with macrophages, activation of monocyte TLRs drives production of cytokines such as IL-1β, IL-6, TNF-α and IL-10. Monocytes are important in host defense responses, immune surveillance and tissue homeostasis. Regulating cell trafficking, sensing of external or danger molecules and cytokine production are three critical functions of monocytes. During infection, CCR2 is required to drive most monocytes from the bone marrow to the blood and from the blood to the site of inflammation [93]. A deficiency of CCR2 in animal models has been shown to reduce monocyte infiltration and inflammation

in many diseases including NAFLD [94]. CCR1 and CCR5 also play a role in tissue invasion. CCR1 plays a dominant role in monocyte trafficking across endothelial layers. The study of monocyte TLR-activated- and chemotactic-responses could provide additional evidence of how monocytes contribute to NAFLD and provides insights for the development of potential therapeutics.

1.3.6 Oxylipin and NAFLD

Oxylipins are polyunsaturated fatty acid (PUFA) oxidation products, that also function as immune modulators. Oxylipins are found in many tissues as well as in the blood. They are produced by immune cells and endothelial cells in the systemic compartment. PUFA, the precursors of oxylipins, constitute an important component of cell membranes. PUFA can be obtained from diet and are classified as omega-3 (n-3) and omega-6 (n-6) PUFAs based on the position of its first carbon-carbon double bond from the methyl end. Omega-6 PUFAs include Linoleic acid (LA), γ-Linolenic acid (GLA), Dihomo- γ-linolenic acid (DGLA) and Arachidonic acid (AA). Omega-3 PUFAs including α-Linolenic acid (ALA), α-Linolenic acid (ALA), Docosahexaenoic acid (DHA) and Eicosapentaenoic acid（EPA）[95]. In general, n-6 PUFA metabolites are pro-inflammatory, while n-3 PUFA metabolites are anti-inflammatory or at a minimum, less pro-inflammatory. In a previous study, investigators sampled and analyzed the lipid composition of liver biopsies from NAFLD patients and healthy volunteers. They found that an increased n-6/n-3 ratio of liver PUFA in NAFLD patients compared with healthy controls [96]. This may be resulted from a high composition of n-6 PUFA in the Western diet which may lead to a higher levels of pro-inflammatory oxylipins [97].

Table 1. Common PUFAs found in human body

PUFA	Common name	General role (Pro- /anti-inflammatory)
Linoleic acid (LA)	18:2n-6	Pro-inflammatory
α-Linolenic acid (ALA)	18:3n-3	Anti-inflammatory
γ-Linolenic acid (GLA)	18:3n-6	Pro-inflammatory
Dihomo-γ-linolenic acid (DGLA)	20:3n-6	Pro-inflammatory
Arachidonic acid (AA)	20:4n-6	Pro-inflammatory
Docosahexaenoic acid (DHA)	22:6n-3	Anti-inflammatory
Eicosapentaenoic acid（EPA）	22:5n-3	Anti-inflammatory

Most oxylipins have a short half-life and are not stored in cells [95], but rather synthesized in situ when needed and act locally. Synthesis of oxylipins (Figure 2) requires several steps including cell activation, release of free PUFA from the cell membrane and oxidation by cellular enzymes. Cell activation can be achieved by induction of TLR signaling in macrophages. After activation, release of free PUFA is mediated by cytosolic phospholipase A2 (cPLA2) [98]. The enzymes that catalyze free PUFA into oxylipins including a variety of Cyclooxygenases (COX), Lipoxygenases (LOX) and Cytochrome P450 (CYP) enzymes. These enzymes are located to certain types of cells, such as macrophage [99].

As immune mediators, pro-inflammatory oxylipins facilitate cytokine and chemokine activity to induce fever, pain and edema at the site of inflammation. In addition, they are involved in immune cell activation. They also decrease pro-inflammatory cytokine production, such as TNF-α and stimulate anti-inflammatory cytokines such as IL-10. Oxylipins have additional functions including platelet aggregation and vasoactivity. Oxylipins are involved in the pathology of many inflammatory diseases including the metabolic syndrome. Evidence from human studies have shown that NAFLD is associated with increased LOX activity. Specific LOX metabolites were increased in simple steatosis and more so in NASH compared to healthy subjects [100]. Another study showed that the LA metabolite 9-HODE levels were increased in NASH patients [101]. The AA metabolite 11,12-diHETrE levels were significantly higher in NASH patients compared with simple steatosis patients [102]. Evidence has shown an increase in pro-inflammatory oxylipins in patients with metabolic syndrome [103]. The AA metabolite 20-HETE was shown contribute to cardio vascular diseases (CVD) by promoting vascular inflammation and

endothelial dysfunction [104]. Drugs that target oxylipin pathways have shown beneficial therapeutic effects on subjects with metabolic syndrome. Aspirin, an inhibitor of COX pathways that inhibit the COX metabolites which promote platelet aggregation is effective in decreasing the atherosclerosis associated with CVD development [97, 105]. Because NAFLD can cause CVD, the pro-inflammatory oxylipins associated with NAFLD development could be a potential drug target in the treatment of NAFLD.

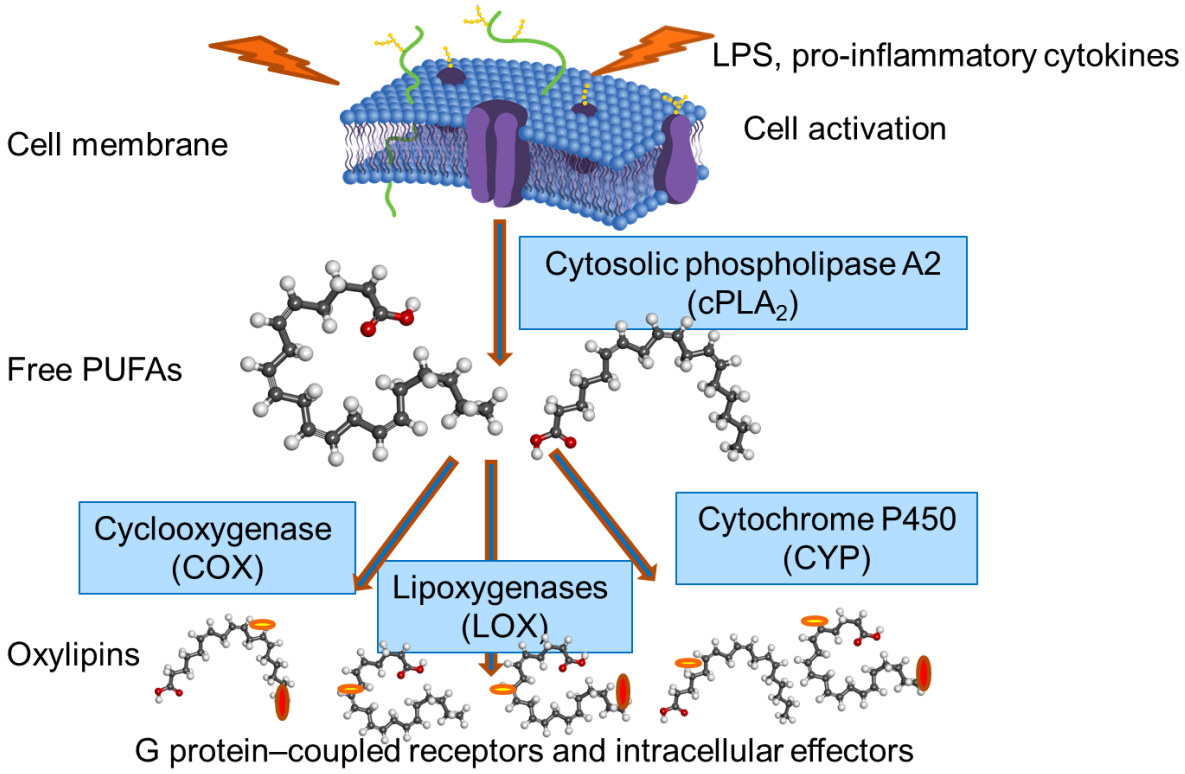


Figure 2. Oxylipin synthesis

Oxylipin formation starts with cell activation. The cell activation leads to release of membrane PUFAs by cPLA2 into cytoplasm. Free PUFAs can undergo metabolism into oxylipins through threes enzymatic pathways. The three pathways involve three main types of enzymes, COX, LOX and CYP. The metabolic oxylipins formed can then activate cells by binding to oxylipin receptors or modulate transcription factors.

1.3.7 Gaps in knowledge

Although emerging data has emphasized the important role of immune signaling, it is difficult to study signaling liver tissue directly. In addition, no studies to date have specifically looked at the role of intestinal derived microbial components and liver steatosis-associated fatty acid TLR activated cytokine response, monocyte TLR and CCR profiles in NAFLD patients. And no studies have linked NAFLD circulating immune functions to the degree of liver apoptosis. Knowledge of the role of oxylipins in NAFLD is even more limited.

1.3.8 Hypothesis

The purpose of our research was to determine whether patients with NASH have increased TLR2 and TLR4 receptor expression in their monocyte/macrophage cell population and/or whether there is increased sensitivity to PAMPs exposure as reflected by changes to TLR2 and TLR4 expression as well as pro-inflammatory cytokine responses. This would help explain the hepatocyte injury associated with NASH. We also endeavored to determine whether differences in chemokine receptor 1 and 2 (CCR1 and CCR2) expression on unstimulated peripheral blood monocytes and following PAMP exposure results in changes in CCR1 and CCR2 expression. This would help explain the inflammatory component of NASH. Finally, because NASH (and perhaps SS) is associated with an increased risk of atherosclerosis and ischemic heart disease we tested the hypothesis that these outcomes could be explained by elevated levels of pro-inflammatory oxylipins and/or low levels of anti-inflammatory oxylipins, the metabolic by products of intercellular fatty acid metabolism pathways.

Based on findings derived from the literature and preliminary data from our laboratory, we

hypothesize that:

1. NAFLD is associated with increased monocyte activities such as increased TLR, CCR expression and elevated TLR induced cytokine response.

2. In NAFLD, increased pro-inflammatory oxylipins associate with disease severity and a pro-inflammatory profile.

2. Materials and Methods

2.1 Solutions

1. Saline

   The saline solution contains 8.5g Sodium chloride powder (Fisher, Whity ON) in 1L Distilled Deionized water. The saline solution was adjusted pH to 7.4 with NaOH (Fisher) and autoclaved to be sterile.

2. Phosphate buffer saline (PBS)

   PBS solution contains 0.25g $NaH_2PO_4 \cdot H_2O$ (Fisher), 1.42 g Na_2HPO_4 (Fisher) and 8.77g NaCl dissolved in 1L DD water and adjusted to pH=7.4.

3. Culture medium (CM)

   CM is mixed by 10% 56°C heat inactivated FBS (Invitrogen Life Technologies, Grand Island, NY), 89% RPMI-1640 (Invitrogen) containing 25mM HEPES buffer and L-glutamine, 0.1% 55m M2-Mercaptoethanolat (Sigma, St. Louis MO) and 1% Antibiotic-Antimicotic PSF (Invitrogen).

4. Bovine serum albumin (BSA, Prolient biologicals, Ankeny, IA) conjugated palmitate (Sigma)

   2.267g BSA was dissolved in 100ml of 150mM NaCl solution at 37°C to make a 0.17mM BSA solution. 30.6mg of Sodium Palmitate was added to 44ml of 150mM NaCl solution and heated to 70 °C. 40 ml of 70 °C palmitate solution was transferred to 50ml BSA solution while stirring at 37 °C for 1 hour and adjusted to final volume of 100ml to achieve a 1mM palmitate working solution.

5. Fluorescence-activated cell sorting (FACS) buffer

FACS buffer contains10g BSA dissolved in 1L saline solution.

6. ELISA Coating buffer

   ELISA Coating buffer contains 1.59g Na_2CO_3 (Fisher), 2.93g $NaHCO_3$ (Fisher) dissolved in 1L DD water and adjusted to pH=9.6 with NAOH.

7. ELISA Blocking buffer (20x)

   ELISA Blocking buffer (20x) contains 34g BSA, 100ml 10x PBS and 10ml 2% Sodium Azide (X) in 1L DD water.

8. ELISA Washing buffer

   2 liters distilled water was added with 20ml 10x PBS and 1ml Tween 20 (Fisher).

9. ELISA Dilution buffer

   ELISA Dilution buffer contains 25ml 20x Blocking Buffer, 100ml 10x PBS and 0.5ml Tween 20 (X) in 1L DD water. The solution was adjusted to pH=7.4 with NAOH solution.

10. ELISA Substrate buffer

    101 mg $MgCl_2$•$6H_20$ was dissolve in H_2O first, then 97 ml Diethanolamine (Sigma) was added and adjusted to 1000ml with D.D Water. pH=9.8 was adjusted with HCL.

2.2 Ethics statement

This study was approved by the University of Manitoba Research Ethics Board. Participation was voluntary and written informed consent forms were signed by all study participants. The collection of blood and all clinical information was conducted by a professional research nurse.

2.3 Cohort definition

Fifty-two adult subjects including healthy controls were recruited. NAFLD patients were recruited on the basis of ultrasound findings consistent with fatty liver and then separated

into two groups based on their ALT levels the day their blood was drawn. Exclusion criteria for all subjects included conditions such as acute or chronic infections (HBV, HCV infection), a history of excess alcohol consumption (>21 units/wk in males and 14 units/wk in famels), usage of medications associated with fatty liver disease (eg. hormones, valproic acid, methotrexate etc), other liver inflammatory disease co-morbidities such as arthritis, and/or signs of acute infection (cold, flu, malaise), malignant tumor(s). In addition, individuals on immunomodulations were excluded. After applying exclusion criteria, 35 NAFLD patients were enrolled in the study. The control group, healthy volunteers matched for age, gender, and ethnicity who had normal ALT levels (ALT< 30 IU/L for men, <25 IU/L for women) and no history of liver disease, alcohol abuse etc.

2.4 Clinical parameters

Subject weight and height was done as standard of care in the liver disease clinics at the Health Science Center. BMI for each subject were computed from the height and weight (height/m^2). Blood pressure was performed in the sitting position using a standard sphynometer. Where available, the results of previous liver biopsies were examined for extent of steatosis, inflammation and fibrosis.

2.5 Clinical Assessment

A comprehensive clinical assessment was obtained from each subject, including demographics, medical history, family medical history and medications used. After collection of medical information, blood was drawn from each subject and sent to Health Science Center Diagnostic Services of Manitoba. Biochemical parameters glucose, creatinine, triglyceride, total cholesterol, LDL, HDL, ALT, AST, and ferritin including fasting levels were measured by routine laboratory assays.

2.6 Plasma collection

30ml of blood was drawn and collected in 10ml purple cap sterile EDTA tube (BD, Franklin Lakes, NJ) from each subject. The blood was then centrifuged at 200g for 8 mins with the brake off. Plasma aliquots were collected immediately into 2ml and 4ml tubes (Eppendorf, Hamburg, Germany) respectively in a biosafety cabinet and stored in a -80^{o}C freezer until analysis for cytokine and oxylipin levels.

2.7 PBMC isolation

Blood remaining after plasma collection was diluted with 0.85% saline (2:1 ratio), mixed and layered on ficoll Histopaque® (Sigma) slowly (3.5ml ficoll/10ml blood-saline solution) in a 15ml tube. Then the tubes were spun at 600g in an IEC centrifuge with brake off for 30mins at room temperature. After centrifugation, plasma was removed except for a small layer on top of the buffy coat. Buffy coat (PBMCs) were suctioned by a 1ml plastic pipette and transferred to 15ml tubes with saline, then washed twice with saline at 300g with high break for 10mins at room temperature. Cells were re-suspended in culture medium (CM) and counted using a hemocytometer. PBMC cell count and viability were detected by staining with trypan blue (Sigma) with a 1 in 10 dilution in culture medium.

CM for all subjects was prepared at the same time aliquoted and stored in -80^{o}C freezer. Isolated, fresh PBMC were used fresh for the vitro cultures, and remaining cells were frozen in 1ml cryotubes and stored in liquid nitrogen tanks for future studies.

2.8 Cell culture for antigen-induced cytokine detection assays

Freshly isolated PBMC were transferred into 96 well round bottom sterile culture plate (Corning Incorporated, Corning, NY) with a final count of 0.25 million per well. LPS

(Sigma), Pam3csk4 (Invivogen) and BSA conjugated palmitate were added into the wells respectively with a final concentration of LPS (20ng/ml, 2ng/ml, 0.2ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) mixed. Conjugated palmitate to BSA (6:1) was used as it had better solubility in aqueous solutions, following the protocol from Seahorse bioscience. Cells in CM alone acted as negative controls. The final volume of each well was 200ul. Each condition was replicated for 5 wells. PBMC were then cultured in a 5% CO_2 humidified incubator at 37ºC for 24hr. The next day, culture supernatant were transferred into empty plates and stored in -80ºC freezer until analyzed for cytokine levels.

2.9 Cell culture for 20-HETE induced cytokine detection assays

Fresh isolated PBMC were transferred into 96 well round bottom sterile culture plate with a final count of 0.25 million per well. 20-HETE (Cayman chemical Ann Arbor, Michigan) at different concentration (0.01nM, 0.1nM, 1nM, 10nM, 100nM) were add into the wells with or without LPS (2ng/ml, 0.2ng/ml). The final volume of each well was 200ul. Each condition was replicated for 5 wells. PBMC were then cultured in a 5% CO_2 humidified incubator at 37ºC for 24hr. The next day, culture supernatant were transferred into empty plates and stored in -80ºC freezer until analyzed for cytokine levels.

2.10 Cell culture for antigen-and 20-HETE-induced monocyte surface marker and intracellular cytokine assay by flow cytometry

Fresh isolated PBMC were transferred into 5ml sterile polystyrene round-bottomed tubes (Falcon). Each tube contained 0.5million PBMC. For the experiment exploring monocyte

surface marker expression with and without stimulation, LPS, Pam3CSK4, BSA conjugated palmitate were added into different tubes (Table 2. Tube 1-4) respectively at a final concentration of (20ng/ml, 200ng/ml and 200mM). For the experiments exploring the influence of 20-HETE on monocyte surface markers, 0.5 million cells in each tube (Table 2. Tube a-d) were cultured with 20-HETE (0.01nM, 0.1nM, 1nM, 10nM, 100nM) respectively with and without LPS (2ng/ml). For the experiment exploring monocyte intracellular cytokine levels with and without stimulation, LPS, Pam3CSK4, palmitate was added into different tubes (Table 2. Tube 5-8) respectively at a final concentration of (20ng/ml, 200ng/ml and 200mM). At the same time, Golgiplug (BD) 1ul/million cells was added to these tubes. All tubes were incubated in a 5% CO_2 humidified incubator at 37ºC. After 12hr, all tubes were washed with 3ml FACS buffer, at 500g for 5 mins at 4ºC. After washing, PBMC were reconstituted in 100ul FACS buffer and stained with Non-monocyte cell markers and Monocyte lineage markers. For the experiments exploring the influence of 20-HETE, LPS, Pam3CSK4 and palmitate on monocyte surface marker expressions, antibodies for toll-like receptor markers and monocyte chemotaxis markers were added to each tube. After staining, these tubes were washed with 3ml FACS buffer. Then 100ul 4% PFA (from a 16% stock, Canemco Inc., Lakefield, Quebec) was added to all tubes and fixed at 4ºC for 30mins. The tubes were finally washed with FACS buffer and reconstituted in 300ul FACS buffer. For the experiment that explored the influence of LPS, Pam3CSK4 and palmitate on monocyte intracellular cytokine levels, after staining with the non-monocyte cell markers and monocyte lineage markers, cells were washed with Perm/Wash™ Buffer (BD) and then fixed and permeabilized with 200ul/tube Fixation/Permeabilization solution (BD) for 20mins at 4ºC. Then the tubes were washed

twice with Perm/Wash™ Buffer and added with Monocyte intracellular cytokine antibodies for 30mins at 4ºC. In the end, tubes were washed twice with Perm/Wash™ Buffer at first then FACS buffer and reconstituted in 300ul FACS buffer. All tubes were place in the dark box at 4 ºC, and analysis was performed on flow cytometer within 3hr.

Table 2. Flow cytometry panel

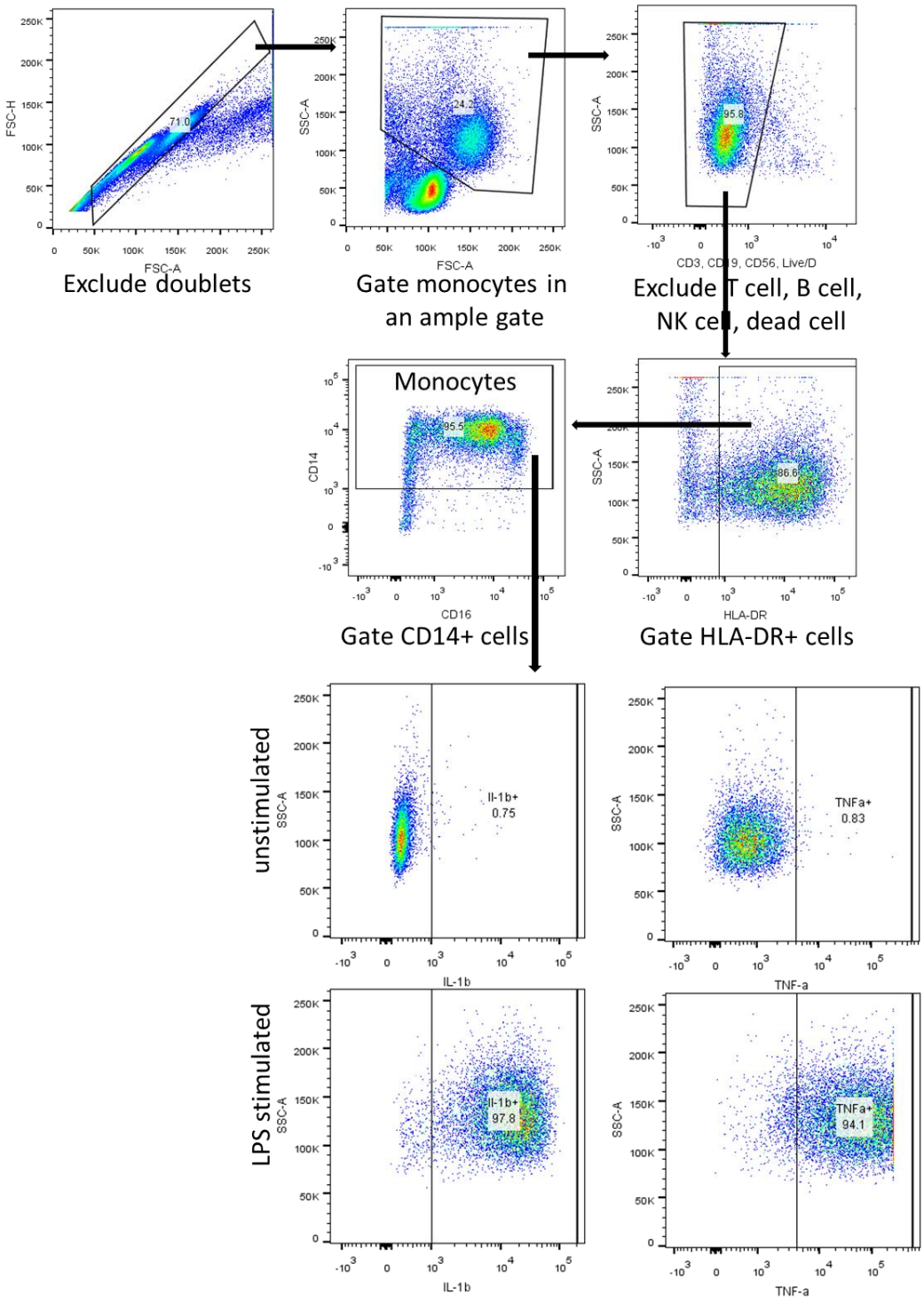
Tube label	Stimulation	Surface staining	Intracellular staining
1	CM	TLR2, TLR4, CCR1, CCR2	
2	LPS	TLR2, TLR4, CCR1, CCR2	
3	Pam3CSK4	TLR2, TLR4, CCR1, CCR2	
4	Palmitate	TLR2, TLR4, CCR1, CCR2	
a	20-HETE (0.01nM) +CM/LPS	TLR2, TLR4, CCR1, CCR2	
b	20-HETE (0.1nM) +CM/LPS	TLR2, TLR4, CCR1, CCR2	
c	20-HETE (1nM) +CM/LPS	TLR2, TLR4, CCR1, CCR2	
d	20-HETE (10nM) +CM/LPS	TLR2, TLR4, CCR1, CCR2	
e	20-HETE (100nM) +CM/LPS	TLR2, TLR4, CCR1, CCR2	
5	CM		IL-1β, TNF-α
6	LPS		IL-1β, TNF-α
7	Pam3CSK4		IL-1β, TNF-α
8	Palmitate		IL-1β, TNF-α

Table 3. Information of flow cytometry antibodies

Antibody	**Clone**	**Conjugated fluorochrome**	**Company**	**Volume used/tube**
Viability Dye	-	FITC	Life technologies	1/19 ul
Non-monocyte cell markers				
CD3	UCHT1	FITC	BD Biosciences	5ul
CD19	HIB19	FITC	BD Biosciences	10ul
CD56	HCD56	FITC	Biolegend	1ul
Monocyte lineage markers				
HLA-DR	L243	PerCP	Biolegend	2ul
CD14	HCD14	APC-Cy7	Biolegend	1ul
CD16	3G8	Brilliant Violet 510	Biolegend	5ul
Monocyte chemotaxis markers				
CCR1	5F10B29	APC	Biolegend	5ul
CCR2	K036C2	Brilliant Violet 421	Biolegend	3ul
Monocyte Toll-like markers				
TLR2	T2.5	PE-Cy7	Biolegend	1.5ul
TLR4	HTA125	PE	Biolegend	5ul
Monocyte intracellular cytokine				
IL-1β	HCD14	APC-Cy7	BD bioscience	3ul
TNF-α	MAb11	APC	BD bioscience	0.5ul

Flow cytometric analysis was performed on an LSRII flow cytometer (BD). The voltage was set at 3-fold of background and saved as the appropriate application setting. Compensation was performed using single color antibody and Facs Compbeads (Invitrogen) for each experiment. One hundred thousand monocyte events were acquired for each tube. Flow cytometry data analysis was performed by Flowjo software (Tree Star Inc., Ashland, OR). Gates were adjusted following fluorochrome minus one conditions (FMO).

Cells were first gated for singlets in a FSC-A and FSC-H. Then gated on monocytes following a negative gate to exclude T-cell, B cell, NK cells by staining their exclusive surface marker CD4, CD19, CD56 and live/dead dye in color FITC. Daughter cells were gated on HLA-DR positive cells and monocytes were gated as the CD14 positive population. CD14 is considered to be lineage marker of monocytes while positive HLA-DR includes immune active monocytes. All analysis of TLR2, TLR4, CCR1 and CCR2 were gated on HLA-DR+, CD14+ monocytes. Median fluorescence intensity (MFI) was also calculated for each marker.


FSC-H
FSC-A
71.0
Exclude doublets
SSC-A
24.2
Gate monocytes in an ample gate
95.8
CD3, CD19, CD56, Live/D
Exclude T cell, B cell, NK cell, dead cell
Monocytes
95.5
CD14
CD16
Gate CD14+ cells
86.6
HLA-DR
Gate HLA-DR+ cells
unstimulated
LPS stimulated
Il-1b+
0.75
IL-1b
TNFa+
0.83
TNF-a
Il-1b+
97.8
TNFa+
94.1

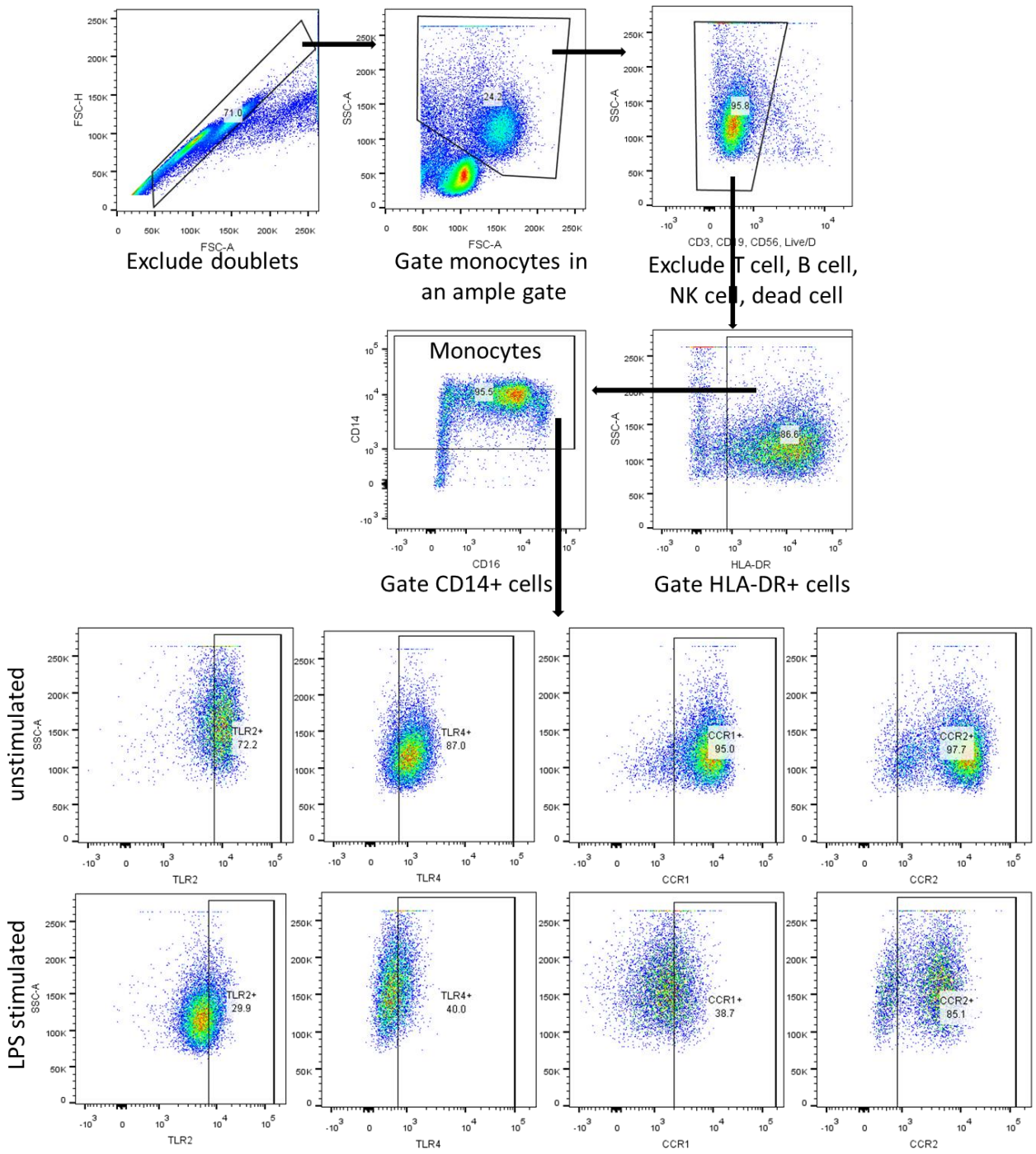


Figure 3. Monocyte TLR2, TLR4, CCR1 and CCR2 gating strategy

Flow cytometric analysis was performed on an LSRII flow cytometer. The voltage was set up as 3-fold of background and saved as the appropriate application setting. Compensation was performed using single color antibody and Facs Compbeads for each experiment. One hundred thousand monocyte events were acquired

for each tube. Flow cytometry data analysis was performed by Flowjo software. Gates were adjusted following fluorochrome minus one conditions (FMO). Cells were first gated for singlets in a FSC-A and FSC-H. Then gated on monocytes following a negative gate to exclude T-cell, B cell, NK cells by staining their exclusive surface marker CD4, CD19, CD56 and live/dead dye in color FITC. Daughter cells were then gated on HLA-DR positive cells. Monocytes were gated as the CD14 positive population. CD14 is considered to be the lineage marker of monocytes while postive HLA-DR includes immune active monocytes. All analysis of TLR2, TLR4, CCR1 and CCR2 were gated on HLA-DR+, CD14+ monocytes. MFI was also calculated for each marker.

2.11 ELISA cytokine detection

Culture supernatants were analysed forIL-1β, IL-6, TNF-α and IL-10 cytokines using enzyme-linked immuno absorbent assays (ELISAs). Paired antibodies and standards were obtained from the companies listed below in Table 4.

Table 4. Information of ELISA antibodies

Antibody (Ab)	Clone	Company	Working dilution
IL-1β capture Ab	JK1B-1	Biolegend	1/1000
IL-1β detection Ab	JK1B-2	Biolegend	1/1000
IL-6 capture Ab	MQ2-13A5	Biolegend	1/250
IL-6 detection Ab	MQ2-39C3	Biolegend	1/500
TNF-α capture Ab	MA b1	BD bioscience	1/250
TNF-α detection Ab	MA b11	BD bioscience	1/1000
IL-10 capture Ab	JES3-9D7	Biolegend	1/200
IL-10 detection Ab	JES3-12G8	Biolegend	1/500
Standard		Company	Working concentration
IL-1β standard		Peprotech	1000 pg/ml
IL-6 standard		Peprotech	8000 pg/ml
IL-10 standard		Peprotech	1000 pg/ml
TNF-α standard		Peprotech	4000pg/ml

Capture Abs were diluted in Coating buffer with the dilution factor as shown in Table 4. The diluted capture antibodies were added into 96 well ELISA plates (Corning) at a final volume of 50ul/well. The plates were then wrapped with plastic film and placed in a moisture box overnight at 4 °C. Next day, the plates were vigorously tapped dry and then blocked with 100µl of Blocking buffer for 1hr at 37 °C. The plates were then washed with Washing buffer using a BioTek Micro Plate washer (BioTek, Winooski, VT), then samples and standard were added to each well and placed in a moisture box overnight at 4 °C. The following day, after washing again with Washing buffer, detection antibodies were diluted in Dilution buffer and added to the plates in a final volume of 50ul/well. Plates were placed in a moisture box overnight at 4°C.The next day, the plates were washed with Washing buffer and coated with Strepavidin-alkaline phosphatase (Jackson Immuno Research laboratories, Pennsylvania, USA) diluted in Dilution buffer. Plates were then incubated at 37°C for 45min. After washing the plates, para-Nitrophenyl phosphate tablets (PNPP, Thermo Fisher scientific) were dissolved in Substrate buffer (5ml/tablet) and added into the plates in a final volume of 50ul/well. Plates were read at wavelength 405-690nm on a BioTek Microplate Reader.

The CK18 M30 test was performed using M30 Apoptosense ELISA kit (Vivalavida AB, Nacka, Sweden) following the manufacturers instructions. All reagents were from the kit. Briefly, solutions were diluted according to the instructions and the plate was coated with mouse monoclonal K18 antibody M5 in advance. Then, 25ul of plasma samples from each subject were added to the plate in duplicate. The specified concentrations of Standard and Assay controls were added into the plate and 75ul of M30 conjugate solution was followed to add into each well. Then the plate was covered with sealing tape and shaken at 600 rpm

for 4hr. After 4hr the plate was washed with Wash Tablet solution 5 times. Then 200ul of TMB substrate was added to each well and incubated in darkness at room temperature for 20min. Next, 50ul of Stop solution were added into each well and mixed well with TMB substrate. The plate was then read using a BioTek microplate reader at 450 wave-length within 30min.

2.12 Statistics

Chi-square (and Fisher's exact) tests were performed with categorical data. Correlations were performed by Pearson correlation test. Kruskal-Wallis test and Dunn's multiple comparisons test were used to determine the differences among and between 3 study groups. Data were shown as mean (±) SE. $P<0.05$ was considered to be statistical significant.

3. Results

3.1 Study demographics

The demographics, medical history and laboratory results of study subjects are shown in Table5. A total of 52 adults were consented for the study between 2014 and 2016. Of these, 43 met the inclusion criteria. Controls were 8 healthy subjects with normal ALT levels. Simple steatosis (those with normal ALT values) and NASH (those with elevated ALT values) patients were further classified according to their plasma ck18 M30 level. The three study groups were similar with respect to age, gender and ethnicity. BMI was higher in NASH subjects compared with HC (Dunn's multiple comparisons test, $p=0.015$). In terms of medical history, the presence of diabetes, hypertension, hypercholesterolemia as well as cardiovascular disease was similar among the three study groups based on their clinical records. All study groups were free of cardiovascular diseases. Blood glucose levels tended

to elevated in NAFLD patients in general including both SS and NASH (Kruskal-Wallis test, p=0.0584). Further comparisons of blood glucose levels between SS and HC, NASH and HC and between NASH and SS did not reach statistic significance (Dunn's multiple comparisons test, p=0.0968, p=0.0725, p>0.9999 respectively). Serum creatinine, a kidney disease marker, triglyceride, total cholesterol and their transportcarrier proteins was similar in all groups.

Blood ck18 M30, ALT and AST that reflect apoptotic and necrotic liver injury were significantly different between the three study groups (Kruskal-Wallis test, all p<0.0001). To further compare the differences between individual study groups, Dunn's multiple comparisons test was performed. As per the definations of SS, NASH and HC, ALT levels were significantly higher in SS and NASH subjects compared with HC subjects (p=0.0025, p<0.0001) and also significanlty higher in NASH subjects compared with SS subjects (p=0.018). Ck18 M30 was significantly higher in SS and NASH subjects compared with HC subjects (p=0.0104, p<0.0001) and also significanlty higher in NASH subjects compared with SS subjects (p=0.0011). AST was significantly higher in SS and NASH subjects compared with HC subjects (p=0.0247, p<0.0001) and also significanlty higher in NASH subjects compared with SS subjects (p=0.0026). Blood ferritin levels can reflect the release of ferritin from tissues, including the liver. In the study patients, ferritin levels were elevated (Kruskal-Wallis test, p=0.0053). A further analysis found ferritin levels significantly higher in NASH subjects compared with HC and SS subjects (p=0.0089, p=0.0270) but similar in SS subjects compared with HC subjects (p=0.6054).

Overall, as expected, the ALT, AST and hepatic apoptosis marker CK18, were elevated in NASH more so than SS and HC subjects. The liver disease-associated marker ferritin, was

also elevated in NAFLD patients, while other clinical features and biochemical markers (other than BMI) were not different between 3 groups.

Table 5. Demographics, medical history and laboratory data of study groups

Parameters	Healthy Control (n=8)	Simple steatosis (n=25)	NASH (n=10)	P value
Demographics				
Age (years)	48 (33-59)	51 (23-55)	56 (43-69)	0.2730
Female (%)	75	44	40	0.2543
Caucasian (%)	86	75	90	0.5581
BMI	26 (20-30)	32 (26-47)	36 (28-51)	**0.0188**
Medical History				
Diabetes (%)	0	8	20	0.3284
Hypertension (%)	13	28	50	0.2118
Hypercholesterolemia (%)	25	28	40	0.7352
Cardiovascular disease (%)	0	0	0	
Laboratory				
Glucose (mmol/L)	4.6 (3.9-6.1)	5.7 (3.6-11.4)	6.7 (4.2-11.6)	0.0584
Creatinine (umol/L)	65 (50-93)	75 (50-110)	75 (43-107)	0.3043
Triglyceride (mmol/L)	1.5 (0.6-2.2)	2.4 (1-5.7)	2.4 (1.2-4.5)	0.3664
Total cholesterol (mmol/L)	4.7 (3.7-5.9)	4.6 (3-6.2)	5.7 (3.8-8.2)	0.1433
LDL (mmol/L)	2.5 (1.7-3.5)	2.4 (0.8-4.2)	3.4 (2-5)	0.2606
HDL (mmol/L)	1.5 (1.1-2.8)	1.3 (0.7-2.2)	1.2 (0.9-1.9)	0.3551
Ck18 M30 (U/L)	38 (10-111)	137 (10-238)	561 (249-894)	**<0.0001**
ALT (IU/L)	18 (13-21)	48 (12-91)	94 (49-209)	**<0.0001**
AST (IU/L)	17 (9-22)	30 (14-65)	57 (22-135)	**<0.0001**
Ferritin (ug/L)	68 (14-118)	200 (12.3-960)	429 (98.6-1117)	**0.0053**

Kruskal-Wallis test, mean and range are shown. Chi-square (and Fisher's exact) test were performed with proportion data.

3.2 Plasma oxylipins were increased in NAFLD patients

3.2.1 Plasma 20-HETE and 8-HETrE were elevated in NAFLD patients

The role of oxylipins in the development of NAFLD is unkown. To explore the role of oxylipins in NAFLD progression, participant plasma oxylipin levels were detected by HPLC-MS/MS operating by Tanja Winter from Dr. Aukema's lab. Fifty one oxylipin metabolites (listed in table 6.) from omega-6 poly unsaturated fatty acid Arachidonic acid (AA), Linoleic acid (LA), γ-LA, α-LA and from omega-3 fatty acid Eicosapentaenoicacid (EPA) and (Docosahexaenoic acid) DHA were compared among HC, SS and NASH groups. As shown in table 6 and Figure 4, the mean levels of AA Cyp metabolite 20-HETE and the γ-LA LOX metabolite 8-HETrE significant increased in NAFLD patients compared with HCs (Kruskal-Wallis test, $p<0.05$). The 20-HETE level was 0.1 ng/ml in HCs, and significantly elevated in NASH and SS subjects, 0.156 ng/ml and 0.178 ng/ml respectively (Dunn's multiple comparisons test, $p=0.006$, $p=0.024$). Although the levels of 20-HETE were higher in both SS and NASH subjects compared with HCs, there were no significant differences between SS and NASH (Dunn's multiple comparisons test, $p>0.999$). 8-HETrE levels were elevated in NASH, 0.023 ng/ml, but not SS subjects compared with HCs, 0.013 ng/ml (Dunn's multiple comparisons test $p=0.037$, $p=0.170$). The level of the AA LOX metabolite Tetranor 12-HETE trended towards being higher in SS and NASH groups compared to controls but did not achieve statistical significance (Dunn's multiple comparisons test, $p=0.106$, $p=0.080$).

Table 6. Oxylipin levels within the 3 study groups, Healthy controls (HCs), Simple steatosis (SS) and Non-alcoholic steatohepatitis (NASH)

Fatty acid	Enzyme pathway	Abbreviation	HC (n=8) ng/ml	SS (n=25) ng/ml	NASH (n=10) ng/ml	p value (Kruskal-Wallis test)	r value (pearson correlation)	p value (pearson correlation)
Arachidonic acid	COX	PGE2	0.089	0.016	0.011			
		TXB2	0.154	0.087	0.060			
	LOX	5-HETE	0.088	0.109	0.132		0.35	**0.025 ***
		8-HETE	0.090	0.138	0.143		0.35	0.052
		9-HETE	0.046	0.058	0.085		0.51	**<0.001 ***
		11-HETE	0.145	0.193	0.207			
		12-HETE	0.118	0.138	0.428			
		15-HETE	0.132	0.144	0.178		0.31	**<0.05 ***
		5-oxoETE	0.043	0.037	0.028		-0.29	0.061
		15-oxoETE	0.009	0.012	0.009			
		Tetranor 12-HETE	0.015	0.020	0.023	0.058	0.27	0.083
	CYP	16-HETE	0.053	0.054	0.062			
		17-HETE	0.016	0.018	0.018			
		18-HETE	0.034	0.041	0.041			
		20-HETE	0.100	0.156	0.178	0.007 *	0.4	**0.012 ***
		5,6-EpETrE	0.011	0.015	0.016			
		11,12-EpETrE	0.006	0.008	0.009		0.28	0.073
		14,15-EpETrE	0.020	0.023	0.021			
		5,6-DiHETrE	0.017	0.021	0.024			
		8,9-DiHETrE	0.018	0.019	0.020			
		11,12-DiHETrE	0.049	0.052	0.068		0.43	**0.005 ***
		14,15-DiHETrE	0.067	0.078	0.089		0.31	**0.046 ***
Linoleic acid	LOX	9-HODE	5.048	6.360	6.607			
		13-HODE	2.892	3.577	3.709			
		9,10,13-triHOME	4.103	3.390	2.609			
		9,12,13-triHOME	10.557	7.357	6.971			
		9-oxoODE	0.150	0.197	0.173			
		13-oxoODE	0.725	0.801	0.735			
	CYP	12,13-EpOME	0.092	0.104	0.096			
		9,10-diHOME	0.180	0.217	0.195			
		12,13-diHOME	0.181	0.212	0.213			
γ-Linolenic acid	LOX	8-HETrE	0.013	0.020	0.023	0.04 *		
		15-HETrE	0.047	0.052	0.060		0.3	0.055

α-Linolenic acid	LOX	9-HOTrE	0.327	0.591	0.442		
		13-HOTrE	0.254	0.426	0.341		
		9-oxoOTrE	0.032	0.045	0.030		
	CYP	12,13-EpODE	0.028	0.035	0.036		
Eicosapentaenoicacid	COX	18-HEPE	0.039	0.073	0.040		
	LOX	12-HEPE	0.025	0.049	0.104	0.27	0.091
		15-HEPE	0.012	0.018	0.016		
Docosahexaenoic acid	LOX	4-HDoHE	0.081	0.141	0.115		
		7-HDoHE	0.031	0.056	0.047		
		8-HDoHE	0.035	0.055	0.056		
		10-HDoHE	0.020	0.033	0.026		
		11-HDoHE	0.027	0.053	0.067		
		13-HDoHE	0.018	0.029	0.032		
		14-HDoHE	0.109	0.179	0.176		
		16-HDoHE	0.035	0.052	0.046		
		17-HDoHE	0.125	0.160	0.142		
	CYP	20-HDoHE	0.073	0.110	0.088		
		19,20-DiHDoPE	0.295	0.348	0.346		

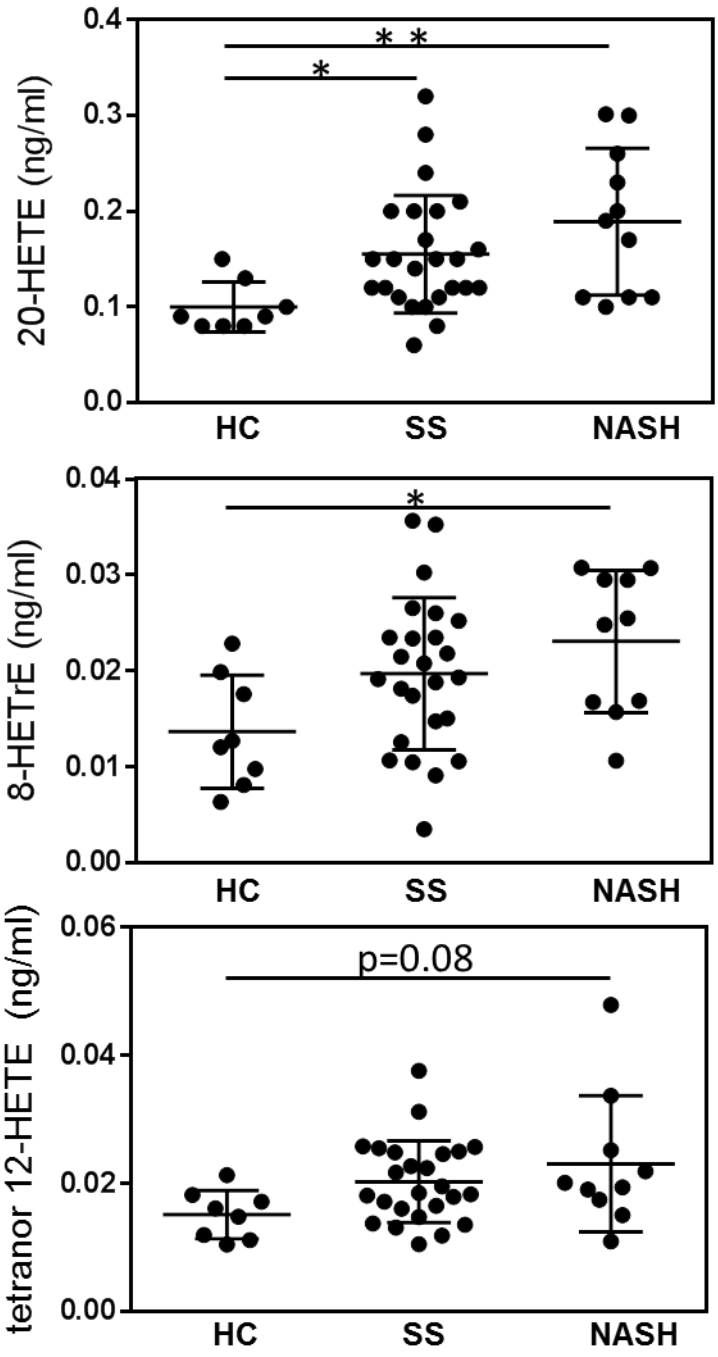


Figure 4. Plasma oxylipin 20-HETE and 8-HETrE levels were increased in NAFLD patients

Subject plasma oxylipin levels were determined by HPLC-MS/MS in study subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis). Each point represents the value from a single patient. Data are represented as mean ± SE, (HC n=8, SS n=25, NASH n=10). Kruskal-Wallis tests were performed, *$p<0.05$, **$p<0.01$.

3.2.2 Plasma oxylipin 5-HETE, 9-HETE, 15-HETE, 20-HETE, 11,12-DiHETrE and 14,15-DiHETrE positively correlated with ck18 level

In order to determine the correlation between various oxylipins and disease severity, Spearman's rank correlations were performed on 51 oxylipins and ck18 levels. The plasma apoptotic cell death biomarker ck18 fragment M30 level, which has reflects the degree of NAFLD associated liver damage was used as a marker of disease severity. Six out of 51 oxylipins positively correlated with plasma ck18 M30 (Figure 5), including 5-HETE, 8-HETE,15-HETE, 20-HETE, 11,12-DiHETrE and 14,15-DiHETrE ($p<0.05$ for all). Of note, these oxylipins are all metabolites of omega-6 polyunsaturated fatty acid.

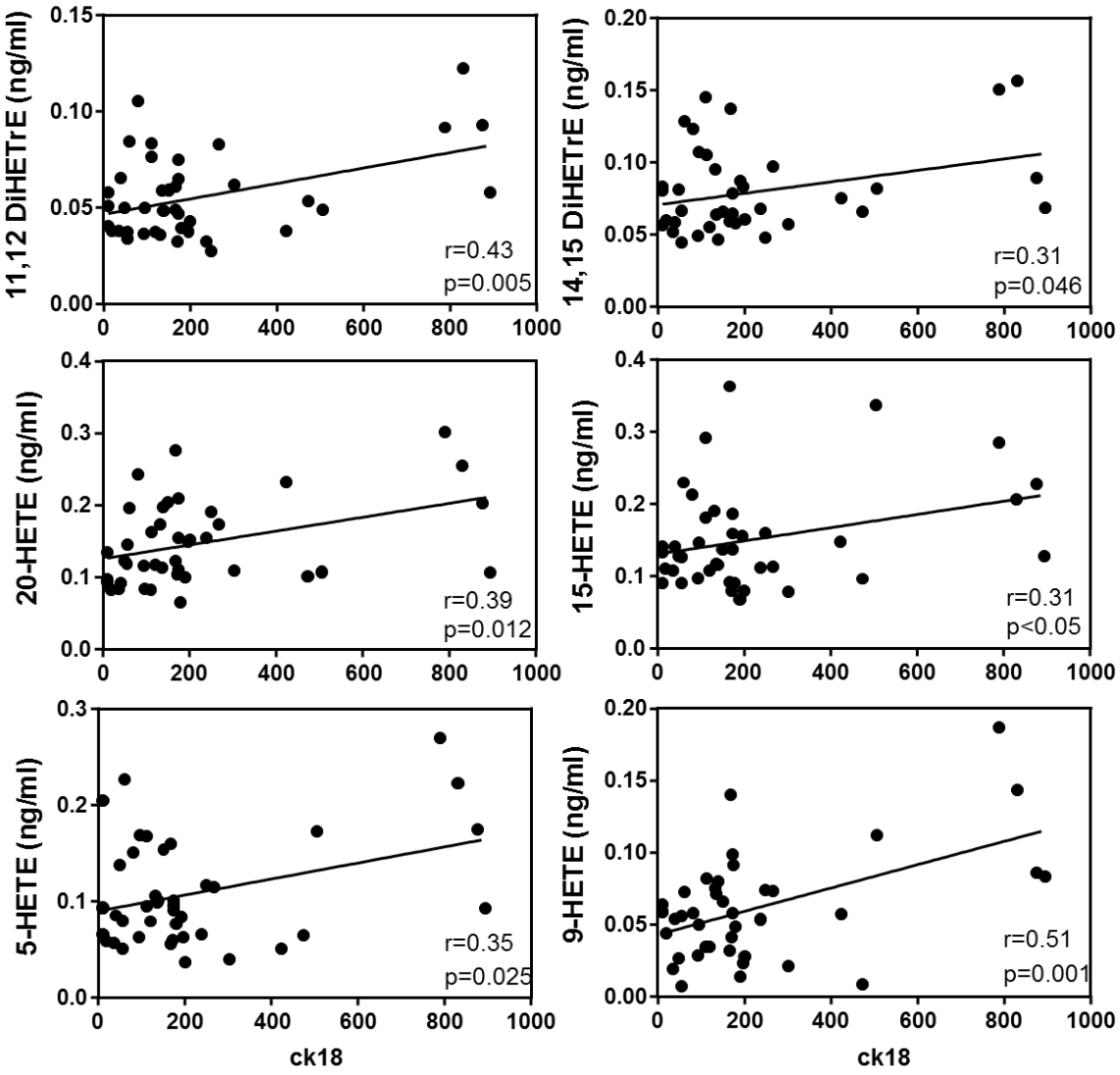


Figure 5. Plasma oxylipin 5-HETE, 9-HETE, 15-HETE, 20-HETE, 11,12-DiHETrE and 14,15-DiHETrE positively correlated with ck18 level

Oxylipin level in subject plasma was determined by HPLC-MS/MS. Pearson correlation between oxylipin levels and plasma ck18 levels in all subjects were performed for 51 oxylipins. Analysis with $p<0.05$ were shown (HC n=8, SS n=25, NASH n=10). Each dot indicates the ck18 level and the described oxylipin for one subject.

3.3 Bacteria PAMP-induced IL-1β and IL-6 levels were increased in NAFLD patients

3.3.1 Overview of cytokine levels of stimulated PBMC supernatant

Like NAFLD, T2D is a metabolic syndrome and resulting from insulin resistence. Previous work from our laboratory revealed that TLR4 induced TNF-α and IL-1β production by PBMC from T2D subjects were elevated compared with non-T2D youth which suggested increased immune activation in T2D [106]. To explore if immune activation is also elevated in NAFLD, we used the TLR2 agonist Pam3CSK4 and TLR4 agonist LPS as well as BSA congugated-palmitate (palmitate) which is an agonist of both TLR2 and TLR4 to activate PBMC. We congugated palmitate with BSA to render it more soluble. We then analysed the induction of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and the immune regulatory/anti-inflammatory cytokine IL-10 production in stimulated PBMC of subjects from the different study groups.

Both pro-inflammatory and anti-inflammatory cytokines were detected at very low levels when stimulated only with CM (negative control) for all subjects. However, cytokine levels significantly increased in a dose dependent manner when exposed to LPS, Pam3CSK4 or palmitate respectively (Figure 6). Compared to Pam3CSK4 and palmitate, LPS was a more potent PBMC stimuli in producing cytokines. To better distinguish cytokine responses in culture, we then analysed groupwise responses to each stimulii (including CM).

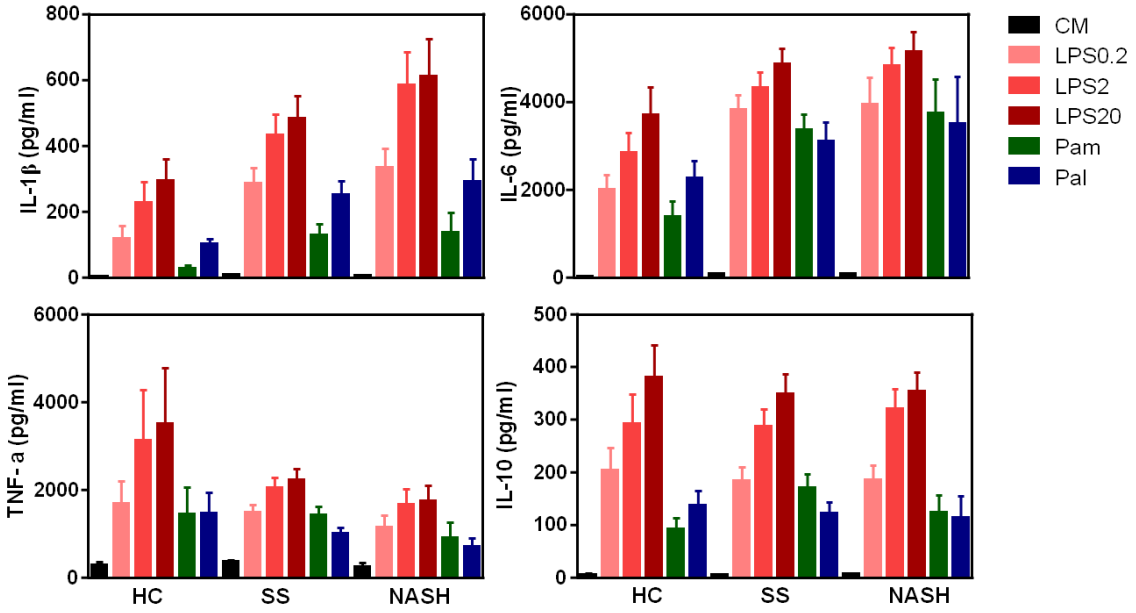


Figure 6. Cytokine levels in stimulated PBMC supernant

Freshly isolated PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with bacteria endotoxins LPS 20ng/ml (LPS20), 2ng/ml (LPS2), 0.2ng/ml (LPS0.2), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 24hr. Cell culture supernatant cytokine IL-1β, IL-6, TNF-α and IL-10 levels were determined by ELISA. Data are represented as mean ± SE, (HC n=8, SS n=25, NASH n=10). Statistic differences between study groups are shown in Figure 7-10.

3.3.2 LPS-stimulated PBMC IL-1β and IL-6 were increased in NAFLD patients

We next compared the induced cytokine levels between the 3 study groups. Baseline levels of IL-1β, IL-6, TNF-α and IL-10 were similar in SS, NASH and HC subjects (Figure 7) When LPS-stimulated cytokine responses were examined, NAFLD subjects (SS and NASH) trended towards higher IL-1β and IL-6 responses compared to HC (Figure 8). LPS2 and LPS0.2 stimulated IL-1β levels were approximately 3-fold in PBMCs from NASH compared to HC subjects ($p=0.0507$, $p=0.0661$). While IL-6 stimulated by LPS 2ng/ml and 0.2ng/ml was nearly 2-fold higher in PBMC from SS compared to HCs ($p=0.0487$ and $p=0.0153$, respectively) and also nearly 2-fold higher in PBMC from NASH than healthy controls ($p=0.0117$ and $p=0.0430$, respectively); however, there was no significant difference between SS and NASH in LPS20 and LPS0.2 stimulated IL-6 levels. LPS stimulated TNF-α and IL-10 levels were similar in the three study groups.

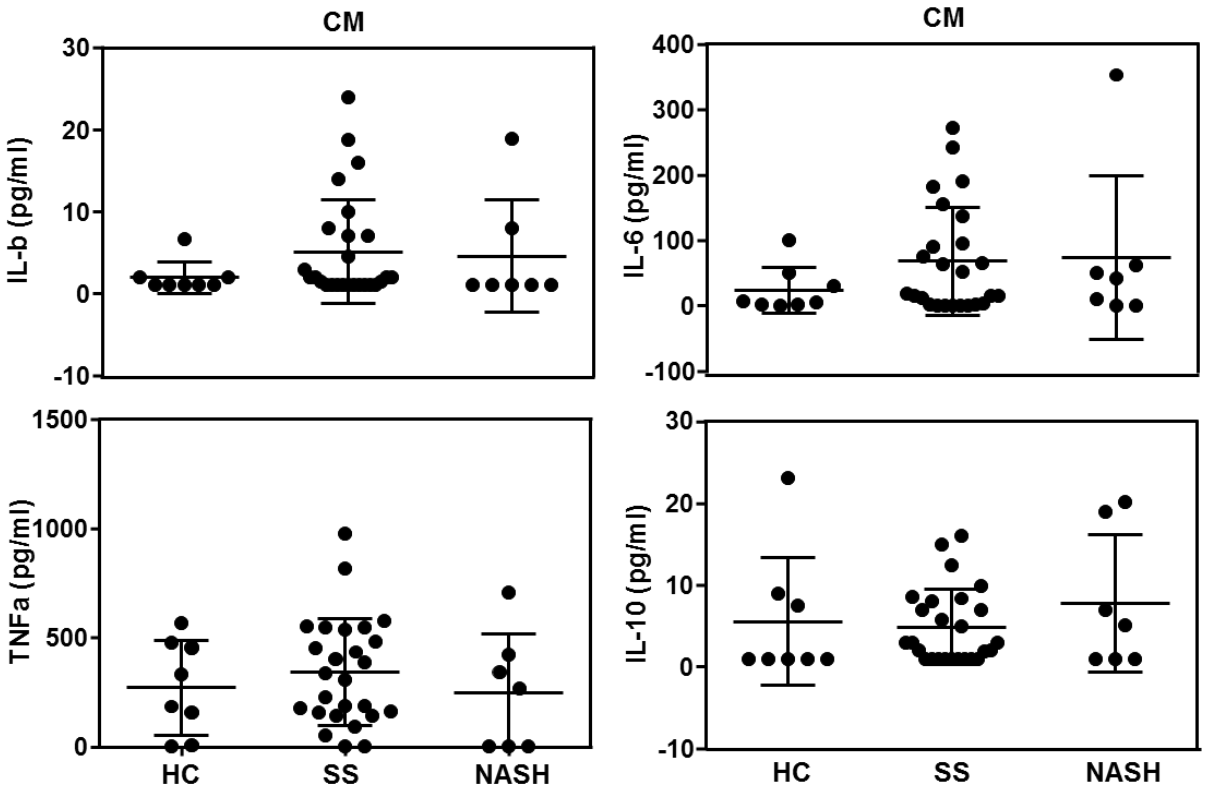


Figure 7. PBMC supernant cytokine levels at baseline were not different among 3 study groups

Freshly isolated PBMC from subjects (HC: Healthy controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured in complete culture medium. Cell culture supernatant cytokine IL-1β, IL-6, TNF-α and IL-10 levels were determined by ELISA. Each dot represents the indicated cytokine level of one subject. Kruskal-Wallis test were performed. Data are represented as mean ±SD, (HC n=8, SS n=25, NASH n=10). No significant differences were observed.

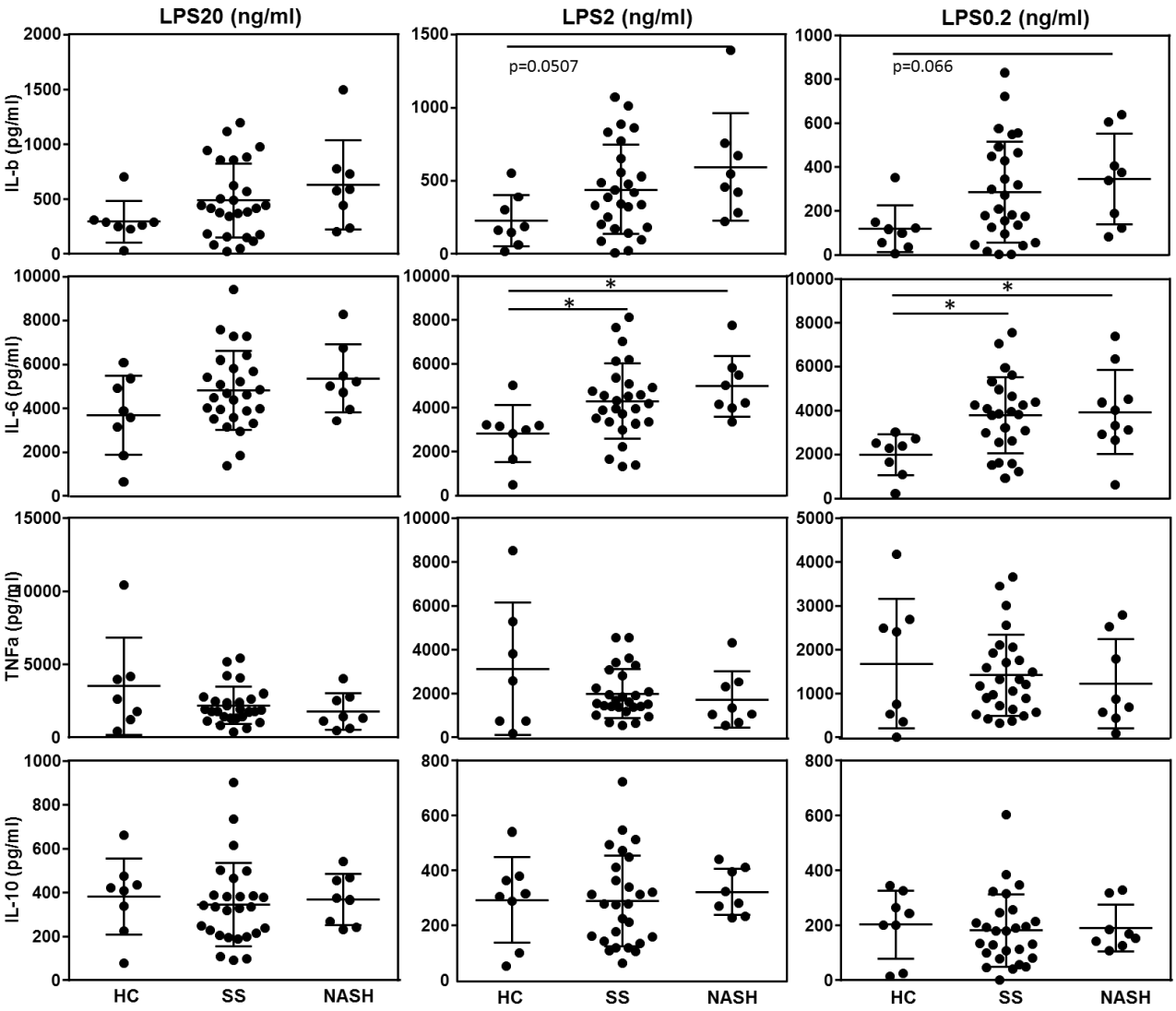


Figure 8. LPS stimulated PBMC IL-1β and IL-6 were increased in NAFLD patients

Freshly isolated PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with bacteria endotoxins LPS (20ng/ml, 2ng/ml, 0.2ng/ml) for 24hr. Cell culture supernatant cytokine IL-1β, IL-6, TNF-α and IL-10 levels were determined by ELISA. Each dot represents the level of the cytokine indicated for one subject. Kruskal-Wallis test were performed, *p<0.05. Data are represented as mean ±SD, (HC n=8, SS n=25, NASH n=10).

3.3.3 Pam3CSK4 stimulated PBMC IL-1β and IL-6 were increased in NAFLD patients

When Pam3CSK4 stimulated cytokine repsonses were examined among the 3 study groups, NAFLD subjects (SS and NASH) trend towards being higher IL-1β and IL-6 responses compared to HC (Figure 9). Pam3CSK4 stimulated IL-1β was 4.8-fold higher in SS compared to HCs ($p=0.0461$) while Pam3CSK4 induced IL-6 levels were 2.4-and 2.8-fold higher were in SS and NASH subjects compared to HCs ($p=0.0267$, $p=0.0627$).There were no significant differences between SS and NASH for both IL-1β and IL-6 levels. Pam3CSK4 induced TNF-α and IL-10 levels were similar among the three study groups.

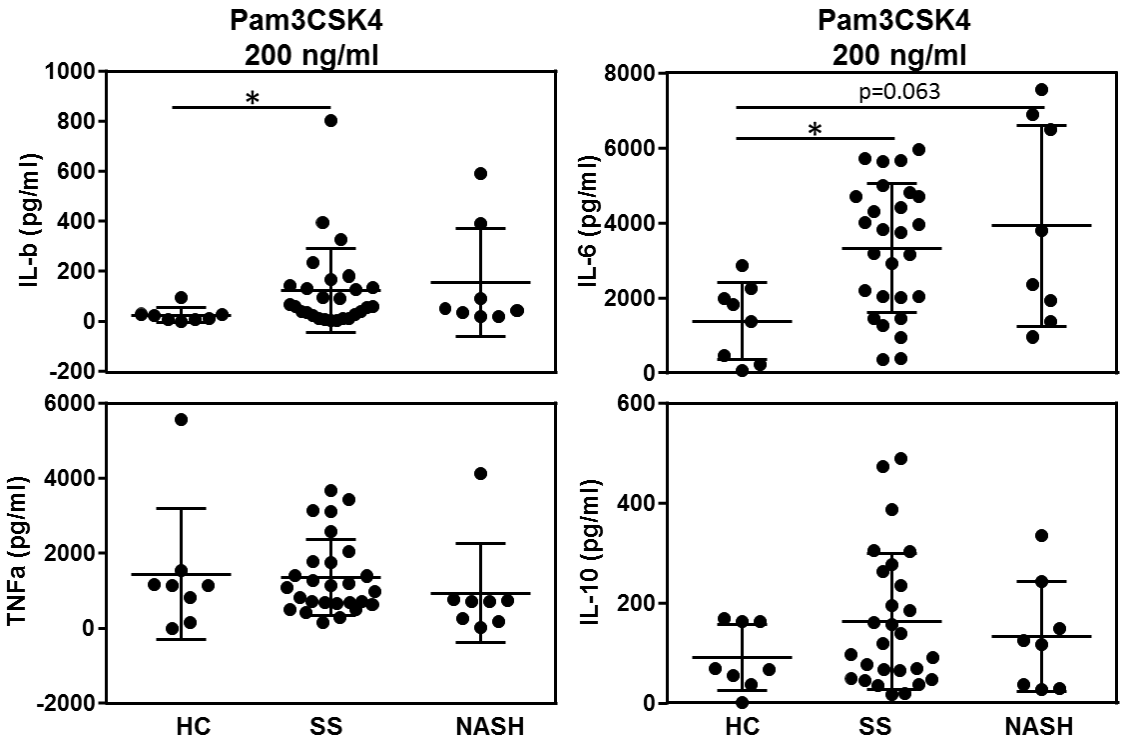


Figure 9. Pam3CSK4 stimulated PBMC IL-1β and IL-6 were increased in NAFLD patients

Freshly isolated PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with bacteria endotoxins Pam3CSK4 (200ng/ml) for 24hr. Cell culture supernatant cytokine IL-1β, IL-6, TNF-α and IL-10 levels were determined by ELISA. Each dot represents the indicated cytokine level of one subject. Kruskal-Wallis test were performed, *$p<0.05$. Data are represented as mean ±SD, (HC n=8, SS n=25, NASH n=10).

3.3.4 Palmitate stimulated cytokines were not different among the three study groups

When palmitate stimulated cytokine responses were examined, NAFLD subjects (SS and NASH) had similar cytokine responses compared to HC (Figure 10). IL-1β, IL-6, TNF-α and IL-10 levels were also similar between SS and NASH subjects.

Cytokine Data Summary

Overall, induction of the pro-inflammatory cytokines IL-1β and IL-6 tended to be greater in SS and NASH subjects compared to HC. However, induction of TNF-α, another strong inflammatory cytokine was similar in all groups as was the immunoregulatory cytokine IL-10.

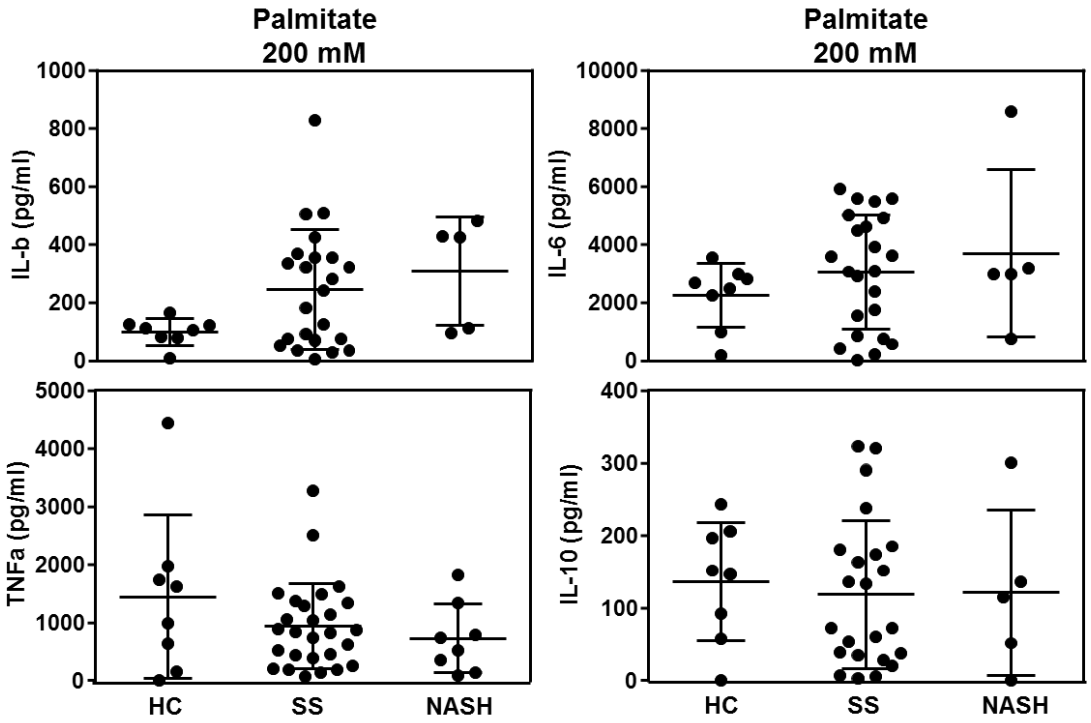


Figure 10. Palmitate stimulated cytokines were not different among 3 study groups

Freshly isolated PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with bacteria endotoxins palmitate (200mM) for 24hr. Cell culture supernatant cytokine IL-1β, IL-6, TNF-α and IL-10 levels were determined by ELISA. Each dot represents the indicated cytokine level of one subject. Kruskal-test were performed. Data are represented as mean ±SD, (HC n=8, SS n=25, NASH n=5). No significant difference was observed.

3.3.5 PAMP-induced pro-inflammatory cytokines IL-1β and IL-6 responses correlate with the disease severity marker, ck18

In order to determine if PAMP-or fatty acid-induced cytokines from PBMCs correlated with NAFLD disease severity, we performed Spearman's rank correlations on subject cytokine levels and ck18 as a marker of NAFLD apoptotic disease (Figure 11a). IL-1β stimulated by different concentrations of LPS positively correlated with plasma ck18 (LPS20 r=0.35, p=0.021; LPS2 r=0.39, p=0.01 ; LPS0.2 r=0.3, p=0.05). Pam3CSK4-induced IL-1β also correlated with ck18 (r=0.31, p=0.04) but not palmitate (r=0.29, p=0.089).

Positive correlations were also observed between PBMC IL-6 levels and ck18 (Figure 11b). Specifically, IL-6 induced by LPS positively correlated with plasma ck18 level (LPS20 r=0.26, p=0.086; LPS2 r=0.32, p=0.039 ; LPS0.2 r=0.32, p=0.037). There was also a positive correlation between Pam3CSK4 induced IL-6 levels and plasma ck18 (r=0.36, p=0.019). Once again, palmitate induced IL-6 showed a positive association with ck18, but did not reach statistical significance (r=0.22, p=0.2).

No significant correlations were observed between LPS, Pam3CSK4 or palmitate induced TNF-α levels and plasma ck18. Neither was any correlation seen between LPS, Pam3CSK4 or palmitate induced IL-10 level and plasma ck18 level (Data not shown).

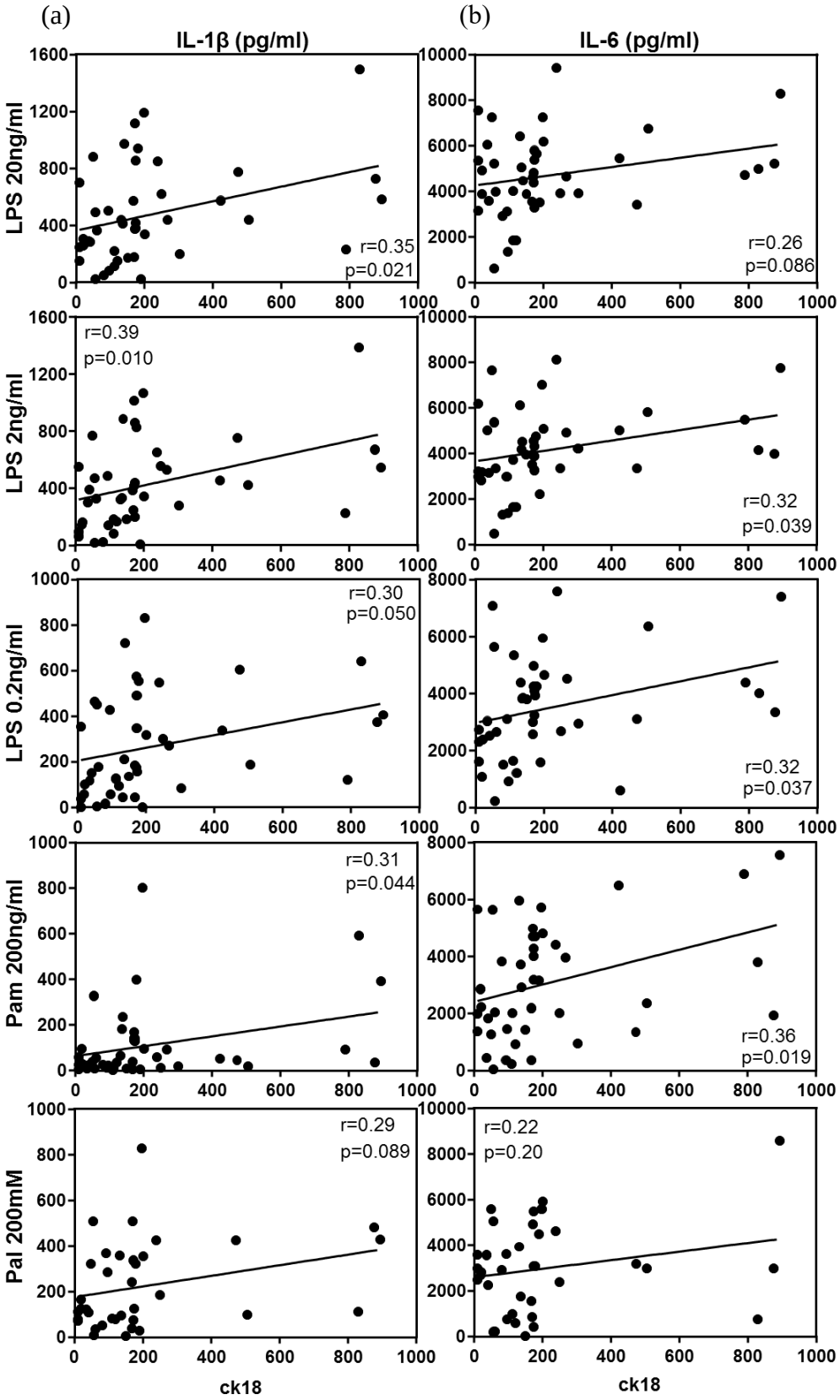


Figure 11. Correlation between timulated PBMC cytokine levels and ck18

Freshly isolated PBMC were cultured with bacteria endotoxins Pam3CSK4 (200ng/ml) for 24hr. Cell culture supernatant cytokine IL-1β, IL-6, TNF-α and IL-10 levels were determined by ELISA. Each dot represents the indicated cytokine level and the plasma ck18 level of one subject. Pearson correlation test were performed, *$p<0.05$. Data are represented as mean ±SD, (HC n=8, SS n=25, NASH n=10).

3.4 Monocyte IL-1β and TNF-α production did not differ between 3 study groups

3.4.1 The percentage of IL-1β+ monocytes or monocyte expressing intracellular IL-1β in response to PAMP signalling did not differ between 3 study groups

To determine if monocytes from NAFLD subjects displayed an elevated pro-inflammatory cytokine capacity, tested intracellular levels of monocyte IL-1β and TNF-α before and after 12hr stimulation with LPS, Pam3CSK4 and palmitate.

We compared the percentage of IL-1β by producing monocytes and the cell-level expression of IL-1β median fluorence intensity (MFI) among the study groups. Median fluorence intensity refers to the fluorescence intensity of all cells that contain intracellular IL-1β and represents the relative expression of IL-1β per cell. At baseline, the percentage of IL-1β+monocytes was similar among HC, SS and NASH subjects (Figure 12). Similarly, the relative per cell intracellular IL-1β levels showed no differences between study groups. Upon stimulation by LPS, Pam3CSK4 and palmitate, the percentage of IL-1β+ monocytes were similar among the 3 study groups and mean expression of IL-1β per cell was also not different among 3 study groups after stimulation.

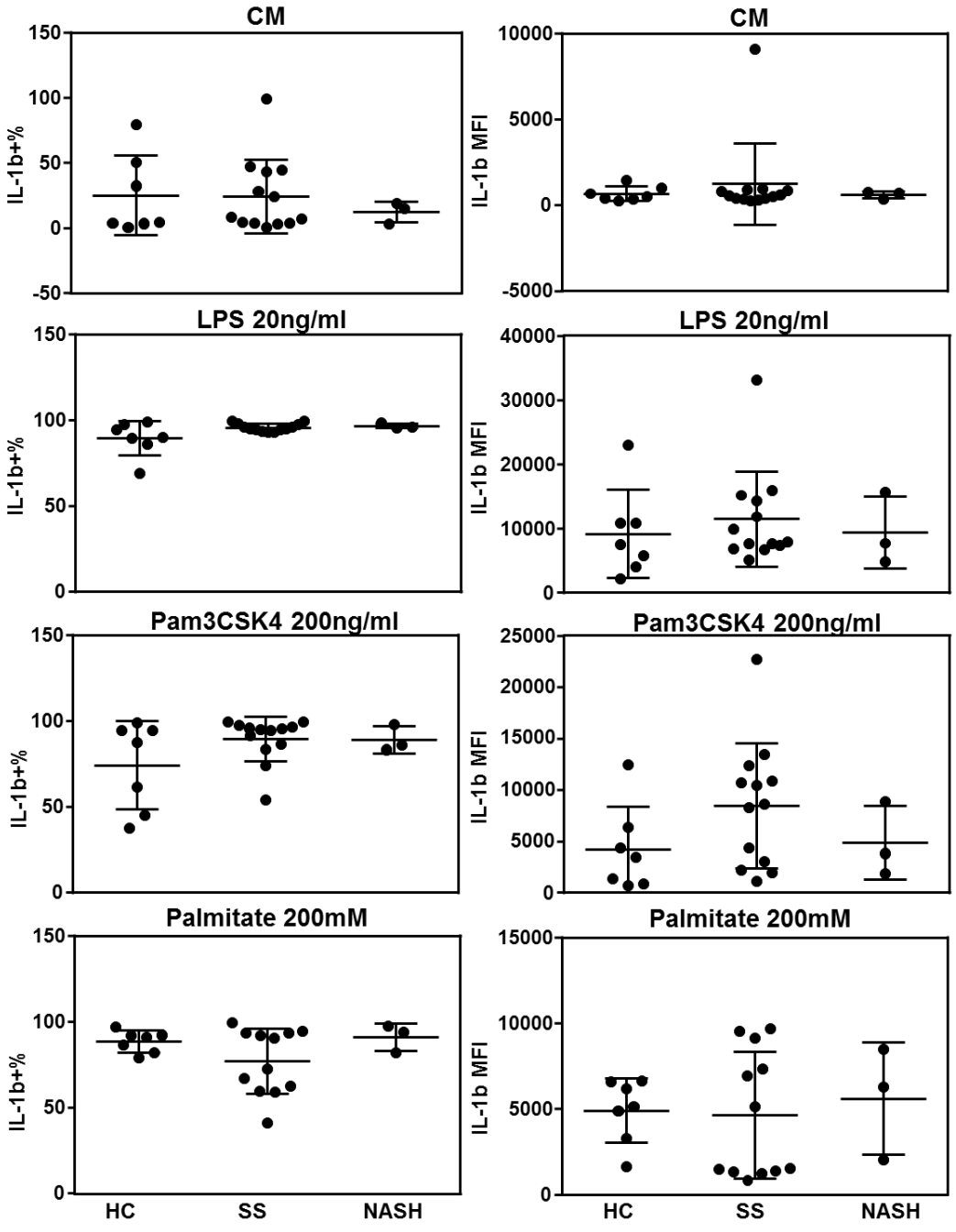


Figure 12. The percentage of IL-1β+ monocytes or monocyte expressing intracellular IL-1β in response to PAMP signalling did not differ among 3 study groups

PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained with antibodies that define monocytes and monocyte intracellular cytokine IL-1β as shown in Table 2. Each

dot represents the expressed percentage/MFI of IL-1β of one subject. Kruskal-Wallis test were performed to compare intracellular IL-1β level among 3 study groups. Data are represented as mean ±SD, no significance was observed (HC n=7, SS n=15, NASH n=3).

3.4.2 The percentage of TNF-α+ monocytes or monocyte expressing intracellular TNF-α in response to PAMP signalling did not differ among 3 study groups

We then compared the percentage of TNF-α+ monocytes and cell-level expression of TNF-α median fluorence intensity (MFI) among the 3 study groups to see if they are altered in SS and NASH patient at baseline and/or upon stimulation.

At basline, the percentage of TNF-α+ monocytes was similar among HC, SS and NASH subjects. In addition, the relative per cell intracellular TNF-α level showed no difference between study groups. Upon stimulation with LPS, Pam3CSK4 and palmitate, the percentage of TNF-α+ monocytes were similar among the 3 study groups as were median TNF-α intracellular levels (Figure 13).

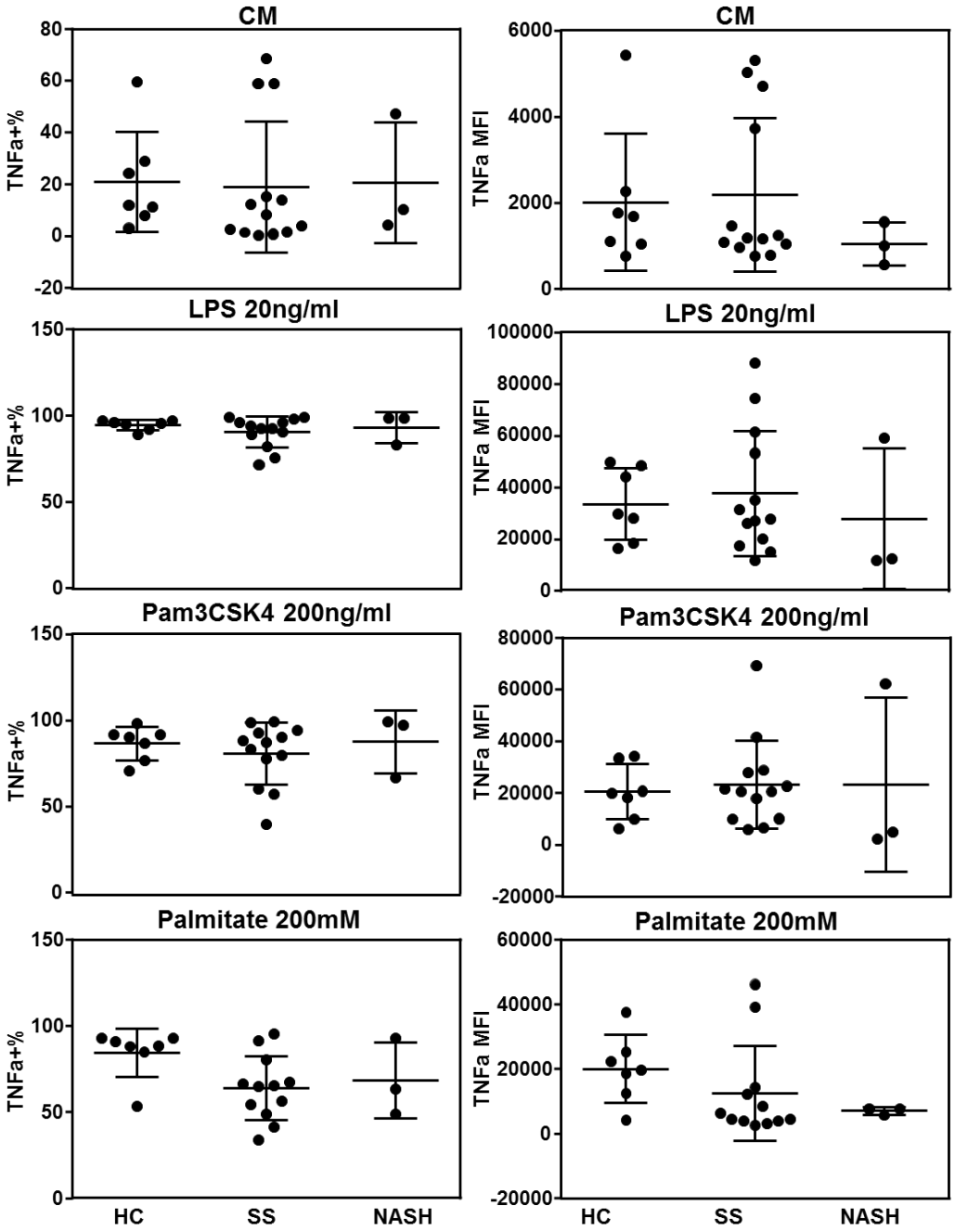


Figure 13. The percentage of TNF-α+ monocytes or monocyte expressing intracellular TNF-α in response to PAMP signalling did not differ among 3 study groups

PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained with antibodies that define monocytes and monocyte intracellular cytokine TNF-α as shown in Table 2. Each

dot represents the expressed percentage/MFI of TNF-α of one subject. Kruskal-Wallis test were performed to compare intracellular TNF-α level among 3 study groups. Data are represented as mean ±SD, no significance was observed (HC n=7, SS n=15, NASH n=3).

3.5 Monocyte TLR2 and TLR4 surface expression did not differ between 3 study groups at basal level or upon stimulation of PAMPs

3.5.1 The percentage of TLR2+ monocytes or TLR2 expression on monocytes did not differ among the 3 study groups at basal level or upon stimulation of PAMPs

In order to explore if the changes in TLR-stimulated cytokine secretion in simple steatosis and NASH subjects was due to the alterations of TLR expression, we determined TLR2 and TLR4 surface expression on monocytes from the 3 study groups before and after simulation with LPS, Pam and Pal. Freshly isolated PBMC were cultured with CM, LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12 hr, then TLR2 and TLR4 expression were determined by flow cytometry. Note that prior to the analysis of the TLR expressions, we analyzed the fraction of the monocyte population (monocyte count/10^5 PBMC) in 3 study groups to determine if monocyte numbers differed among the 3 study groups. We found that the monocyte fraction *ex vivo* was equivalent in HC, SS and NASH subjects (Figure 14).

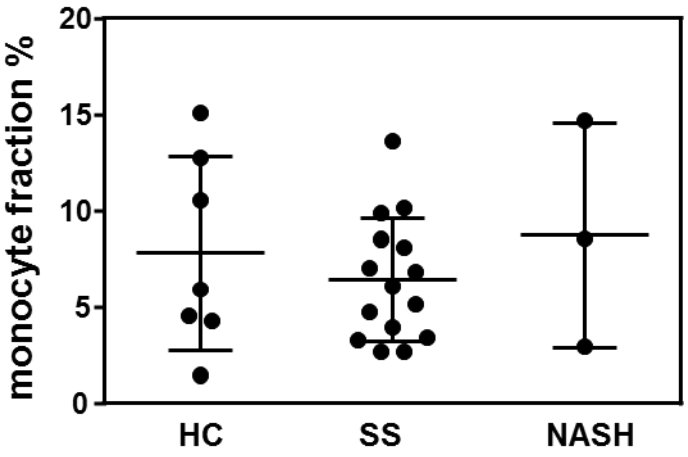


Figure 14. Description of monocyte fraction at baseline

Monocytes from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were gated as described in methods. Monocyte fraction was computed by dividing the monocyte count by 10^5 (acquired PBMC number for each tube). Each dot represents the monocyte fraction of one subject. Kruskal-Wallis testwere performed to detect difference among 3 study groups, data are represented as mean ±SD. No significant difference was observed. (HC n=7, SS n=15, NASH n=3).

Next, we evaluated the level of TLR2 expression-induced by stimulation with LPS, Pam3CSK4 and palmitate in all subjects regardless of study status to demonstrate that monocytes are capable of responidng to PAMPs and refect by the change of TLR2 expression. At baseline, the percentage of monocytes that express TLR2 was relatively low, but detectable (Figure 15). Upon stimulation with LPS, Pam3CSK4 and palmitate, TLR2 expression increased by nearly 2-fold ($p<0.0001$, $p<0.001$, $p<0.0001$ respectively) regardless whether the stimuli was a TLR2 or TLR4 agonist.

TLR2 MFI was low at baseline, and increased per cell upon stimulation of LPS, Pam3CSK4 and palmitate by 1.3-fold ($p<0.001$, $p<0.01$, $p<0.001$ respectively). All 3 stimuli LPS, Pam3CSK4 or palmitate induced the expression of TLR2 on monocyte.

To summarize, data above reveal that all monocytes are capable of responding to PAMP signalling and upregulate TLR2.

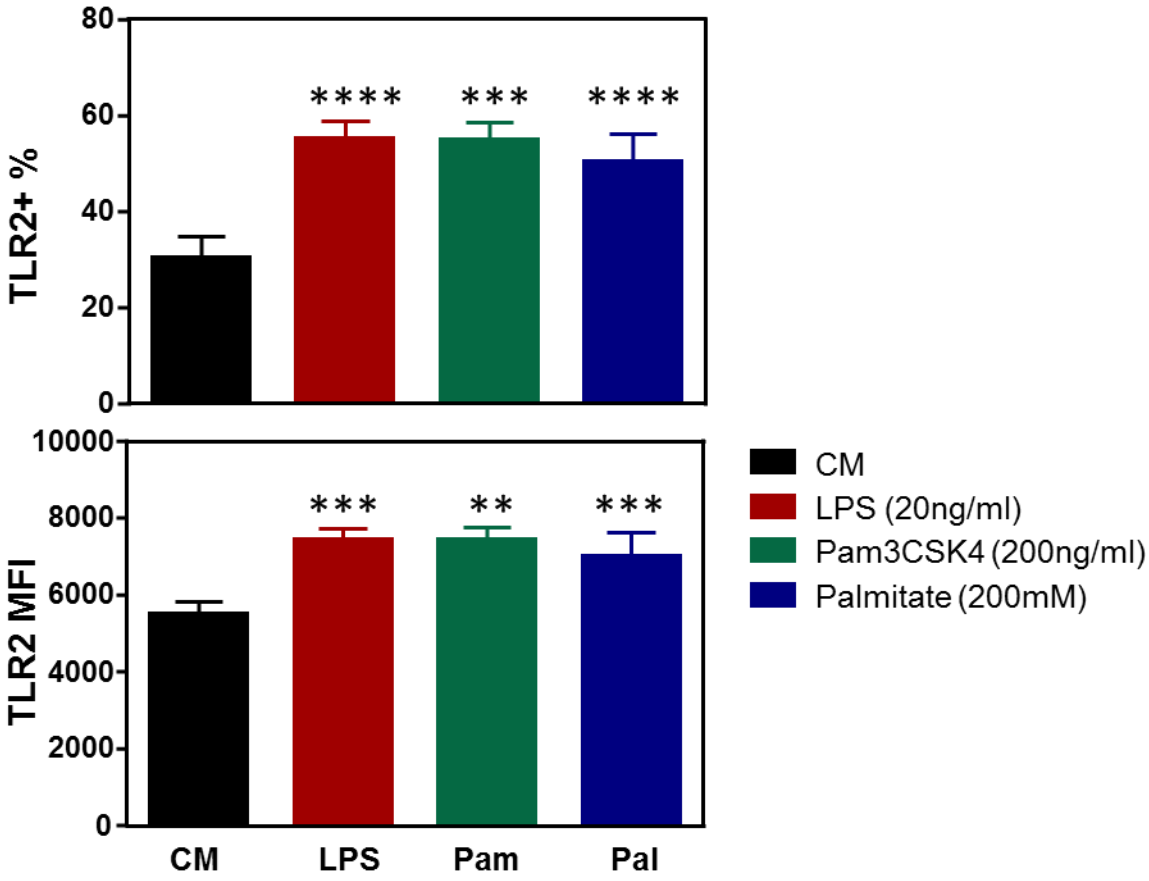


Figure 15. TLR2 expression on monocyte at baseline and upon PRR stimulation

PBMC from subjects were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte TLR2+ gates were defined as described. Dunn's multiple comparisons test were performed to compare TLR2 expression between CM and LPS, Pam3CSK4 and palmitate stimulated monocytes. Data are represented as mean ±SE, **p<0.01, ***p<0.001, ****p<0.0001 (n=25 including HC n=7, SS n=15, NASH n=3).

We compared the percentage of TLR2+ monocytes and median TLR2 expression per monocyte among the 3 study groups to see if monocyte responsiveness is altered in SS and in NASH patients relative to controls at baseline and/or upon stimulation (Figure 16). At baseline, the percentage of TLR2+ monocytes was similar among HC, SS and NASH subjects. In addition, the relative mean TLR2 expression per cell showed no difference between study groups. An analysis comparing the fold-changes of the percentage of TLR2+ monocytes upon stimulation (LPS, Pam3CSK4 and palmitatemitate values stimulated divided by non-stimulated CM value) among 3 study groups also showed no differences. Similar analyse of the MFI intensity were also conducted. No significant differences were observed (data not shown).

Overall, monocytes are capable of responding to PAMP signalling and upregulate TLR-2; however, the percentage of TLR2+ monocytes and TLR2 expression per monocyte after stimulation did not differ among the 3 study groups.

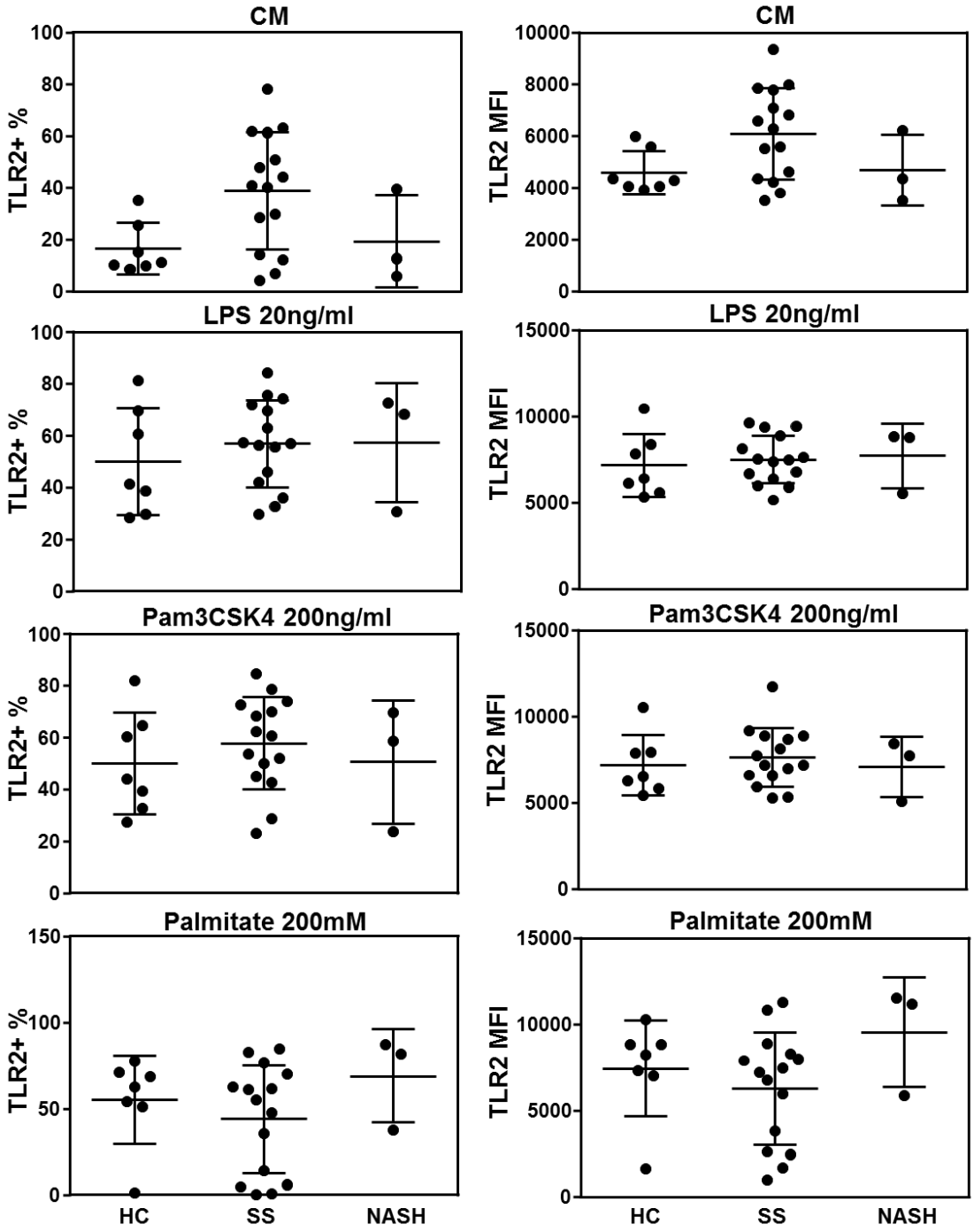


Figure 16. The percentage of TLR2+ monocytes or TLR2 expression on monocytes did not differ among 3 study groups

PBMC from from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were

stained with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte TLR2+ gate was defined as described. Each dot indicated the interested TLR2 level of one subject. Kruskal-Wallis test were performed to compare TLR2 expression among 3 study groups. Data are represented as mean ±SD, no significance was observed (HC n=7, SS n=15, NASH n=3).

3.5.2 The percentage of TLR4+ monocytes or TLR4 expression per monocytes did not differ among the 3 study groups at basal level or upon stimulation of PAMPs

Next, we evaluated the level of TLR4 expression induced by stimulation with LPS, Pam3CSK4 and palmitate in all subjects regardless of study status to demonstrate that monocyte are capable to respond to PAMPs and leading to change of TLR4 expression (Figure 18). At baseline, the percentage of monocytes that express TLR4 was relatively low, but detectable. Upon stimulation with LPS, Pam3CSK4 and palmitate monocyte, TLR4 expression appeared differently based upon the stimulation. At baseline, about 50% of monocytes expressed TLR4. Upon stimulation with LPS, monocytes that expressed TLR4 decreased approximately 20% ($p<0.05$). Interestingly, upon stimulation by Pam3CSK4 the pecentage of monocyte that expressed TLR4 increased slightly. Finally, upon stimulation by palmitate, the percentage of monocytes that expressed TLR4 did not change. When we evaluated MFI we found the relative expression of TLR4 per monocyte decreased upon stimulation with LPS compared to unstimulated. However, upon stimulation with Pam3CSK4 and palmitate the relative expression of TLR4 did not differ from unstimulated monocytes.

Overall, changes in monocyte TLR4 expression differ with different PAMP stimulation.

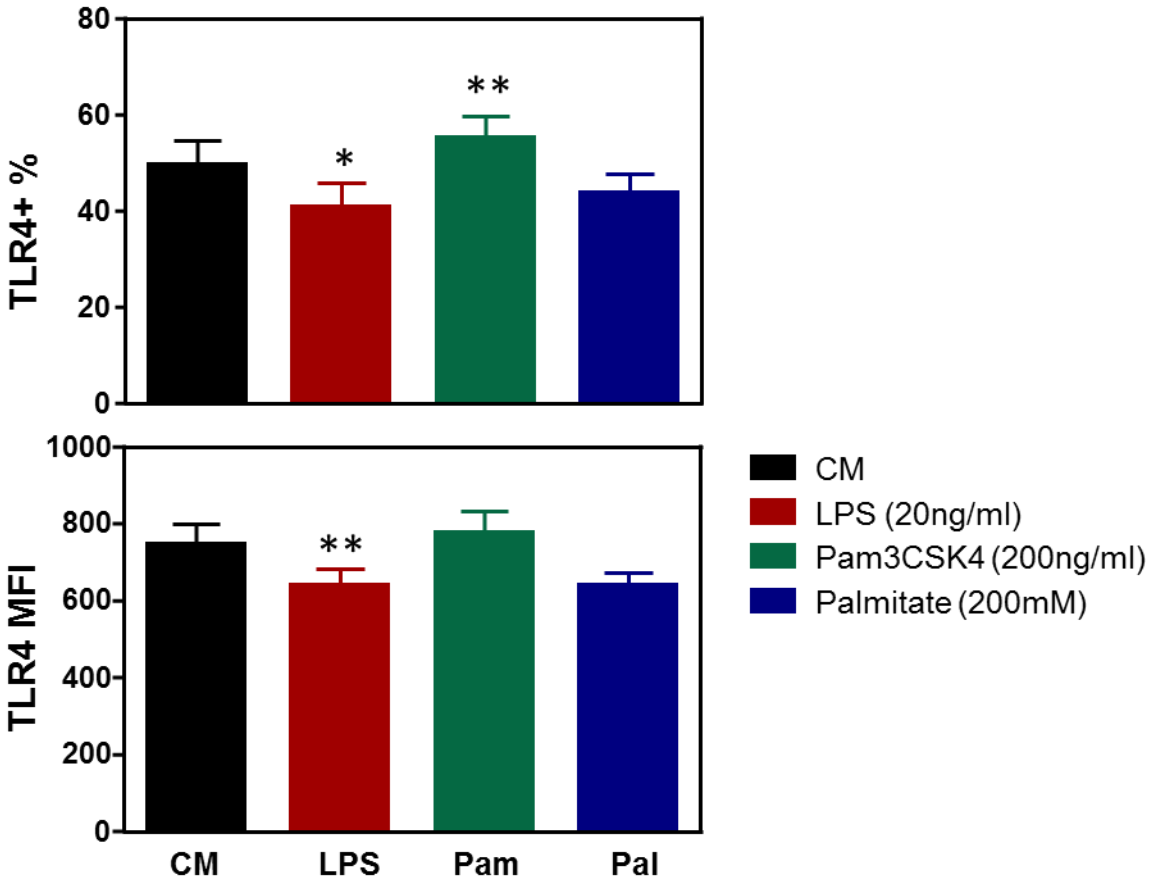


Figure 17. Description of TLR4 expression on monocyte

PBMC from subjects were stained with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte TLR4+ gate was defined as described. Dunn's multiple comparisons testwere performed to compare TLR4 expression between CM and LPS, Pam3CSK4 and palmitate stimulated monocytes. Data are represented as mean ±SE, *p<0.05, **p<0.01, (n=25 including HC n=7, SS n=15, NASH n=3).

We then compared the percentage of TLR4+ monocytes and TLR4 expression on monocytes among the 3 study groups to see if they are altered in SS and/or NASH patients relaive to HC at baseline and/or upon stimulation.

At baseline, the percentage of TLR4+ monocytes was similar among HC, SS and NASH subjects. Similarly, relative TLR4 expression showed no difference between study groups. Upon stimulation with LPS, Pam3CSK4 and palmitate, the percentage of TLR4+ monocytes were similar among the 3 study groups. Relative TLR4 expression level was also not different among 3 study groups after stimulation. An analysis comparing the fold-change in percentages of TLR4+ monocytes upon stimulation (LPS, Pam3CSK4 and palmitate stimulated values divided by non-stimulated CM value) among the 3 study groups also showed no differences. Similar analysis of the MFI intensity were also conducted**.** No significant difference was observed (data not shown).

Overall, the changes in monocytes TLR4 expression were different upon different PAMP stimulation. However, the percentage of TLR4+ monocytes and TLR4 expression per monocytes did not differ among the 3 study groups.

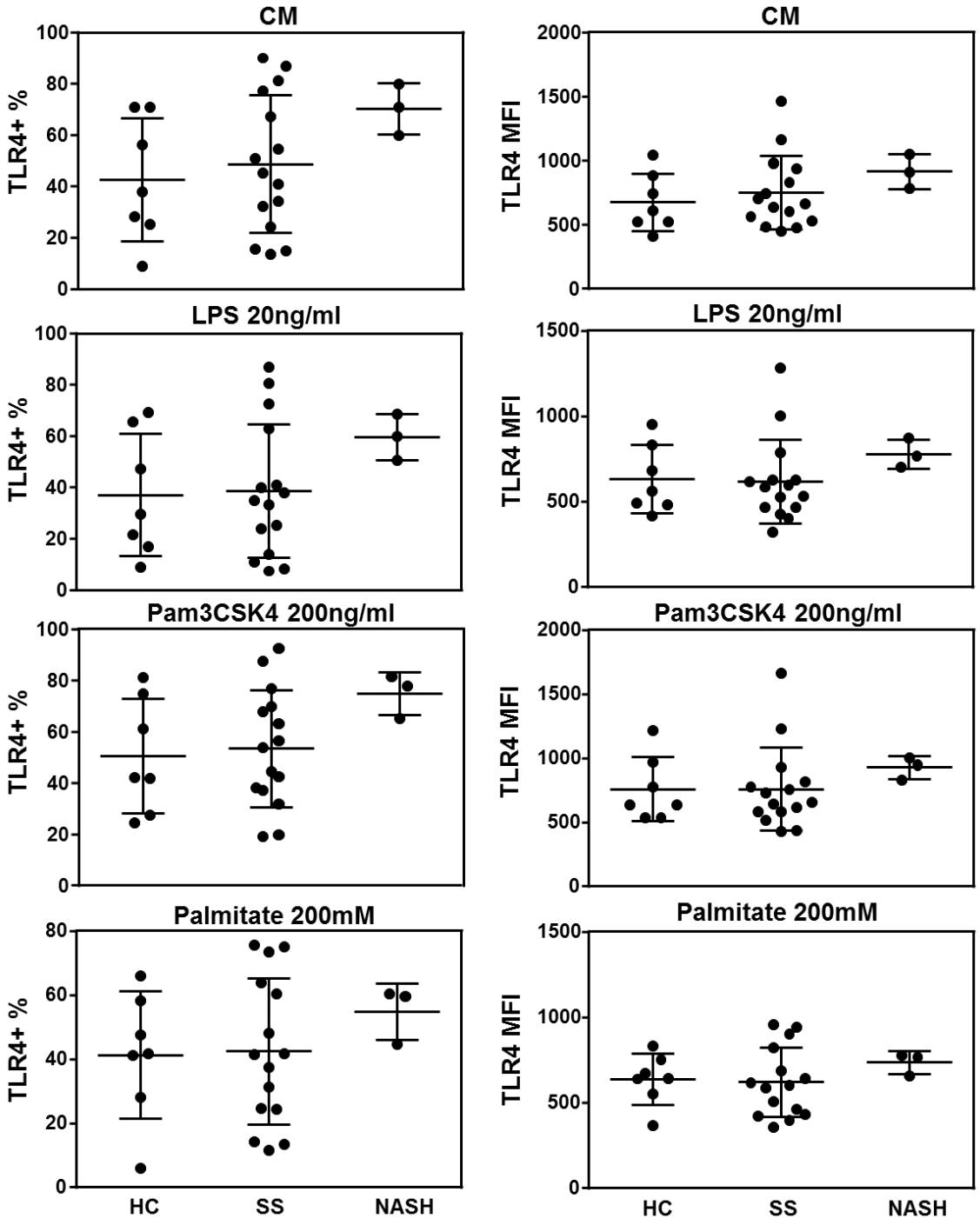


Figure 18. The percentage of TLR4+ monocytes or TLR4 expression on monocytes did not differ among 3 study groups

PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained

with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte TLR4+ gate was defined as described. Each dot indicated the interested cytokine level of one subject. Kruskal-Wallis test were performed to compare TLR4 expression among 3 study groups. Data are represented as mean ±SD, (HC n=7, SS n=15, NASH n=3).

3.6 PAMP induced down-regulation of monocyte CCR1 correlated with plasma ck18 level

Monocyte chemotaxis is critial for recruitment to an inflammed liver, and a potential driver of NAFLD. The monocyte CCR1 plays a dominant role in monocyte trafficking across endothelial layers and CCR2 is required to drive most monocytes from the bone marrow to the blood and from the blood to the site of inflammation [93]. In order to explore if monocyte CCR1 and CCR2 expression are different among the 3 study groups with and without PAMP stimulation, we determined the expression of the monocyte chemokine receptor CCR1 and CCR2 at baseline and upon the stimulation with LPS, Pam3CSK4 and palmitate as we did for TLR2 and TLR4. Freshly isolated PBMC were cultured with CM, LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12 hr and CCR1 and CCR2 expression were determined by flow cytometry.

3.6.1 The percentage of CCR1+ monocytes or CCR1 expression on monocytes did not differ among the 3 study groups at basal level; however PAMPs-induced alteration of monocyte CCR1 expression negatively correlates with plasma ck18

At baseline,from all subjects, 90% of monocytes expressed CCR1 (Figure 19) . Upon stimulation by all 3 stimuli, CCR1 expression down-regulated by approximately 40% (all $p<0.0001$). Similarly, upon stimulation with LPS, Pam3CSK4 and palmitate, the relative expression of CCR1 per monocyte decreased by 2 to 3-fold in comparison with non-stimulated monocytes ($p<0.0001$, $p<0.001$, $p<0.0001$).

To summarize, monocyte CCR1 expression per cell was down-regulated by all 3 stimuli.

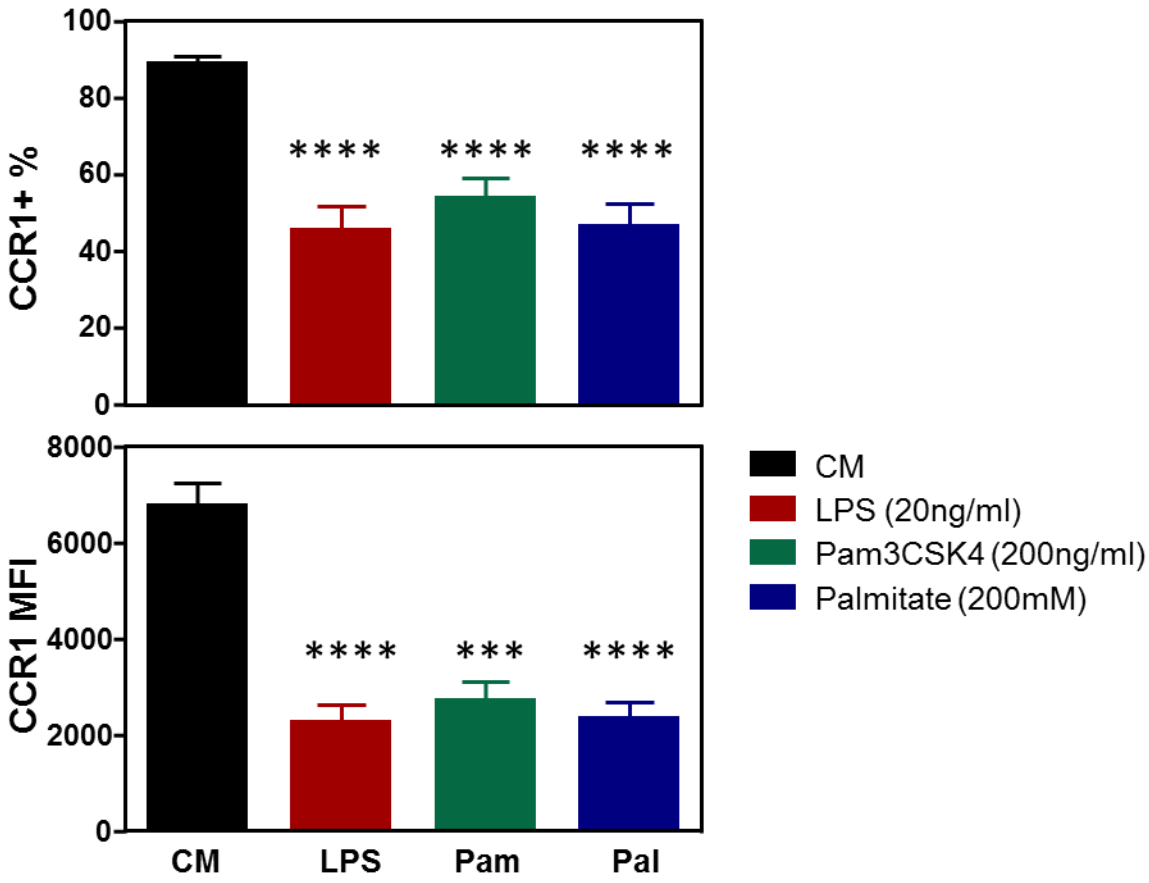


Figure 19. Description of CCR1 expression on monocyte

PBMC from subjects were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte CCR1+ gate was defined as described. Dunn's multiple comparisons testwere performed to compare CCR1 expression between CM and LPS, Pam3CSK4 and palmitate stimulated monocytes. Data are represented as mean ±SE, ***p<0.001, ****p<0.0001, (n=25 including HC n=7, SS n=15, NASH n=3).

We then compared the percentage of CCR1+ monocytes and CCR1 expression on monocytes among the 3 study groups to see if they were altered in SS and NASH patients relative to HC at baseline and upon stimulation (Figure 20). At baseline, the percentages of CCR1+ monocytes were similar among HC, SS and NASH subjects. Similarly, the relative CCR1 expression showed no difference between study groups. Upon stimulation by LPS, Pam3CSK4 and palmitate, the percentage of CCR1+ monocytes also did not show differences among the 3 study groups. The CCR1 expression level was also not different among the 3 study groups after stimulation. An analysis comparing the fold-change of the percentage of CCR1+ monocyte upon stimulation (LPS, Pam3CSK4 and palmitate stimulated values divided by non stimulated CM value) among the 3 study groups also showed no differences. A similar analysis of the MFI intensity was also conducted**.** No significant differences were observed (data not shown). To summarize, the monocyte CCR1 expression was not different in NAFLD patients compared with HCs.

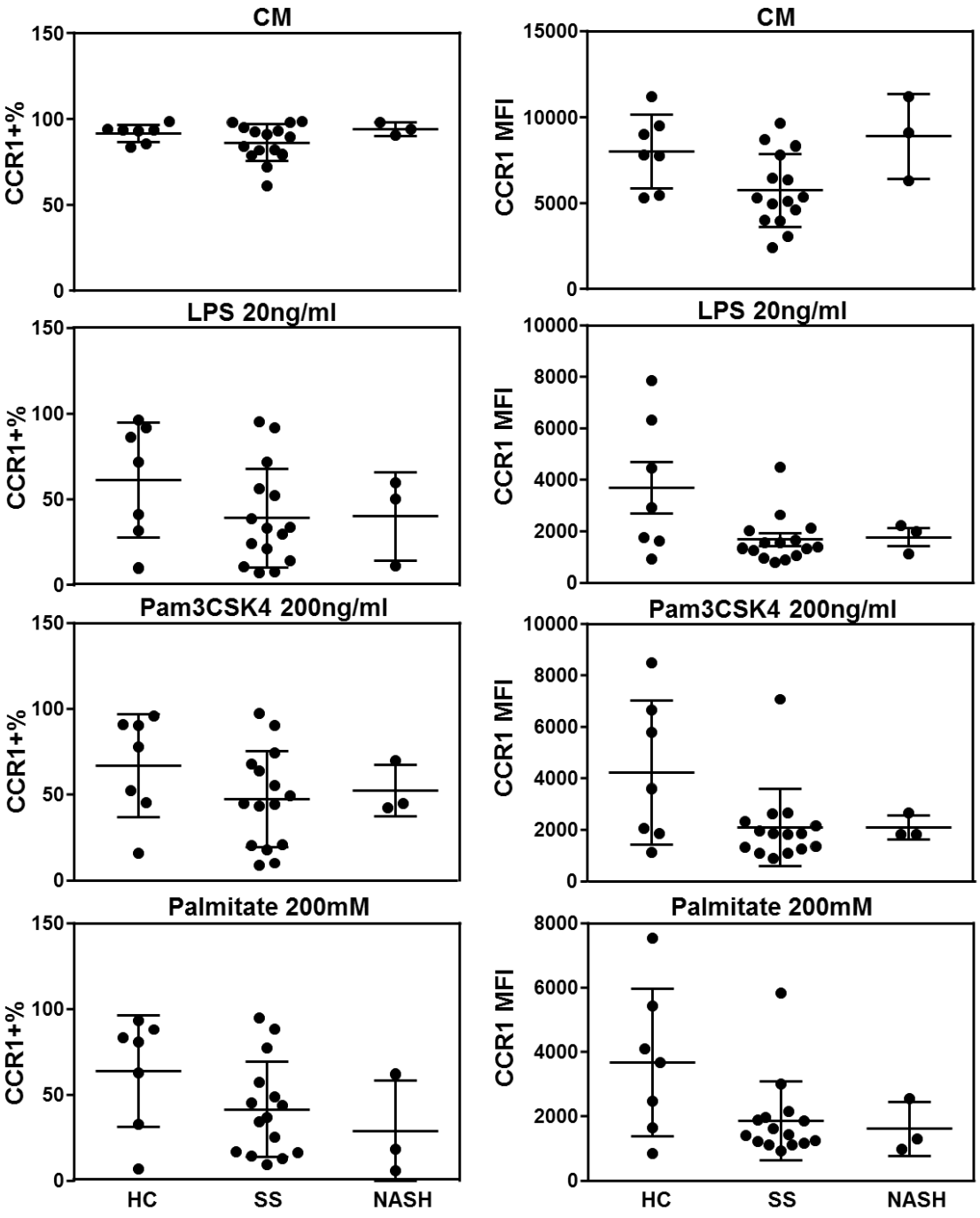


Figure 20. The percentage of CCR1+ monocytes or CCR1 expression on monocytes did not differ among 3 study groups

PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte CCR1+ gates were defined as

described. Each dot indicated the CCR1 level of one subject. Kruskal-Wallis test were performed to compare CCR1 expression among 3 study groups. Data are represented as mean ±SD, (HC n=7, SS n=15, NASH n=3).

Although there were no differences in CCR1 expression after stimulation with the 3 different antigens, we found that after stimulation, the CCR1 MFI in HC was evenly distributed from 1000 to 8000 but CCR1 MFI of SS and NASH was predominantly in the 1000-3000 range (the difference with in the groups was not statistically significant) (Figure 20). To explore if the degree of down-regulation of CCR1 induced by stimulation was different among 3 study groups, we then compared degree of down regulation ofmonocyte CCR1 MFI by subtracting the baseline expression level from the stimulated levels. We found that the degree of down regulation was 1.6-fold greater in NASH subjects compared to SS subjects upon stimulation with palmitate with a mean reduction of approximately 4000 MFI versus a mean of approximately 7000 MFI (p=0.045). LPS induced down regulation of CCR1 also tended to be greater in NASH subjects, approximately 4000 MFI, compared to SS subjects, approximately 7000 MFI (p=0.068) (Figure 21 a). An analysis comparing the fold-change of down regulation of CCR1 MFI upon stimulation (LPS, Pam3CSK4 and palmitate stimulated values divided by non stimulated CM value) among the 3 study groups showed no significantly differences (data not shown).

We next performed pearson correlations comparing the degree of CCR1 down regulation and the liver disease indicator ck18. Significant correlations between the down regulation of CCR1 and ck18 levels following LPS, Pam3CSK4 and palmitate stimulation were observed (r=-0.51, p=0.01; r=-0.53, p=0.007; r=-0.41, p=0.04) (Figure 21 b).

Overall, monocyte CCR1 expression down regulated on exposure to all 3 stimuli. However, the percentage of CCR1+ monocytes or CCR1 expression per monocyte did not differ between the 3 study groups. Nonetheless, the degree of CCR1 down regulation induced by all 3 stimulis correlated with plasma ck18 levels.

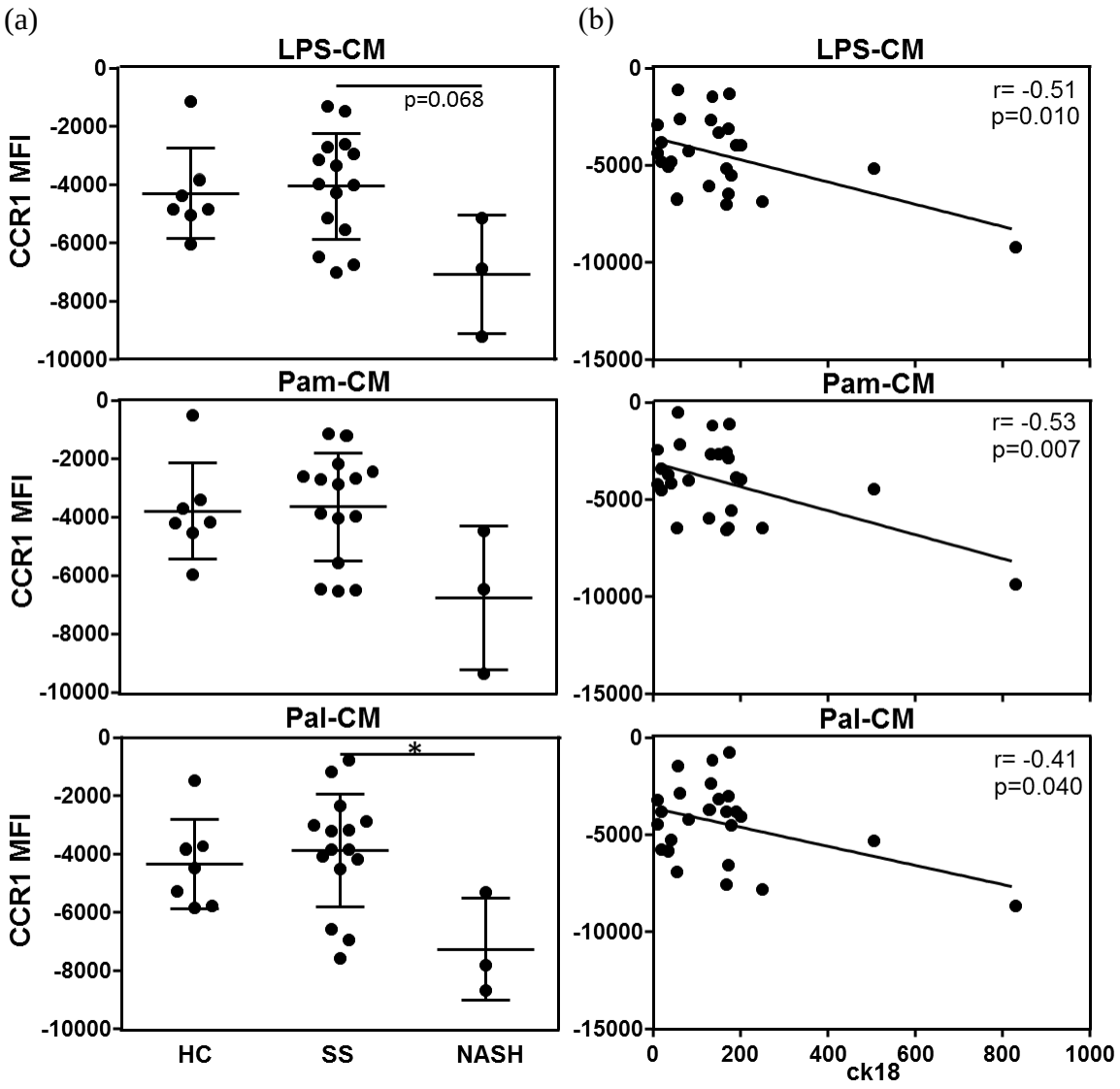


Figure 21. LPS, Pam3CSK4 and palmitate induced alteration of monocyte CCR1 expression negatively correlated with ck18

PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. Alteration of CCR1 expression was determined by subtracting baseline CCR1 from stimulated CCR1 expression. Kruskal-Wallis test were performed to compare altered CCR1 expression among 3 study groups. Each dot indicated the degree of down regulation of CCR1 and/or ck18 level of one subject. Data are represented as mean ±SD*$p<0.05$. Pearson correlations were performed between altered CCR1 expression and ck18 level (HC n=7, SS n=15, NASH n=3).

3.6.2 The percentage of CCR2+ monocytes or CCR2 expression on monocytes did not differ among 3 study groups at basal level or upon stimulation of PAMPs

As with CCR1, 90% of monocytes were found to express CCR2 (Figure 22). Upon stimulation with Pam3CSK4 and palmitate, the percentage of monocytes that expressed CCR2 significantly decreased but by less than 10% ($p<0.0001$, $p<0.05$). Upon stimulation with LPS, Pam3CSK4 and palmitate, the relative expression of CCR2 per monocyte decreased by 2 to 3-fold in comparison with non-stimulated monocytes (all $p<0.0001$). To summarize with the percentage, monocyte CCR2 expression did not appreciably change after antigen stimulation, the amount of CCR2 per cell was significantly down regulated after stimulation.

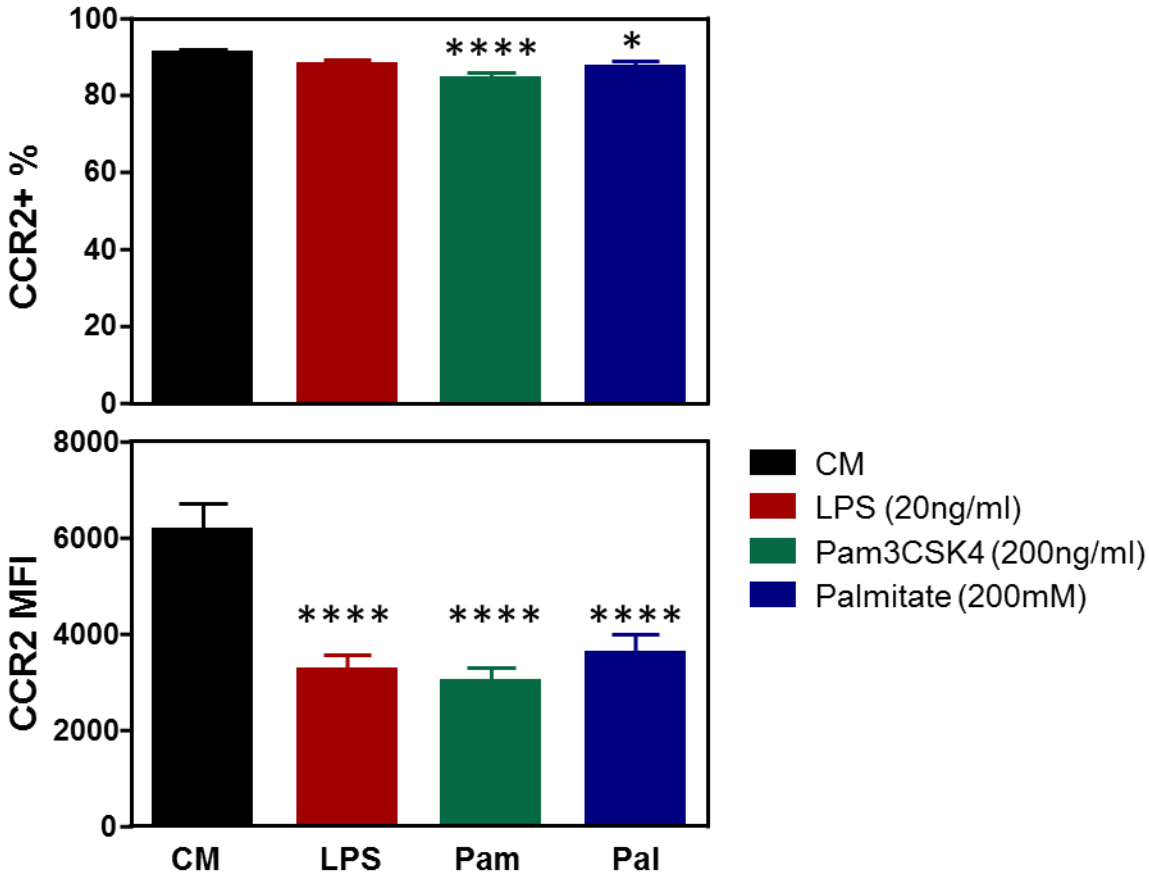


Figure 22. Description of CCR2 expression on monocyte

PBMC from subjects were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained with fluorochrome-conjugated antibodies as shown in Table 2. Monocyte CCR2+ gate was defined as described. Dunn's multiple comparisons testwere performed to compare TLR2 expression between CM and LPS, Pam3CSK4 and palmitate stimulated monocytes. Data are represented as mean ±SE, *p<0.05, ****p<0.0001, (n=25 including HC n=7, SS n=15, NASH n=3).

We then compared the percentage of CCR2+ monocytes and CCR2 expression per monocyte among the 3 study groups to see if they are altered in SS and NASH patients relative to HC at baseline or upon stimulation (Figure 23). At baseline, the percentage of CCR2+ monocytes was similar among HC, SS and NASH subjects. Similarly, the relative CCR2 expression per cell showed no difference between study groups. Upon stimulation by LPS, Pam3CSK4 and palmitate, the percentage of CCR2+ monocytes also did differ among the 3 study groups. The relative CCR2 expression levels was also not different among the 3 study groups after stimulation. An analysis comparing the fold-change of the percentage of CCR2+ monocytes upon stimulation (LPS, Pam3CSK4 and palmitate stimulated values divided by non-stimulated CM value) among the 3 study groups were similar. MFI intensities and correlation analysis between the degree of down regulation of CCR2 per cell and ck18 levels were also conducted. No significant differences were observed (data not shown). Overall, monocyte CCR2 expression was down regulated by all 3 stimulis. However the percentage of CCR2+ monocytes and CCR2 expression on monocytes did not differ among 3 study groups. Moreover there were not correlatins between the extent of down regulation and ck18 levels.

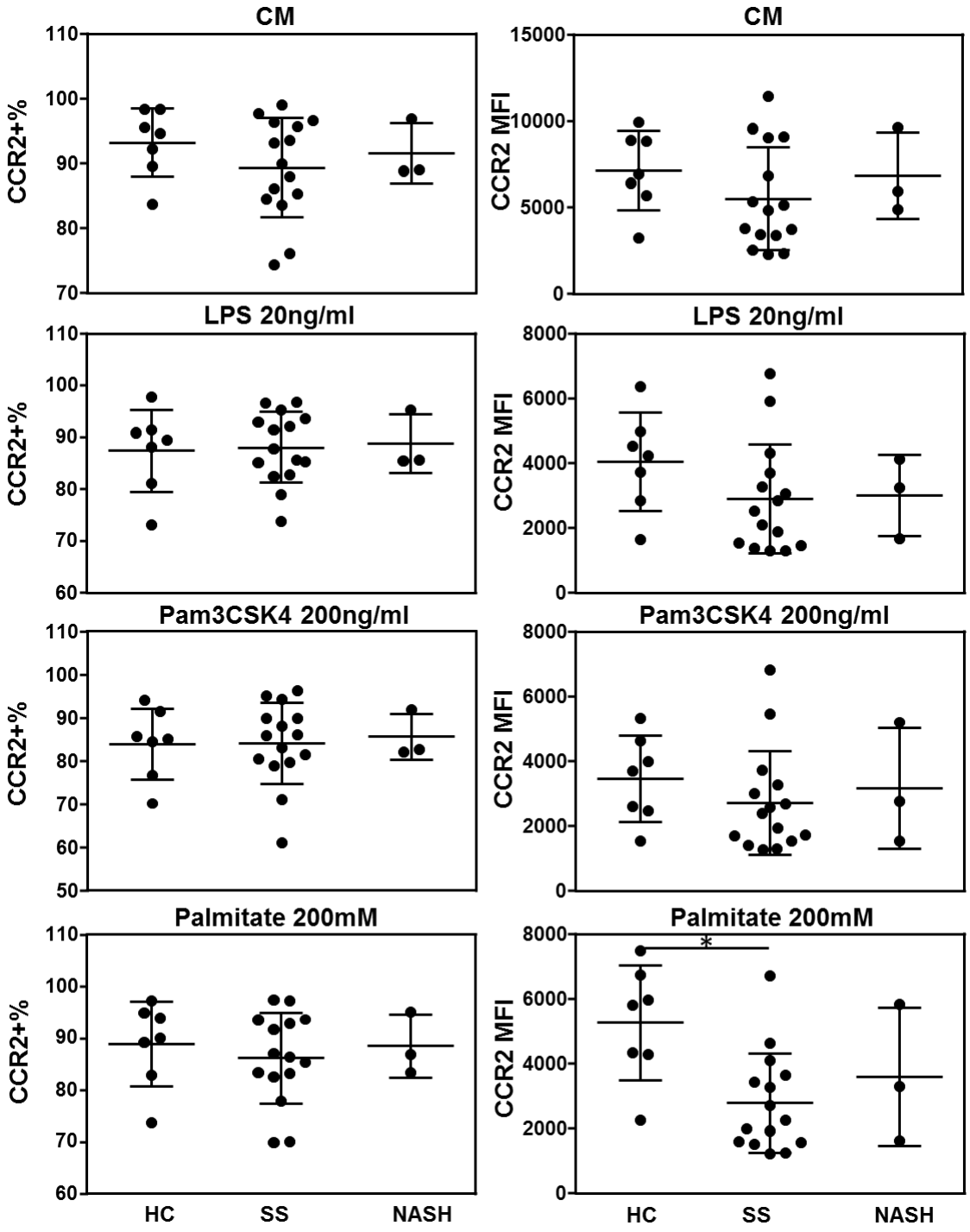


Figure 23. The percentage of CCR2+ monocytes or CCR2 expression on monocytes did not differ among 3 study groups

PBMC from subjects (HC: Health controls, SS: Simple steatosis, NASH: Non-alcoholic steatohepatitis) were cultured with LPS (20ng/ml), Pam3CSK4 (200ng/ml) and palmitate (200mM) for 12hr. PBMC were stained

with fluorochrome-conjugated antibodies as shown in Table 2. Each dot indicated the percentage of cell that express CCR2 or CCR2 expression per cell one subject. Monocyte CCR2+ gate was defined as described. Kruskal-Wallis test were performed to compare CCR2 expression among 3 study groups. Data are represented as mean ±SD, *$p<0.05$, (HC n=7, SS n=15, NASH n=3).

3.7 20-HETE did not influence PBMC cytokine production and monocyte chemokine receptor expression

3.7.1 20-HETE did not influence PBMC cytokine production

The current literature suggest that some oxylipins can induce cytokine production [107]. As 20-HETE levels were found to be significantly elevated in SS patients and NASH patients and positively correlated with ck18 levels, we evaluated the effects of 20-HETE on PBMC cytokine production. Additional, studies have suggested that 20-HETE is asscociated with immune cell trafficking [108, 109]. Thus we hypothesised that 20-HETE could influence PBMC cytokine production and may modulate monocyte chemokine receptor CCR1 and CCR2 expression.

We cultured PBMC from subjects from the three study groups with CM only and different concentrations of 20-HETE for 24hr, and determined if 20-HETE enhanced LPS induced cytokine production by adding 2 different concentrations of LPS (0.2ng/ml, 2ng/ml). The production of IL-1β, IL-6, TNF-α and IL-10 in PBMC supernatents was examined by ELISA.

20-HETE treatment alone, in varying concentrations did not induce production of any of the above four cytokines found in CM (Figure 24). PBMC cultured with both 20-HETE and LPS did indeed produce cytokines, but more likely as a result of exposure to LPS not 20-HETE, since the induced cytokine levels were similar between LPS alone and LPS with different concentrations of 20-HETE. Similar to HC subjects, the supernant from PBMC of SS and NASH subjects cultured with 20-HETE did not have 20-HETE responses above that of CM, and again cytokines were induced by LPS in the presence of 20-HETE in both groups. Thus, under these conditions, 20-HETE did not induce any cytokine response in

any study group.

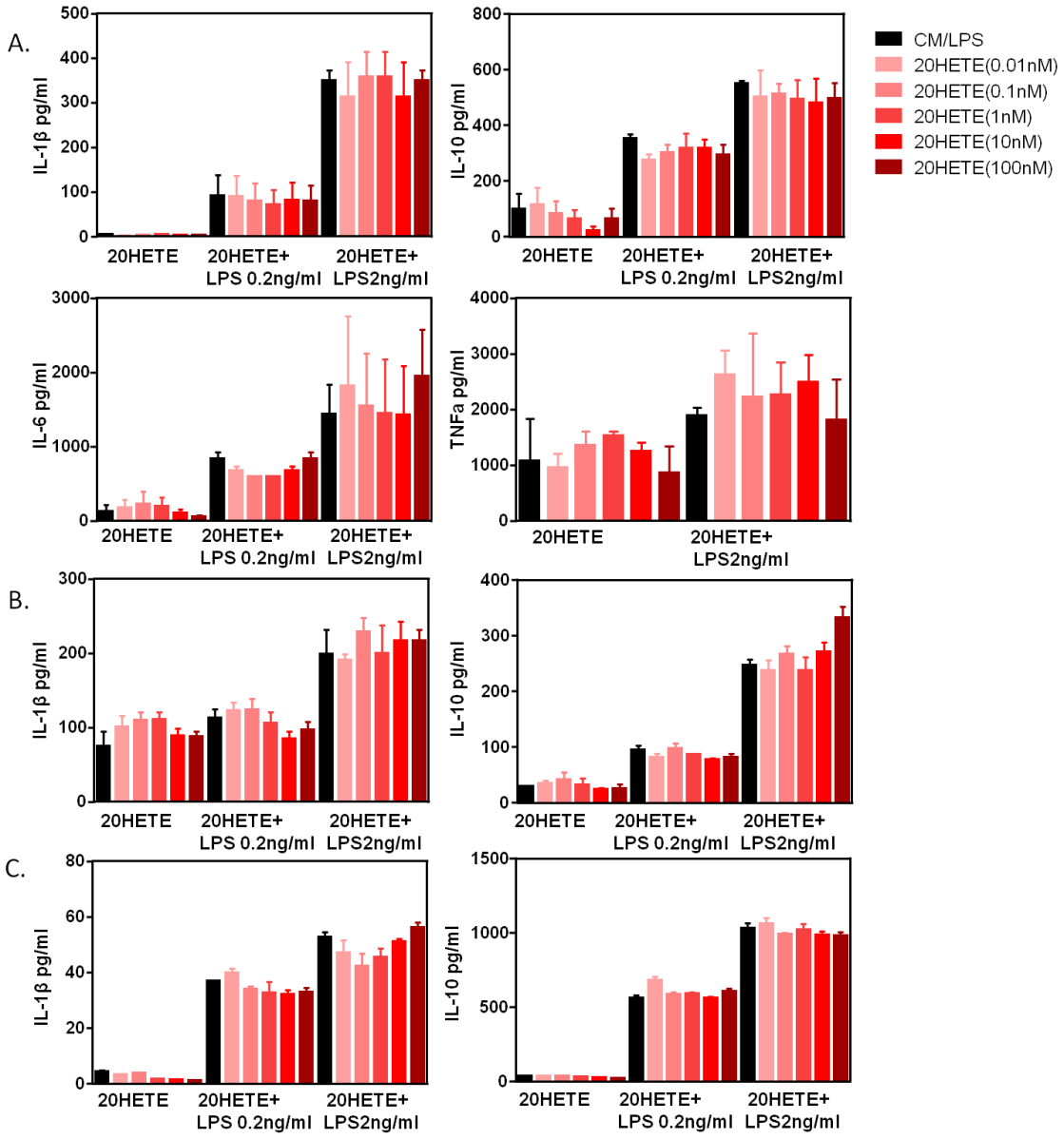


Figure 24. 20-HETE did not influence PBMC cytokine production

Freshly isolated PBMC from A. Healthy cotrol (n=3), B. Simple steatosis (n=2), C. NASH (n=2) were cultured with different concentration of 20-HETE (0.01nM, 0.1nM, 1nM, 10nM, 100nM) with or without LPS (0.2ng/ml, 2ng/ml) for 24hr. Supernatant cytokin levels were determined by ELISA. No dose dependent effect were observed.

3.7.2 20-HETE did not influence monocyte chimokine receptor CCR1 and CCR2 expression

Studies have suggested that 20-HETE is asscociated with immune cell trafficking [108, 109]. Thus we hypothesised that 20-HETE treatment could influence monocyte chemokine receptor CCR1 and CCR2 expression. We cultured PBMC from HC with different concentrations of 20-HETE alone or in combination with LPS for 12hr. PBMC were then stained for the expression of CCR1 and CCR2 (Figure 25). However, we did not observe any effect of 20-HETE on the percentage of CCR1+ monocytes or CCR1 expression. 20-HETE also did not have any effect on LPS induced down regulation of CCR1. Similar results were obtained for CCR2, 20-HETE did not have an effect on either CCR2 expression or LPS induced down regulation of CCR2.

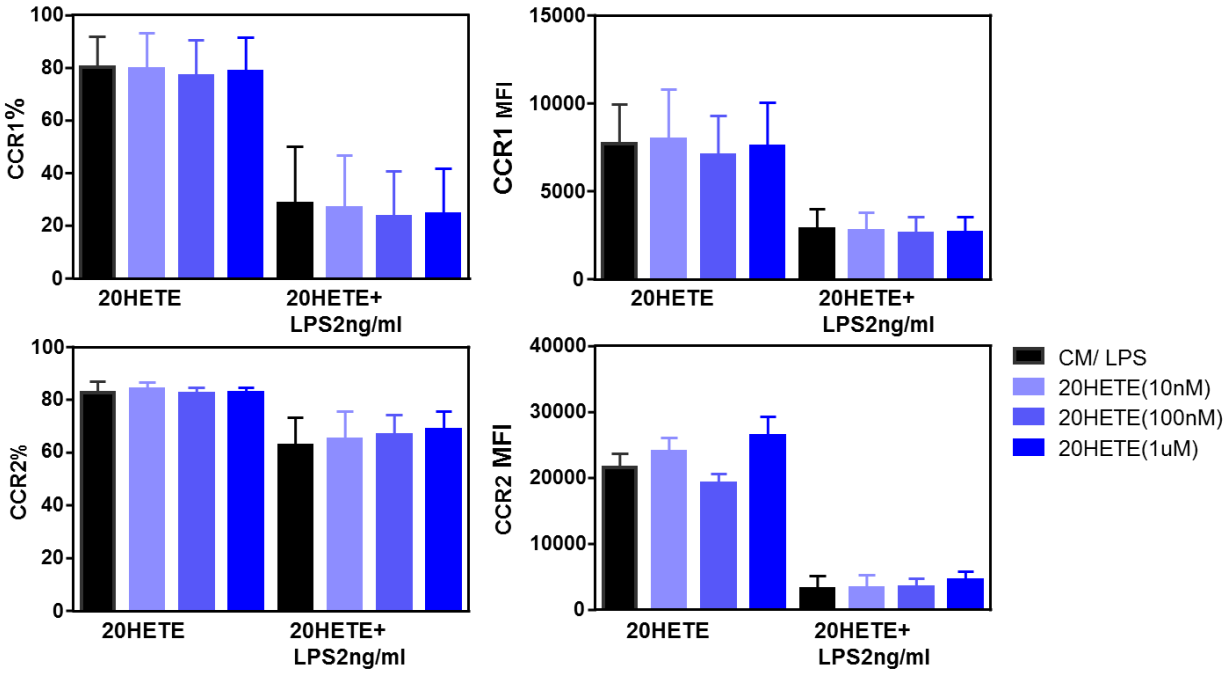


Figure 25. 20-HETE did not influence monocyte chimokine receptor CCR1 and CCR2 expression

Freshly isolated PBMC from healthy cotrol (n=2)were cultured with different concentration of 20-HETE (0.01nM, 0.1nM, 1nM, 10nM, 100nM) with or without LPS (2ng/ml) for 24hr. Monocyte CCR1 and CCR2 expression were determined by flow cytometry as previously discribed. No dose dependent effect were observed.

4. Discussion

4.1 Plasma oxylipin levels

Lipids and lipid metabolism have been shown to be important in NAFLD pathogenesis [110]. Several studies have emphasized the role of altered lipid composition in NAFLD patients. As precursors of oxylipins, alterations in of lipid composition and may lead to alterations in oxylipin composition and levels. Blood, similar to many other tissues and systems, contains secreted oxylipins.

Oxylipins, as immune mediators, are able to modulate immune responses. They can be categorized into pro-inflammatory and anti-inflammatory oxylipins. Pro-inflammatory oxylipins activate immune cells and induce the expression of inflammatory cytokines and chemokines. Anti-inflammatory oxylipins decrease pro-inflammatory cytokines and enhance anti-inflammatory cytokine production [111]. By comparing blood oxylipin levels between HC and NAFLD patients as well as SS and NASH, we would be able to determine if NAFLD is associated with altered oxylipin profiles.

We have previously found that serum oxylipin levels were significantly different between T2D patients, a condition accompanied with NAFLD and healthy controls. In that study, 15/84 detected oxylipins demonstrated a 2-fold higher concentration in the T2DM group relative to healthy controls. In addition, 20-HETE and 12,13-EpOME positively correlated with ALT levels suggest an association with liver function. In our current study, 51 of oxylipins were detected from plasma of all subjects. Plasma concentrations of 20-HETE and 8-HETrE levels were significantly higher in the NAFLD group compared with controls ($p<0.05$). That 12,13-EpOME did not show any difference among the 3 study groups

suggests that despite the T2D/NAFLD association, different pathogenic mechanisms are induced.

20-HETE, a potent vasoconstrictor, was significantly increased in both SS and NASH patients. Increasing concentrations of 20-HETE have been associated with cardiovascular diseases, hypertension and renal diseases. The mechanism appears to induce oxidative stress [112]. In heart disease, elevated 20-HETE was associated with increased mitochondrial pathway activation and increased ROS production both of which promote cardiomyocyte apoptosis [113, 114]. Studies have also shown that 20-HETE facilitates neutrophil accumulation and contributes to inflammation in endothelium and tissues [115, 116]. Although the role of 20-HETE in NAFLD progression is not known, it may play a role in linking NAFLD and cardiovascular diseases and help to explain why NAFLD is an important risk factor for cardiovascular diseases.

As shown in our results, 8-HETrE was also elevated in the NASH cohort of NAFLD patients; however, there are few studies describing its function or association with diseases. Our findings could provide novel insights on the role of oxylipins, derived via the LOX pathway from γ-linoleic acid.

Our results also showed positive associations between hepatic apoptosis as reflected by ck18 levels and some of the AA metabolites. 5-HETE, 9-HETE, 15-HETE, 20-HETE, 11,12-DiHETrE, 14,15-DiHETrE were all positively correlated ($p<0.05$) with ck18 levels in plasma and 8-HETE, Tetranor12-HETE, 11,12-EpETrE, 15-HETrE and 12-HEPE showed positive associations with plasma ck18 ($p<0.1$). Each of these oxylipins have pro-inflammatory properties except for 12-HEPE, are derived from EPA. Additionally, 5-oxoETE, the metabolite of 5-HETE which may be considered as anti-inflammatory

oxylipin since it decreased the pro-inflammatory oxylipin 5-oxoETE showed a negative association with ck18. But how do these oxylipins contribute to NAFLD progression is not known.

11,12-DiHETrE is a vasodilator. It was demonstrated to vasodilate pressurized arteries potently in a murine model [117]. Our finding that 11,12-DiHETrE correlated with disease severity are in keeping with the results from Loomba et al [102]. They previously proposed that 11,12-DiHETrE is a potential biomarker able to distinguish SS from NASH. 14,15-DiHETrE, another CYP dihydroxy product from AA, which has similar functions [117-119] with 11,12-DiHETrE was also elevated in parallel with ck18 levels and hence, could be a potential NAFLD biomarker.

12-HETE is a powerful pro-inflammatory product, that promotes inflammation by stimulating protein kinase C (PKC) to boost the production of inflammatory cytokines [120]. Both 5-HETE and 8-HETE can induce immune chemotaxis activities [121]. Evidence has also shown that 5-HETE is able to induce eosinophil chemotaxis activities [122] and neutrophil degranulation in humans [123]. Increased levels of 5-HETE, 8-HETE, 12-HETE and 15-HETE are seen in other inflammatory diseases, for example in rheumatoid arthritis [120]. 15-HETE was described to enhance caspase 3 activation [124]. Patients with high levels of 12-HETE and 15-HETE had a lower survival rates in pulmonary arterial hypertension [125]. In the condition of pollen allergy, a second challenge of antigen significantly elevated 15-HETE level in a IgE dependent manner [126]. These properties are particularly relevant to NAFLD because previous studies have shown improvements in liver histology inflammation scores by taking Pentoxifylline, a medication that decreases lipid oxidation while limiting plasma 8-HETE, 9-HETE, 11-

HETE and 15-HETE levels in NASH patients [127]. In terms of NAFLD, Puri et al. have found that 5-HETE, 8-HETE, 15-HETE and 11-HETE were elevated in both SS and NASH patients [100]. This suggests that in addition to AA-derived oxylipins other common immune mediators for example cytokines, play role in liver inflammation, and may be a potential therapeutic target.

Among AA products 20-HETE was significantly elevated in both SS and NASH subjects. In order to know if the AA oxylipin 20-HETE has an effect on NAFLD development, we explored if 20-HETE influences immune cell function. However, our experiments that culture PBMC with 20-HETE showed 20-HETE did not induce or promote LPS-stimulated IL-1β, IL-6, TNF-α and IL-10 production or influence monocyte CCR1, CCR2 expression in either HC or NAFLD subjects. Although we were unable to demonstrate that 20-HETE influences immune cell cytokine production and certain chemokine receptor expression, immune cells, or other immune cells activation in NAFLD. Moreover, 20-HETE in the circulation is able to enter into the liver and act directly on liver immune cells, hepatocytes and endothelial cells. The influence of 20-HETE on these cells should be further explored. Presently, 20-HETE is at least a potential disease marker of NAFLD.

On the other hand, many of these oxylipins have direct effects on the circulation system. 20-HETE has vasoconstriction effect and could also lead to dysfunction of endothelial cells [116]. 11,12-DiHETrE 14,15-DiHETrE are potent vasodilators. 5-HETE, 12-HETE and 15-HETE were shown to elevate thrombin-induced human platelet aggregation [128]. 15-HETE has both vasodilation and vasoconstriction features depending on different conditions [129]. Studies have shown that the peak and mean portal vein velocity was decreased in NAFLD and negatively correlated with steatosis severity [130]. If decreased

flow is causative rather than an effect of fatty liver, the abnormal vasoactive oxylipin profiles in our study could be a potential contributor to NAFLD development.

In summary, elevated pro-inflammatory oxylipin levels in NAFLD patient blood reflects systemic inflammation. Elevation of certain oxylipins was associated with hepatic apoptosis. Thus, it is conceivable that oxylipins traffic to the liver act as immune modulators, activate and alter hepatic perfusion.

4.2 TLR2 and TLR4 activated PBMC cytokine levels

Due to the specific location of the liver, blood from the gut, contains byproducts of intestinal microbes, that enter the liver through the portal vein. TLR2 and TLR4 are 2 dominant toll-like receptors that can recognize and be activated by bacteria components. Activated immune cells especially macrophage/monocytes, produce pro- and anti-inflammatory cytokines. These cytokines are important immune mediators that are able to induce amplify or attenuate inflammation. In our study, cytokines IL-1β and IL-6 from PBMC activated by both TLR4 ligand LPS and TLR2 ligand Pam3CSK4 were elevated in either SS subjects or NASH subjects or elevated in both study groups compared with HCs. These results suggest an increased susceptibility of PBMC to TLR4 and TLR2 activation. Based upon our studies, causality is not apparent. Either PBMC from patients with NAFLD are more sensitive to this signaling, or these subjects are more responsive due to non-specific illness. Another possible explanation is that the development of NAFLD causes changes to cytokine production. The cytokine responses showed no difference between groups upon stimulation by the highest concentration of LPS which is 20ng/ml. Thus, maximal stimulation may mask population specific change in TLR responses.

Palmitate is an agonist for both TLR2 and TLR4 and the IL-1β and IL-6 responses to

palmitate were similar between 3 study groups. This could because of differences between the TLR4 agonist LPS and palmitate as well as between TLR2 agonist Pam3CSK4 and palmitate. Even though they share the same receptor, downstream signaling may be different. In SS and NASH subjects, palmitate induced cytokine production and its upstream signaling transduction did not change compared with HC subjects.

The balance of pro- and anti- inflammatory cytokines is important in immunoregulation. Anti-inflammatory cytokines could suppress the synthesis of pro-inflammatory cytokines, promote synthesis of pro-inflammatory cytokine receptor antagonists and induct more anti-inflammatory cytokine production, thus ameliorating inflammation and limiting tissue injury caused by inflammation. Of the many anti-inflammatory cytokines, IL-10 is central. Lower levels of IL-10 are observed in several inflammatory diseases such as multiple sclerosis [131] and inflammatory bowel disease. In addition, IL-10 can directly regulate the synthesis of TNF-α[132], such that upregulation of IL-10 lowers the levels of TNF-α. In our study, we did not detect any difference of stimulated IL-10 levels among 3 study groups regardless of TLR4 or TLR2 stimulation. The findings suggest that stimulated anti-inflammatory cytokine by PBMC may not change during NAFLD progression. This may be one reason that TNF-α levels did not differ among the 3 study groups.

In order to determine if the increased pro-inflammatory response was associated with hepatic apoptosis, we performed the Pearson correlation analyses between IL-1β and IL-6 and plasma ck18 levels for all subjects. In agreement with our previous findings, we found that LPS induced IL-1β and IL-6 positively correlated with plasma ck18 levels. Similar correlations were found with Pam3CSK4 induced IL-1β and IL-6. This indicates that increased PBMC cytokine responses are associated with hepatic injury as reflected by

apoptosis markers.

In order to explore if the increased PBMC cytokine production is associated with increased monocyte cytokine production directly, we evaluated IL-1β that was elevated in SS or NASH subjects and one cytokine TNF-α that did not different among 3 study groups. However, neither intracellular IL-1β nor TNF-α levels differed by analysis of intracellular staining in the monocyte population among the 3 study groups. This could because that the difference we observed in PBMC cytokine production could be the contribution of cells of PBMC other than monocytes, however in our flow experiments the lymphocyte population did not show IL-1β or TNF-α positivity upon 12hr stimulation. Another reason could be the different time of stimulation, for the assay that detected supernatant cytokines, PBMC were cultured for 24hr. However, for the assay that measure monocyte intracellular cytokines, cells were cultured for 12hr. Another possible explanation could be the different forms of the cytokines between these 2 assays. The cytokines detected from PBMC were secreted which could be accumulate in the cell supernatant to a high level, while the maximal monocyte intracellular cytokine is limited and easy to reach maximum upon stimulation. Also, the subject number of these 2 assays was different.

Taking together with the results for cytokine production, PBMC from NAFLD patients showed an elevated production of IL-1β and IL-6 but not TNF-α and immunoregulatory IL-10 levels after LPS and Pam3CSK4 stimulation. In addition, the LPS and Pam3CSK4 stimulated IL-1β and IL-6 positively correlates with ck18 level. These results suggest an increased PBMC pro-inflammatory response and a stable anti-inflammatory response upon stimulation. And the increased PBMC pro-inflammatory responses correlate with hepatic apoptotic activities.

A possible speculation is that during liver injury, PBMC or monocytes are recruited to the liver. The degree of liver apoptosis is associated with the quantity of PBMC cytokine production upon stimulation of bacterial products. Patients with NAFLD tend to have a greater PBMC cytokine production and thus may be more vulnerable to NAFLD development.

4.3 Monocyte TLR2 and TLR4 expression

Within 24 hours the stimulated cytokine production of PBMC is mainly produced by monocytes [106]. In order to determine if the elevated IL-1β and IL-6 production is due to increased proportion of monocyte in PBMC from the different study groups. We evaluated the monocyte count per one hundred thousand cells for the 3 groups. In a Korean study including 794 subjects, an elevated monocyte fraction was found in NAFLD patients compared with healthy controls using an automatic blood cell counter [92]. However, in our study, when we used a more stringent classification, excluded immune inactivated HLA-DR-positive cells, and define CD14 positive cells as monocytes, there is no difference of monocyte count among 3 study groups.

In order to know if the elevated IL-1β and IL-6 production induced by TLR4 and TLR2 agonist was due to changes in TLR expression, we tested TLR4 and TLR2 surface expression on monocytes. Increasing evidence has shown that hepatic TLR2 and TLR4 expression contributes to NAFLD development especially on Kuppfer cells. In a murine model deficiency of TLR2 or TLR4 showed a protective role against NAFLD development[70-72, 74, 75].However, the role of expression of TLR2 and TLR4 on Kuppfer cell precursor bone-marrow derived monocytes remained unclear in NAFLD patients.

In recent studies, TLR2 mRNA expression in adipose tissue and in PBMC were found to be elevated in obese and overweight subjects compared with lean subjects [133]. Obese subjects with the metabolic syndrome features hypertension and abnormal blood lipid levels had an elevation of TLR2 expression on circulation monocytes[134]. In this study, TLR2 expression did not differ among 3 study groups suggesting that in contrast to obesity or other metabolic syndromes, the liver form of metabolic syndrome, NAFLD may have a different immune profile reflected by unchanged TLR2 levels on monocytes.

Pam3CSK4 cultured with PBMC significantly induced monocyte TLR2 expression. This is likely due to the activation of Nuclear factor-κB (Nf-kB) pathway [135]. However, TLR2 expression at baseline or after stimulation did not show any difference among 3 study groups. This result suggests that TLR2 expression on monocyte may be not associated with NAFLD. Studies have shown that TLR4 expression on monocytes was elevated in a metabolic syndrome study in groups such as those suffering with obesity [133]. However in our study, at baseline, TLR4 expression was similar in 3 study groups. Upon stimulation of LPS, TLR4 expression was significantly decreased. This is because of internalization of TLR4 after recognition of LPS [136]. Pam3CSK4 induced the expression of TLR4 similar to TLR2 and likely through the Nf-kB pathway. TLR4 expression remained unchanged with palmitate stimulation. Palmitate can stimulate via both TLR2 and TLR4. The unchanged TLR4 expression could due to the opposite effects of TLR signaling during TLR2 and TLR4 activation. Palmitate stimulated TLR2 may activate Nf-kB pathways that induced TLR4 expression. At the same time, palmitate stimulated TLR4 can cause internalization of surface TLR4 that decreases TLR4 expression resulting in a lack of change in TLR4 surface expression in the end.

In terms of association with NAFLD, after stimulation, both the percentage of TLR4+ monocytes and the median TLR4 expression were still similar between 3 study groups. These results suggest that TLR4 on monocyte may not be associated with NAFLD development.

4.4 Monocyte CCR1 and CCR2 expression

Chemotactic responses are important in immune cell trafficking to the liver. We tested the surface expression on monocytes of the chemokine receptors CCR1 and CCR2. At baseline, the level of CCR1 did not differ among the 3 study groups. Upon TLR2 and TLR4 stimulation, CCR1 expression decreased dramatically. The decrease of CCR1 expression has been found by others to be due to the de novo synthesis and release of high concentrations of CCR1 ligands such as CCL3, CCL4 and CCL5 which act in an autocrine manner and leads to a decrease of its cognate receptor [137]. However, regardless of the three stimulations, the levels of CCR1 did not differ between the 3 groups.

We then compared the degree of down regulation of CCR1 expression after different stimulation conditions. We found that upon stimulation of palmitate, the down regulation of CCR1 expression was greater in the NASH subjects compared to SS subjects ($p=0.045$). LPS induced the degree of down regulation of CCR1 on monocytes from NASH subjects also had a trend to be greater than that in SS subjects ($p=0.068$). Interestingly, a Pearson correlation analysis showed that the change of CCR1 median surface expression induced by all 3 stimulation conditions correlated with plasma ck18 levels. In brief, subjects with higher ck18 levels are associated with a greater CCR1 reduction upon stimulation, which suggests that in NAFLD, hepatic apoptosis may be associated with an increased response by circulating monocyte/PBMC of CCR1 ligands upon TLR2 and/or TLR4 stimulations.

CCR2 levels were similar in the 3 groups at baseline. About 90% of monocytes expressed CCR2. Upon stimulation of LPS and Pam3CSK4, the percentage of monocytes that express CCR2 decreased to a small extend compared with non-stimulated monocytes. Similar to CCR1 we found CCR2 surface expression per cell significantly decreased after stimulation. Previous studies have shown that the decrease of CCR2 has a different mechanism from the decrease of CCR1. One study showed that LPS could reduce CCR2 mRNA half-life thus decrease its cell surface expression [138]. Another study showed that the reduction of CCR2 was due to the activation of serine proteinases and tyrosine kinase [139]. After stimulation, only palmitate stimulated monocyte CCR2 expression was significantly lower in SS subjects compared with HC subjects. This suggests that NAFLD may associate with mechanisms that could decrease monocyte CCR2 expression more than healthy subjects upon stimulation.

Bacterial products and fatty acids are found in higher concentrations in the liver than in the blood. According to our results, once monocytes are recruited into the liver, stimulated by bacterial products, will decrease surface CCR1 and CCR2 expression resulting in reduced trafficking out of the liver. For CCR1, the decreased expression is associated with increased chemokine production. CCR1 expression in NASH patients tended to decrease more than SS and HC, which suggest that monocytes from NASH subjects may produce higher level of chemokines than SS and HC subjects. This could provide a new view of NAFLD development, an increased ability of chemokine production and increased monocyte concentrations is associated with NASH development.

On the other hand, decreases of chemokine receptors decrease the monocyte ability to traffic outside the liver. Monocytes from NAFLD patients tend to have a lower CCR1 and

CCR2 expression than healthy control upon stimulation, thus monocytes from NAFLD patients may tend to stay in the liver.

Taken together with the data for cytokine production, our results suggest that in NAFLD, upon stimulation, there is both an increased PBMC pro-inflammatory response and a reduced trafficking out of the liver. Further that the increased PBMC pro-inflammatory responses as well as the degree of down regulation of chemokine receptor CCR1 correlated with hepatic apoptotic activities.

4.5 Influence of 20-HETE on PBMC cytokine production and CCR1, CCR2 expression

Plasma 20-HETE was significantly increased in both SS and NASH study groups. In order to determine if 20-HETE is able to impact monocyte cytokine production and chemokine receptor expression, we cultured PBMC from HC and NAFLD study groups with different concentration of 20-HETE. To determine if 20-HETE is acting in conjunction with other stimulation, we cultured different concentration of 20-HETE with 2 concentrations of LPS. However, neither 20-HETE alone nor 20-HETE with LPS had any influence PBMC cytokine production or monocyte CCR1 and CCR2 expression. These results suggest that 20-HETE may not influence the PBMC cytokine production and chemokine receptor expression that we have tested.

5. Global summary

In our study, we compared innate immune features and responses between SS, NASH and HC subjects. We evaluated monocyte TLR2 and 4 expression, activated monocyte/PBMC cytokine IL-1β, IL-6, TNF-α and IL-10 production and monocyte chemokine receptor

CCR1 and 2 expression in different conditions including at baseline and upon stimulation with the gut-derived PAMP antigens, LPS, Pam3CSK4 and the steatosis associated-fatty acid, palmitate. In addition, we also looked at immune mediators, oxylipins, in the 3 study groups and determined if certain oxylipins could influence immune responses.

Our results showed that bacterial antigen induced pro-inflammatory cytokine IL-1β and IL-6 levels but not TNF-α from circulatory PBMC's were significantly higher in NAFLD patients, either SS/NASH or both compared to healthy subjects. Further that stimulated IL-1β and IL-6 levels were significantly correlated with the hepatic apoptosis marker ck18.

For monocyte chemokine receptor expression, upon stimulation with PAMPs, both CCR1 and CCR2 expression were found to be decreased. And the degree of down regulation of CCR1 was significantly correlated with ck18. For plasma oxylipin levels, compared with HC, 20-HETE levels were significantly higher in both SS and NASH subjects, and plasma 8-HETrE levels were significantly higher in NASH subjects. In addition, plasma 5-HETE, 8-HETE, 15-HETE, 20-HETE, 11,12-DiHETrE and 14,15-DiHETrE positively correlated with ck18 levels.

Our results suggest that NAFLD is associated with increased PBMC activation, reflected by increased IL-1β and IL-6 production. These increased responses correlated with the degree of hepatocyte apoptosis. These results suggested that even at the same level of PAMP exposure, the immune system reacts differently in terms of cytokine production. The immune cells of NAFLD patient showed increased responsiveness. In addition, we for the first time identified a correlation between PAMP stimulation and the down regulation of CCR1 and NAFLD hepatic apoptosis. Our results suggested that in NAFLD, there is an increased tendency for monocytes to remian in the liver, i.e. the site of the antigen

stimulation. The hyper-rensponsive PBMC's and/or increased ability of monocyte to remian in the liver may be due to host genetics, the disease environment or unkown reasons. Further studies are needed to find out the mechanizms of these activities. Immune pathways that are associated with these activities could be potential drug targets to treat NAFLD.

Pro-inflammatory oxylipin 20-HETE and 8-HETrE was significantly elevated in the plasma of NAFLD patients, suggesting a systemic inflammation in NAFLD and the link of NAFLD to cardiovascular diseases. 5-HETE, 8-HETE, 15-HETE, 20-HETE, 11,12-DiHETrE and 14,15-DiHETrE significantly correlated with hepatic apoptosis marker ck-18 suggesting that they are potiental disease indicators of NAFLD.

6. Future Directions

For future studies, more participants would be ideal in each group. Increasing the sample size would boost the statistical power and decrease the probability of false negative findings. We would recruit at least 20 participants of each group so that we will get about 80% of power (alpha = 0.05, two-tail).

In order to better understand the mechanism of the immune reactions in the process of liver injury, we would recruit a group of NAFLD patients without any other metabolic syndrome. This will give us a sense of how NAFLD progresses without the interfer once of the systemic environment due to metabolic disorder. By comparing the differences in responses between patients suffering from NAFLD only and NAFLD patients with metabolic syndrome, we would be able to determine the impact of metabolic syndrome diseases on the progression of NAFLD.

Based on the alteration in PBMC cytokine production responses in NAFLD patients, we could measure more cytokines in terms of inflammation markers to have a comprehensive

cytokine map that is changed in NAFLD patient. Additionaly, we could isolate and culture monocytes, let them differentiate into macrophages and determine the phenotype of these macrophage to see if the differentiated macrophages are more M1 or M2 polarized.

In order to directly study immune cells in the liver, Kupffer cells from high fat, high sucrose induced NAFLD mice could be isolated and compared with those from same strain of mice with a normal diet. Kupffer cell cytokine production, chemokine production and chemokine receptor expression could be determined at baseline level and upon stimulation. Kupffer cells could also be co-cultured with fibroblasts to see their ability of activating fibroblast which is critical for liver fibrosis.

The mechanism of increased cytokine production of IL-1β and IL-6 could be determined at the molecular level to see if the signal pathways of transcription and/or translation is altered in NAFLD.

We could also determine the plasma levels and the levels of oxylipin produced by monocyte/macrophage similar to experiements described in this Thesisto better understand the role of oxylipins in NAFLD progression.

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