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Circulating peripheral blood Mucosal-Associated-Invariant T cells predict stages of liver fibrosis in patients with Non Alcoholic Fatty Liver Disease

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Dedication

I dedicate my thesis to my family, my husband, and my children, especially Amal, who all gave me unconditional love and support. To my parents whose love, encouragement and prayers made me able to get this thesis done. I also dedicate this work to my teachers, especially Dr. Rania Abu Seir, who helps and encourages me, to my supervisor Dr. Johhny Amer and friends at Al-Quds University and Hadassah Medical Center. Without all of you, the completion of this work would have not been possible.

Dania Bakri

Declaration:

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institute.

Signed:

Dania Issa Abd elftah Bakri

Date: 5\5\2018

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Abstract

Background: Mucosal-associated invariant T (MAIT) cells comprise a subpopulation of T cells that can be activated by bacterial products and cytokines to produce TNFα, IL-17, and granzymes. Little is known about the role of MAIT cells in fibrosis progression in fatty liver disease. **Aim**: To assess frequency and phenotype of MAIT cells and their correlation with liver fibrosis severities in NAFLD patients.

Methods: twenty five NAFLD patients and five healthy donors were recruited. Age, Body mass index, alanine aminotransferase, serum-fasting-insulin levels, hemoglobin A1c, homeostatic model assessment for insulin resistance score, triglycride, high density lipoprotein, low density lipoprotein and c-reactive protein were correlated with fibrosis-scoring in adult NAFLD cases lacking metabolic-syndrome or other liver etiologies. *In vitro,* peripheral blood samples were obtained from both the patients and healthy. Then using direct human T cell isolation kit, $CD3^+$ T cells were extracted. Using Flow cytometry, MAIT cells were identified by the expression of CD161 and TCR Vα7.2. For immune phenotyping, markers like TNFα, CD69, CD38 and IL17 were used. Then the serum cytokine levels were detected using quantibody human cytokine array 1. Using Flow cytometry, the expression of insulin receptor were determined in three patients with cirrhosis.

Results: a total of 25-cases fulfill inclusion/exclusion criteria, all were males, mean age at biopsy 39.3±9.8y, BMI 29.19±9. Seven had cirrhosis as their fibrosis stage were 4. Serum HOMA-IR found the most significant predictor for histological severity. Healthy donors had 12.68%±1.11 MAIT cells which was significantly decreased in peripheral blood of patients with NAFLD 10.62%±1.37, 3.9%±0.71, 1.66%±0.08 and 1.2%±0.21 with fibrosis grades of F1, F2, F3 and F4, respectively. Moreover, MAIT cells from patients showed higher expression of activation markers such as CD69 and CD38 as compared to heathy donors. MAIT cells had also elevated IL-17 expression, a pro-fibrogenic cytokine as well as TNFα, a pro-inflammatory cytokine. These results were linearly correlated with fibrosis severities. Singnificant elevation was founded in the inflammatory cytokines including IL-12 that known to activate MAIT cells. Downregulation in insulin receptor was observed in patients with cirhosis as compered with healthy donors.

Conclusions: Decreased expressions of peripheral blood MAIT cells in NAFLD patients were inversely correlated with the fibrosis stage. Our data suggest MAIT cells might contribute to the development of fibrosis in NAFLD and represent a novel diagnostic target.

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List of abbreviations

AST	Aspartate Transaminase
ALT	Alanine Transaminase
ABC	ATP binding cassette
APC	Allophycocyanin
BCL2	B Cell Lymphoma 2
BMI	Body Mass Index
CRP	c-reactive protein
CLOCK	Circadian Locomotor Output Cycles Kaput
CT	Computed Tomography
CRN	Clinical Research Network
CD	Cluster of Differentiation
CCR	C-C Chemokine Receptor
CXCR	C-X-C Chemokine receptor
CXCL	C-X-C Chemokine Ligand
DENV	Dengue virus
FFA	Free Fatty Acid
FITC	Fluorescein Isothiocyanate
FTO	Fat mass and obesity-associated
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
Gr	Granzyme
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HbA1c	Hemoglobin-A1c
HCV	Hepatitis C Virus
HLA-DR	Human Leukocyte Antigen- antigen D Related
HSC	Hepatic Stellate Cells
HIV	Human Immunodeficiency Virus
HMOX-1	heme oxygenase-1
IRS	Insulin Receptor Substrate
IFN-γ	Interferon γ
IgG	immunoglobulin G
MAIT	Mucosal Associated Invariant T
MHC	Major Histocompatibility Complex
MR1	MHC class I-Related protein 1
MRI	Magnetic Resonance Imaging
mAbs	Monoclonal Antibodies
MMP9	Matrix Metallopeptidase 9
NAFLD	Non Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic SteatoHepatitis
NAS	NASH Activity Score
NKG2D	Natural Killer Group2 member D
NOD	Non Obese Diabetic
NF-κβ	Nuclear Factor Kappa Beta
5-OP-RU	5-(2- oxoethylideneamino) -6-D-ribitylaminouracil
5-OE-RU	5-(2-oxopropylideneamino) -6-D-ribitylaminouracil

PD-1	Programmed Death-1
PBS	Phosphate Buffer Saline
PE	Phycoerythrin
PNPLA3	Patatin-Like Phospholipase Domain Containing 3
PerCP	Peridinin Chlorophyll Protein Complex
PB	Pacific Blue
PBMCs	Peripheral Blood Mononuclear Cells
PXR	Pregnane X Receptor
pg/ml	Picograms per Milliliter
PLZF	Promyelocytic Leukemia Zinc Finger
PMA	Phorbol 12-Myristate 13-Acetate
RORγt	Retinoic Acid-Related Orphan Receptor γt
ROS	Reactive Oxygen Species
Sags	Superantigens
STAT3	Signal Transducer And Activator Of Transcription 3
T-bet	T- Box Expressed in T cells
T2D	type2 diabetes
T1D	type 1diabetes
TCR	T Cell Receptor
TG	Triglycerides
TE	Transient Elastography
TNF-α	Tumor Necrosis Factor α
US	Ultrasonography
Vα7.2/ Jα33	Variable region α7.2, Joining region α33
Vβ13	Variable region β13

Chapter One

Introduction

1.1 Background

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disease that ranges from simple steatosis to the more progressive form terms as non-alcoholic steatohepatitis. NASH is the major cause of chronic liver fibrosis characterized by excessive extracellular matrix accumulation in liver cells which is accompanied by inflammation and injury and can lead to cirrhosis, liver failure and eventually carcinoma (Zhang *et al.*, 2018).

NAFLD is the most common chronic liver disease with estimated prevalence 6-35% worldwide (Cobbina & Akhlaghi, 2017). The prevalence of NASH is 3%-5% in the general population which is considered as the leading cause of morbidity and mortality from liver disease (Ashtari *et al.*, 2015). NAFLD is associated with metabolic syndromes such as obesity, diabetes, insulin resistance and dyslipidemia. It is plausible that the prevalence of NAFLD is increasing because of the obesity epidemic (Oh *et al.*, 2016).

A liver biopsy is the gold standard method for diagnosis of NAFLD. However, the sampling error is a major concern; especially that only 1/50000 of the whole liver tissue is sampled during a liver biopsy. Therefore, there is a need for the noninvasive method used for the detection of patients with NAFLD (Amer *et al.*, 2018).

The liver is the immunologic organ that is in continuous exposure to microbial and food antigens from the gut. The presence of MAIT cells in mucosal barrier, the highly enrichment of MAIT cells in the liver and their rapid antimicrobial function render MAIT cells a plausible population that might contribute to both the maintenance of liver and the inflammation of liver in disease (Bolte & Rehermann, 2018; Kurioka *et al.*, 2016).

MAIT cells represent the most abundant subset of non-conventional T cells. MAIT cells express semi-invariant T cell receptor (TCR Vα702-Jα33\12\20). Furthermore, MAIT cells are restricted to nonpolymorphic major histocompatibility complex (MHC) class Ib molecule, MHC class I-related protein 1 (MR1), that presented non-protein antigens, that include derivatives of riboflavin and folic acid synthesis pathways in bacteria, mycobacteria, and yeast. MAIT cells can be activated by TCR dependent manner as conventional T cell. Moreover, it can be activated like other innate T cells by cytokines dependent manner such as interleukin12 (IL-12) and IL-18. Upon activation, these cells express pro-inflammatory cytokines such as interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) and they release cytotoxic and pro-inflammatory granzymes, such as granzyme B and perforin (Kurioka *et al.*, 2016; Xiao & Cai, 2017)

MAIT cells have a wide tissue distribution, they comprise 5% of T cells in peripheral blood, 60% in the jejunum, and they present also in colon, lung, cervix, kidneys, ovaries, and others. MAIT cells represent the major T cell population in the liver in about 20%-50% of T cells, this high frequency suggests that they have a role in the immunity of liver and in the pathophysiology of liver inflammation in diseases (Gapin, 2014).

However, the circulating MAIT cells decreased in the obese patients and type 2 diabetes (T2D) patients, their activation is increased as defined by CD25 and CD69 expression and their production of IL-17, IL-2 and GrB are markedly increased (Magalhaes *et al.*, 2015b). MAIT cells have the ability to express IL-17 under the control of cytokines IL-1β, IL-23 and IL-7 that found to be released by the hepatic stromal cell, confirm the link between liver and MAIT cells (Ussher *et al.*, 2014).

1.2 Nonalcoholic fatty liver disease

1.2.1. NAFLD definition

Non-alcoholic fatty liver disease (NAFLD) is a spectrum disease defined by excessive fat accumulation in the form of triglycerides (steatosis) in more than 5% of hepatocytes that is not a result of alcohol consumption or other causes such as viral hepatitis or medications (Paschos & Paletas, 2009; Zhang *et al.*, 2018). Most NAFLD patients are asymptomatic but 30% of them progress to the aggressive form non alcoholic steatohepatitis (NASH) in which excessive steatohepatitis accompanied with liver cell injury and inflammation that characterized by ballooning, apoptosis/necrosis, Mallory's hyaline and giant mitochondria and eventually fibrosis (Khedmat & Taheri, 2011; Zhang *et al.*, 2018)

Non-alcoholic steatohepatitis (NASH) was firstly described in 1980 by Ludwig et al. (Pascale *et al.*, 2010). NASH firstly was considered as a benign condition until that more studies revealed that NASH can be progressive and may lead to cirrhosis and liver failure (Zhang *et al.*, 2018). NASH hepatic injury is similar to alcoholic fatty liver, however, NASH seems to progress more slowly and is histologically less severe than steatohepatitis caused by alcohol (Vernon *et al.*, 2011).

NAFLD is the most common chronic liver disease worldwide with estimated NAFLD prevalence in Western countries 20-30%, whereas 12%-24% in Asian countries (Ashtari *et al.*, 2015). 20% of NASH may progress to cirrhosis. NASH increases the risk of hepatocellular carcinoma and liver failure by 10-folds and doubling the risk of cardiovascular diseases (Zhang *et al.*, 2018). NASH increases the liver-related mortality by 3-fold than the general population (Pappachan *et al.*, 2017).

1.2.2. NAFLD etiology

There is no precise known etiology of NAFLD. However, the major risk factors of NAFLD development are well established, including age, gender, insulin resistance, type 2 diabetes mellitus, increased ferritin levels and genetic factors (Byrne & Targher, 2015). NAFLD has been reported in all age groups. However, a higher prevalence of NAFLD and fibrosis are reported with increasing age that might attribute to the risk factors present in elderly (Mishra & Younossi, 2012). Although there is a discrepancy between studies in the

predominant gender, NAFLD is found to occur more frequently in men (Khedmat & Taheri, 2011).

Metabolic syndrome is defined by the presence of three or more of the following factors: elevated waist circumference, elevated triglycerides or on drug treatment for elevated triglycerides, reduced HDL level, hypertension or on antihypertensive drug treatment, Type 2 diabetes, Family history of diabetes, overweight, cardiovascular complications, impaired fasting glucose or on antidiabetic drug treatment and insulin resistance (Paschos & Paletas, 2009). There is a strong association between NAFLD and metabolic syndrome. Therefore, the prevalence of NAFLD and NASH are increasing as obesity became an epidemic worldwide (Vernon *et al.*, 2011).

1.2.3. NAFLD pathophysiology

The second hit hypothesis was firstly proposed in which hyperglycemia and insulin resistance lead to steatosis, is the first hit, and the oxidative stress and pro-inflammatory cytokines can lead to hepatocyte apoptosis, recruitment of inflammatory cells and may progress to fibrosis, is the second hit (Khedmat & Taheri, 2011; Zhang *et al.*, 2018). However, the pathogenesis of NAFLD was observed to be more complex and depending on genetic, nutritional and environmental factors. The development of NAFLD and progression of NASH is based on the timing and combination of the factors (Buzzetti *et al.*, 2016).

Abnormal steatosis in hepatocytes is an essential process in the pathgenesis of NAFLD. The source of these lipids is from the systemic circulation, de novo synthesis, reduced degradation and reduced export from hepatocytes. Storing lipids as triglycerides is not directly hepatotoxic, whereas diacyl glycerols, cholesterol, phosphatidylcholines, and certain saturated fatty acids (FAs) are particularly toxic (Kara *et al.*, 2018).

Insulin resistance (IR) is a key factor in NAFLD development. Insulin resistance increases adipocyte lipolysis by decreasing its inhibitory action on hormone-sensitive lipase in adipose tissue, leading to increasing free fatty acid (FFA) in circulation, thus enhance liver uptake of FFA (Paschos & Paletas, 2009). Hyperinsulinemia enhances de novo lipogenesis and inhibiting β oxidation that lead to lipid accumulation in the liver (Buzzetti *et al.*, 2016).

In addition, insulin resistance reduces plasma adiponectin levels that increase insulin sensitivity and reduce inflammation and increase adipocyte cytokine leptin that plays a role in reducing body weight and fat mass (Pappachan *et al.*, 2017). Homeostatic model assessment for insulin resistance (HOMA-IR) is a sensitive and specific method for measuring IR. HOMA–IR measurement based on serum fasting glucose and insulin levels (Buzzetti *et al.*, 2016).

Steatosis activate the transcription factor nuclear factor kappa b (NF-κβ) which lead to increase the production of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL-6) and IL-1β which results in the recruitment and the activation of kupffer cells, thus lead to inflammation condition (Cobbina & Akhlaghi, 2017).

Other potential process may interfere with the pathogensis of NAFLD is the alterations in the composition and functions of the gut microbiota have been found to be associated with NAFLD development (Pappachan *et al.*, 2017). Gut microbiota can activate toll- like receptor 4 (TLR-4) which is an activator to NF- κβ and can also intervene in the metabolism of the secondary bile acids such as deoxycholic acid that found to be associated with the pathogenesis of hepatocellular carcinoma (Byrne & Targher, 2015).

Mitochondrial dysfunction which may happen as a result of excess fatty acid in the liver, leads to the formation of lipid-derived toxic material and overproduction of reactive oxygen species (ROS) in which they can activate kupffer cell and hepatic stellate cell causing necrosis, inflammation, and fibrosis (Buzzetti *et al.*, 2016).

Studies have identified many genetic changes that may be associated with the susceptibility and progression of NAFLD. Among these are polymorphisms involved in the circadian rhythm (CLOCK transcription factor), the signal transducer and activator of transcription 3 (STAT3), the multidrug resistance-associated protein gene (ABCC2), the nuclear pregnane X receptor (PXR), heme oxygenase-1 (HMOX-1), Fat mass and obesity-associated (FTO) and PNPLA3 gene (Pappachan *et al.*, 2017; Sookoian & Pirola, 2017). PNPLA3 gene is found in adipocytes and hepatocytes. It is thought to help regulate the lipogenesis and lipolysis and the development of adipocytes. The PNPLA3 gene variation associated with NAFLD is thought to lead to increased production and decreased breakdown of fats in

the liver. Research is ongoing to determine how the genetic changes contribute to the development of NAFLD and its complications (Kara *et al.*, 2018).

1.2.4. NAFLD diagnosis

A liver biopsy is the gold standard method for staging the disease. However, due to its invasiveness, procedure complications, histopathologist variation and the cost, many other methods were found to diagnose NAFLD (Torres & Harrison, 2008). Liver aminotransferase AST and ALT levels are the most commonly used methods for screening of NASH. The ALT levels are higher than the AST levels in most cases, however, many individuals with severe NASH have more elevated AST serum level than ALT (Khedmat & Taheri, 2011). Other non invasive method is serological markers, such as serum autoantibodies. Antinuclear antibodies are strongly associated with insulin resistance, reactive oxygen species (ROS), adiponectin and leptin as markers for insulin resistance, increased c-reactive protein (CRP), cytokeratin 18 marker for hepatic apoptosis. Moreover, imaging techniques are commonly used in NAFLD diagnosis such as ultrasonography (US) which has high sensitivity, but without accurate staging of steatosis and with obesity decreasing sensitivity of the method. Computed tomography CT is accurate and sensitive, however, cannot distinguish simple steatosis from steatohepatitis. Magnetic resonance imaging (MRI) is the most accurate method for diagnosis and quantification of hepatic steatosis, with specificity, sensitivity and safety of MRI making it an ideal methodology to assess and monitor hepatic steatosis. The limitations are the cost and the inability to distinguish between NASH and NAFLD. Transient elastography (TE) provides an estimation of liver stiffness and it is accurate in diagnosis of severe liver fibrosis but not in the early stages. These methods are unreliable in the differentiation between NAFLD and NASH (Paschos & Paletas, 2009; Torres & Harrison, 2008) .

1.2.5. NAFLD scoring system

To predict the progression of liver fibrosis, scoring systems using a combination of clinical and laboratory parameters were established such as BAAT system based on BMI, age, ALT and serum TG. Fibrotest that used markers of collagen synthesis and degradation, including α2-macroglobulin, Haptoglobin, total bilirubin and apolipoprotein A1 and NAFLD fibrosis score used age, body mass index, platelet count, albumin level and AST\ALT ratio (Oh *et al.*, 2016; Torres & Harrison, 2008).

A liver biopsy is a reliable method for diagnosis, staging and predicts the prognosis of NASH. Systems to standardize the interpretation of the histological diagnosis were established (Oh *et al.*, 2016). Burnt system, classified NAFLD into three types: mild, moderate and sever based on steatosis, hepatocellular ballooning degeneration, lobular and portal vein inflammation. In addition, this system classified liver fibrosis into four stages (Oh *et al.*, 2016). Activity Score (NAS), proposed by the NASH Clinical Research Network (CRN) is calculated by the sum of scores of steatosis (0-3), lobular inflammation (0-3) and hepatocyte ballooning (0-2) with final score ranging from 0 to 8. If NAS score was less than 3 then it is correlated with non-NASH, whereas score of 3-5 is considered suspicious NASH and more than 5 is definitive NASH (Juluri *et al.*, 2011).

1.2.6. NAFLD treatment:

There is no fully effective treatment for NAFLD. However, to improve the steatosis and prevent the progression, treatment of the underlying risk factors are intervened, including lifestyle intervention such as weight loss, increase physical activity, diet (Pappachan, Babu, Krishnan, & Ravindran, 2017). A 7%-10% weight loss is required to improve hepatic inflammation but the minority of patients are able to do (Zhang *et al.*, 2018). Antioxidants drug, insulin-sensitizing drugs such as Metformin, thiazolidinediones, and lipid-lowering drugs are used to improve the metabolic conditions (Torres & Harrison, 2008).

1.3 Mucosal-associated invariant T (MAIT) cells

1.3.1. MAIT cell discovery

Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T lymphocytes, first described in 1993 by Porcelli et al. as a new T cell population expressed invariant TCR-α chain Vα7.2Jα33 (Gapin, 2014; Napier *et al.*, 2015; Xiao & Cai, 2017). In 1999, Treiner et al. observed that these cells are conserved within mammalian species. Later, in 2003, Treiner et al. gave the name MAIT cells for them as they found that they are enriched at the mucosal tissue, including the gut lamina propria. They also described the monomorphic class I-related MHC molecule, MR1, as the restricted antigen presenting molecule for MAIT cells (Xiao & Cai, 2017). Two studies in 2010, Le Bourhis et al. and Gold et al. showed the antimicrobial activity of MAIT cells against bacteria and fungi (Gapin, 2014; Howson *et al.*, 2015; Kurioka *et al.*, 2016; Napier *et al.*, 2015; Reantragoon

et al., 2016). In 2012, the discovery of MR1 ligands by Kjer-Nielsen et al. which were vitamin B_2 pathway metabolites, led to conclusion that MAIT cells are activated by microbes possess the riboflavin pathway (Chiba *et al.*, 2017; Hinks, 2016; Howson *et al.*, 2015).

1.3.2. MAIT cell an innate-like T lymphocyte

MAIT cells are non-conventional T cells act as a bridge between the adaptive and innate immunity. Like conventional T cells, they are TCR and MHC dependent cells, however, they express a semi-invariant TCR-*α* chain that has a limited diversity, and they are restricted to non-polymorphic MHC class I-related molecule, MRI, that presents vitamin B_2 derivatives unlike to conventional T cells that are activated by peptide antigen presented by MHC-I or MHC-II (Brozova *et al.*, 2016; Howson *et al.*, 2015; Xiao & Cai, 2017). MAIT cells like conventional T cells undergo TCR rearrangement and positive selection in thymus but unlike conventional T cells that require an antigenic activation in the periphery to gain the effector function. MAIT cells acquire it in the thymus that makes MAIT cells similar to innate immune cells in the speed of response (Napier *et al.*, 2015). Despite that MAIT cells are developed in the thymus, they have a distinct development pathway from the conventional T cells (Chandra & Kronenberg, 2015). Phenotypically, MAIT cells egress the thymus naïve as the conventional T cells then in the periphery they gain the memory phenotype (Howson *et al.*, 2015; Napier *et al.*, 2015). MAIT cells also produce pro-inflammatory cytokine and cytotoxic molecules upon activation in similar to conventional $CD8^+$ effector T cells (Chandra & Kronenberg, 2015; Napier *et al.*, 2015).

1.3.3. MAIT cell phenotype

MAIT cells are subset of αβ T cells characterized by a semi-invariant TCR-*α* chain with highly abundant in human Vα7.2-Jα33 although Vα7.2-Jα12 or Vα7.2-Jα20 are also found by a minority of MAIT cells, and associated with β chains predominance with Vβ13 and Vβ2 (Brozova *et al.*, 2016; Kurioka *et al.*, 2016; Xiao & Cai, 2017). This invariant TCR and MR1 are conserved in mammalian species (Xiao & Cai, 2017).

MAIT cells have a naïve phenotype after migration from thymus, in the periphery they exhibit memory phenotype CD45RO+, CCR7-, CD62Llo, $CD95^{hi}$, $CD122^{int}$, $CD127^{hi}$, this memory phenotype is termed as effector memory-like due to the absent of CD62L and

CCR7, and the quick response to stimulation that resemble to conventional effector memory T cells (Berkson & Prlic, 2017; Howson *et al.*, 2015; Kurioka *et al.*, 2016; Napier *et al.*, 2015; Reantragoon *et al.*, 2016; Ussher *et al.*, 2014).

MAIT cells also express the transcription factor promyelocytic leukemia zinc finger (PLZF) which is responsible for the innate-like effector function in nonconventional T cells (Berkson & Prlic, 2017; Napier *et al.*, 2015). Other transcription factors express on MAIT cells are Helios, Eomes, T-bet that associated with IFN-γ production and retinoic acid-related orphan receptor (ROR) γt which associated with the ability to produce interleukin-17 (IL-17) (Kurioka *et al.*, 2016; Ussher *et al.*, 2014). NKG2D is transcription factor also express by MAIT cells that activate NK receptor and enhance the cytotoxicity in NK cell (Brozova *et al.*, 2016).

The high and early expression of the C-type lectin-like receptor is used in the definition of MAIT cells with co-expression of TCR α chain Vα.7.2+ CD161++ in many studies (Kurioka *et al.*, 2016). MAIT cells can also be defined by the unique high expression of IL-18R (Ussher *et al.*, 2014). MAIT cells also express cytokine receptors, including IL-7α, IL-12R, IL-18R, IL-2Rβ and IL-23R, activation markers such as, CD25, CD69 and CD38, and tissue homing receptors such as, CCR2, CCR5, CCR6, CXCR6, CCR9, CD103 and α4β7 integrin which indicate their ability to migrate to tissue as well as to the site of infection(Howson *et al.*, 2015; Kurioka *et al.*, 2016; Reantragoon *et al.*, 2016; Xiao & Cai, 2017). The proliferation marker Ki67 is found to be expressed by MAIT cells which indicate the proliferation ability of MAIT cells in the presence of IL-1 and IL-8 (Reantragoon *et al.*, 2016).

MAIT cells have three subsets: the predominance CD8+ with 95%of MAIT cell express the homodimer form CD8αα than CD8αβ unlike to the CD8+conventional T cell that expresses CD8 αβ only (Brozova *et al.*, 2016; Kurioka *et al.*, 2016). Other subsets are double negative DN and only 2-11% of MAIT cells in blood express CD4 (Kurioka *et al.*, 2016; Xiao & Cai, 2017). These subtypes are shown to have discrepancies in their frequency and function. As DN MAIT cells are more resistant to aging than CD8+ MAIT cells, but CD8+ MAIT cells produce more INF-γ and IL-17 than DN MAIT cells. Moreover, the CD8+ MAIT cells, mainly produce Th1 cytokine such as INF-γ, whereas

CD4+ subset produces both Th1 and Th2 cytokine, like INF-γ and IL-4 (Brozova *et al.*, 2016; Lee *et al.*, 2014). MAIT cells are a heterogeneous group in their phenotype and activation response that might render to the diversity of β chain, despite the early consideration that MAIT cells are homogeneous group due to the conserved MR1 and the limited ligands are known to date (Dias *et al.*, 2017). It is also found that the circulating MAIT cell frequencies increased with age to reach the adult levels, then decline with age irrespective of the gender (Lee *et al.*, 2014).

1.3.4. MAIT cells tissue distribution

The expression of the chemokine receptor CCR9, CXCR6 and gut homing integrin α4β7 and $CCR9^{lo}$, conferring MAIT cells' ability to home in tissue especially in the liver, lung, and intestine. Due to the lack of CCR7 and CD62L expression, MAIT cells are not qualified to home in lymph nodes (Kurioka *et al.*, 2016).

MAIT cells are enriched in the intestine with different frequency in different anatomical location, a higher frequency found in the jejunum of about 60%of T cells, colon (10% of T cells), and lower frequency in ileum and rectum. MAIT cells also enriched in the liver with about 20-50% of T cells, in the blood of 1-10% of T cells, in lung, kidney, prostate, ovaries (Howson *et al.*, 2015; Kurioka *et al.*, 2016).

It was found that MAIT cells differ in their phenotyping according to the tissue. For instance, a study about the analysis of the α-chains found out that in tissue the predominance was Vα7.2-Jα12, whereas Vα7.2-Jα33 was in the blood (Howson *et al.*, 2015). Another study revealed that fetal mucosal tissue and the female genital tract MAIT cells produce more IL-17 and IL-22 than MAIT cells in blood when stimulated with either bacteria or phorbol 12-myristate 13-acetate (PMA) /ionomycin (Berkson & Prlic, 2017; Pappachan *et al.*, 2017). Moreover, almost all liver MAIT cells found to express high levels of the activation marker CD69, CD38, and HLA-DR and be more active than those in blood, this reflects the continuous antigen exposure (Howson *et al.*, 2015; Kurioka *et al.*, 2016).

1.3.5. MAIT cell activation

MAIT cells are functionally defined as innate-like T cells that can rapidly produce pro-inflammatory cytokine mainly Th1 type cytokines (INF-γ, TNF-α) and Th-17 type cytokines (IL-17, IL-22), and cytolytic products such as granzymes mainly GrA, K, B and perforin which give them the ability to kill the infected cells (Howson *et al.*, 2015; Kurioka *et al.*, 2016; Xiao & Cai, 2017). They also have the ability to help B cells (Xiao & Cai, 2017). Since MAIT cells produce IL-17 and IL-22, it is suggested that they have a role in epithelial integrity and tissue homeostasis (Berkson & Prlic, 2017).

MAIT cells can be activated by the MR1 dependent manner in which antigen-presenting cells present metabolites derived from the riboflavin biosynthetic pathway of bacteria and yeast interact with MHC class I-related protein (MR1). Therefore, MAIT cells are activated by bacteria and yeasts possessing the riboflavin synthetic pathway, including *Mycobacterium. abscessus, E.coli, Salmonella. enterica, Klebsiella. pneumonia, Staphylococcus. aureus, Staphylococcus. epidermidis, Pseudomonas. aeruginosa, Lactobacillus. acidophilus, Candida. glabrata, C. albicans, and Saccharomyces. Cerevisiae (Kurioka et al., 2016).* MR1 widely expressed across a various cells including professional antigen presenting cells, epithelial cells and monocytes (McWilliam & Villadangos, 2017; Riva *et al.*, 2018). The majority of MR1 in the absence of ligand resides in the endoplasmic reticulum ER, and in the presence of the ligand, MR1 has a conformational change, including binding to β2-microglobulin and then the complex MR1-β2-microglobulin-ligand egress to the cell surface to present the legend to MAIT cells (McWilliam & Villadangos, 2017). MR1 present the pyrimidine intermediates from the riboflavin pathway after the condensation with glyoxal and methylglyoxal molecules derived from metabolic pathways including glycolysis (Keller *et al.*, 2017). The most potent MAIT cells activating ligands are pyrimidine compounds 5-(2-oxopropylideneamino) -6-D-ribitylaminouracil (5-OP-RU) and 5-(2- oxoethylideneamino) -6-D-ribitylaminouracil (5-OE-RU) (McWilliam & Villadangos, 2017). MR1 can present molecules derived from the folic acid pathway, in addition to riboflavin pathway intermediates, however, these molecules do not activate MAIT cells. Drugs and drug-like molecules can also be presented by MR1 and can affect the function of MAIT cells (Keller *et al.*, 2017). A study by Dias et al. suggest that MAIT cells can adjust their response to fit the different antigenic microbes. In this study they found a different response of MAIT

cells toward *E.coli* and *C.albicans*, in which the response against *E.coli* showing most rapid production of INF-γ and TNF-α than did in *C.albicans* (Dias *et al.*, 2017).

MAIT cells also shown to be activated in bacterial and viral infections which lack the riboflavin pathway, and in the non-infectious diseases that confirm the activation of MAIT cells through MR1 independent manner. MAIT cells can be stimulated by IL-18 in synergy with other inflammatory cytokines such as IL-12, IL-15, IL-7 and IL-23 (Kurioka *et al.*, 2016; Wong *et al.*, 2017; Xiao & Cai, 2017). Upon activation of MAIT cells in viral infection, they are found to produce copious amounts of INF-γ, an antiviral cytokine, TNF-α which have an antiviral activity such as reduce spreading of hepatitis C virus (HCV) and granzyme B that license cell for the cytotoxic function (Ussher *et al.*, 2018). For the acquisition of the effector function of MAIT cells in a MR1 dependent manner, the synergy between the inflammatory cytokine signals and TCR signals is needed for efficient effector response. This synergy is critical since MAIT cells present in mucosal tissue that have commensal bacteria may produce the riboflavin metabolites. However, activation through inflammatory cytokine is sufficient to induce the effector function of MAIT cells (Berkson & Prlic, 2017; Slichter *et al.*, 2016).

A new study by Shaler et al. revealed that the activation of MAIT cells can be triggered by superantigens (SAgs) produced by *Staphylococcus. aureus* and *Streptococcus. pyogenes* in TCR Vβ dependent manner and more predominantly through cytokine manner mainly through IL-18 and IL-12 by producing INF-γ, TNF-α, and IL-2 but not IL-17. They also found that MAIT cell responsiveness to SAgs is more rapid and higher than conventional T cells and other non-conventional T cells including invariant natural killer T (iNKT) cells and γδ T cells. However, they found that the hyperinflammatory condition of MAIT cells toward SAgs renders them to be anergy which means they become unresponsive to bacterial Ag-MR1complex. These findings demonstrate the role of MAIT cells in antimicrobial immunity and the potency in utilizing MAIT cells in therapy of SAgs related diseases (Sandberg *et al.*, 2017; Shaler *et al.*, 2017).

A newly revealed function of MAIT cells is the ability to help B cells by inducing B cell plasmablast and antibody production. A study by Micheal et al. found that MAIT cells upon activation release cytokines promote this function including, IL-2 which induce B-

cell differentiation and proliferation, IL-6 that enhance production of IgG, IL-10 promote B-cell survival, proliferation, and antibody production, IL-21 promotes B-cell proliferation and IFN-γ augment antibody production and entry into the S phase in human B cells (Bennett *et al.*, 2017).

1.3.6. MAIT cells in diseases

Many studies suggestes the important roles of MAIT cells in diseases, including infectious and noninfectious diseases. In 2010, Le Bourhis et al. and Gold et al. studies gave the first evidence in the antimicrobial role of MAIT cells. These studies found that in *M.tuberculosis* infection, the circulating MAIT cells were lower than the healthy subjects. Le Bourhis et al. study showed that MAIT cells frequency in ascites of a tuberculosis patient is higher than those in ascites from patients with malignancy. These findings suggest that MAIT cells might migrate to the site of infection (Le Bourhis *et al.*, 2010; Reantragoon *et al.*, 2016).

In addition, Grimaldi et al. found that the early depletion in the frequency of peripheral MAIT cells in intensive care unit patients with sepsis was associated with increased susceptibility to secondary infection (Grimaldi *et al.*, 2014). In Booth et al. *in vitro* study, MAIT cells were found to be less frequent in the blood of *H.pylori* patients than in healthy individuals, and MAIT cells of patients were able to release Th1 and Th2 cytokines and to kill infected macrophages (Reantragoon *et al.*, 2016). Another study on patients with scrub typhus infection caused by *Orientia.tsutsugamushi* revealed a decrease in the frequency of MAIT cells in the blood of patients compared to healthy which correlate with the disease severity (Berkson & Prlic, 2017).

Although MAIT cells are activated through recognition of MR1 presenting vitamin B2 metabolites which are lacking in viruses, MAIT cells have the antiviral activity through a combination of IL-18 and other pro-inflammatory cytokines. An *in vivo* and *in vitro* study by Wilgenburg et al. revealed the ability of MAIT cell to be activated by dengue virus (DENV), hepatitis C virus (HCV) and influenza virus in MR1-independent manner (van Wilgenburg *et al.*, 2016). Many studies showed the depletion in peripheral MAIT cell frequency in the blood of human immunodeficiency virus (HIV) patients. Moreover, circulating MAIT cells in HIV patients have a reversible dysfunction that restored after

anti-retroviral therapy (Reantragoon *et al.*, 2016). Bolte et al. found that the frequency of MAIT cells was lower in the blood and liver of HCV patients when compared with controls. In addition, they found that the frequency of liver MAIT cells in those patients was inversely correlated with liver inflammation and fibrosis (Bolte *et al.*, 2017).

MAIT cells also play a potent role in the non-infectious diseases that proved in many studies. The frequency of peripheral MAIT cells in ankylosing spondylitis found to be lower than the healthy controls. However, synovial fluid of ankylosing spondylitis patients have a higher frequency of MAIT cells than in healthy controls, which reflect their recruitment to the inflamed joints. In addition, they also found that MAIT cells in synovial fluid highly express IL-17 phenotype in response to IL-7 activation (Gracey *et al.*, 2016). Chiba et al. suggest that MAIT cells frequency is reduced in peripheral blood of patients with systemic lupus erythematosus, which may occur due to activation-induced cell death. Moreover, they observed that MAIT cell activation reflected the disease severity (Chiba *et al.*, 2017). The depletion in the circulating MAIT cell frequency that was associated with the enrichment of MAIT cells in the inflamed tissue was observed in many conditions, including inflammatory bowel disease, multiple sclerosis, alcoholic liver disease and nonalcoholic steatohepatitis (Gapin, 2014). It is plausible that MAIT cells may have a role in cancer as they can produce INF-γ which enhance tumor-specific T cell responses and IL-17 that promote the expansion and accumulation of immunosuppressive neutrophils and myeloid-derived suppressor cells (Kurioka *et al.*, 2016). In addition, MAIT cells found to express the ATP binding cassette (ABC) B1 drug resistance transporter which allows MAIT cells to efflux drugs and to persist during chemotherapy of acute myeloid leukemia or breast cancer (Ussher *et al.*, 2014).

1.3.7. MAIT cells in diabetic and obese patients

Due to MAIT cell's location in mucosal tissue that makes them in close to the microbiota, and the fact that MAIT cells depend on microbiota in their development, it was expected that MAIT cells may have alterations in dysbiotic condition (Constantinides, 2018). MAIT cell share the ability to maintain the gut integrity and homeostasis through the production of IL-17 and IL-22. Alteration in microbiota is observed in patients with type 1diabetes (T1D), type2 diabetes (T2D) and obesity that could lead to indirectly induce MAIT cell activation. In addition, the plasma levels of methylglyoxal, a molecule that condenses with

an intermediate of riboflavin pathway to form pyrimidines which are the most potent MAIT cells activating ligands, are found to be elevated in T1D and T2D patients (Magalhaes *et al.*, 2015a).

Rouxel et al. study revealed a decrease in MAIT cell frequency in the blood of children with recent onset of T1D when compared to healthy children. This depletion might be due to the migration of MAIT cells to the site of infection, in this case, is the pancreas. They also showed the expression of activation and exhaustion markers including, CD25, programmed death-1 (PD-1) and BCL2. Moreover, they also found an increase in the frequency of MAIT cells in the pancreas of nonobese diabetic (NOD) mice, as well in their production of INF-γ and granzyme B which was found to directly contribute to pancreatic β cell killing (Rouxel *et al.*, 2017).

In T2D and obesity, circulating MAIT cells were found to be reduced in frequency, but had a higher IL-17 production. In addition MAIT cells in adipose tissue of obese patients showed a higher frequency and activation phenotype represented by the upregulation of CD25 and CD69 expression, as compared to lean controls. Similary, IL-17 production in adipose tissue of obese patients was increased in correlation with insulin resistance that might participate to the obesity-related inflammation and insulin resistance (Carolan *et al.*, 2015; Magalhaes *et al.*, 2015b). After Bariatric surgery, the frequency and function of MAIT cells in peripheral blood of obese patients was found to be restored (Magalhaes *et al.*, 2015b).

1.3.8. MAIT cells in liver

The liver is the immunologic organ that is in continuous exposure to microbial and food antigens from the gut. The presence of MAIT cells in mucosal barrier, the high enrichment of MAIT cells in the liver and their rapid antimicrobial function render MAIT cells a plausible population that might contribute to both maintenance of liver and inflammation of liver in diseases (Bolte & Rehermann, 2018; Kurioka *et al.*, 2016).

MAIT cells express the tissue homing chemokine receptors CCR6, CXCR6, and the integrin αEβ7 in health and disease condition. However, in inflammation the intrahepatic MAIT cells expresses a higher level of CXCR3, a ligand for IFN inducible chemokine

CXCL9, CXCL10 and CXCL11 that are upregulated on inflamed liver sinusoids (Jeffery *et al.*, 2016). Upon liver inflammation, MAIT cells can be activated by IL-18 that present at high levels in the inflamed liver, and by the MR1 dependent manner in which biliary epithelium cells can act as non-professional antigen presenting cells in addition to macrophages and liver B cells to release IFN-γ, TNF-α, IL-17 and granzyme B that drive killing of infected cells (Jeffery *et al.*, 2016).

In a study of MAIT cells in alcoholic liver disease, Riva et al. have reported depletion in blood MAIT cells accompaned with increasing the expression of the activation marker and impaired antimicrobial function that correlated with the disease severity. In addition, they refer this observation to the dysbiosis and loss of gut integrity that was well characterized in alcoholic liver disease patients (Riva *et al.*, 2018). In chronic liver diseases, MAIT cells exhibit reduction in their frequency and impaired antimicrobial function which reduces of the liver immunity function and increase of bacterial infection susceptibility a major cause of mortality in cirrhosis (Bolte & Rehermann, 2018). Tang and colleagues found that MAIT cells function is regulated by IL-1, IL-23 and-7 that is secreted by hepatocytes during liver fibrosis. IL-17^{+} MAIT cells found to play a role in the pathogenesis of various chronic diseases, including alcoholic, metabolic, viral and autoimmune liver disease (Toubal & Lehuen, 2016). Bottcher et al. findings showed that MAIT cells induce hepatic stellate cells (HSC) proliferation and activation in which HSC produced a large amount of extracellular matrix protein that contributes to fibrosis. In addition, they found that this pro-fibrogenic role of MAIT cells is dependent on IL-17 and cell-cell contact between MAIT cells and HSCs (Bottcher *et al.*, 2018). For their phenotype, function, and contribution in diseases, MAIT cells represent novel therapeutic, diagnostic and prognosis marker that is under investigation.

1.4 Problem Statement

NAFLD is a spectrum disease range from simple steatosis to steatosis with liver injury and inflammation that is termed as NASH. NASH has complications of cirrhosis and hepatocellular carcinoma (HCC). Around 13%of HCC is developed from NAFLD disorder (Paschos & Paletas, 2009). The prevalence of disease is rapidly increasing since it is associated with metabolic syndrome, especially obesity, diabetes mellitus and insulin resistance. In all stages of fibrosis, the disease is asymptomatic and lab test doesn't

necessary reflect liver injury as ALT could only reflect acute liver disease and its levels are reduced along disease progressions (Oh *et al.*, 2016). MAIT cells may play a role in the inflammation of the liver. However, the intervention of MAIT cells in the progression of NAFLD has not been explored. Thus, MAIT cells create an area for research to be used as diagnostic, prognostic and prospectively therapeutic tool.

1.5 Study justification

A liver biopsy is the gold standard method for the diagnosis of NAFLD. However, it has some limitations: It is an invasive method that holds a pain and a high risk to the patients . The cost of liver biopsy is high. The sample taken could lack accuracy and may not reflect the stage of the disease Inexperience pathologist (Pappachan *et al.*, 2017). The research currently aims to focus on a MAIT cell as a cell population marker with good diagnostic and prognostic approach. MAIT cells are an attractive target that may interfere with the pathogenesis of NAFLD disease. MAIT cells are still not studied thoroughly and their function is not fully understood.

1.6 Study Aims

- To determine the frequency and phenotyping characteristics of MAIT cells in different stages of fibrosis in NAFLD patients as compared with healthy donors.
- To evaluate the possibility of MAIT cells to predict the severity of liver fibrosis in NAFLD patients.

1.7 Hypothesis

- MAIT cells could be of a great significance in the diagnosis of NAFL disease and in the prediction of liver fibrosis stage in patients with NAFLD.

Chapter Two

Materials and Methods

2.1 Materials

Materials and chemicals used in the experiment are:

1. Sodium Citrate blood collection tubes or heparin blood collection tubes
2. Plain blood collection tubes
3. Phosphate buffer saline (PBS) (biological industries)
4. Conical tubes
5. 5mL polystyrene plastic tubes to apply in a flow cytometer
6. Centrifuge
7. Pipettes
8. Ficoll-paque (isolation of mononuclear cells from human peripheral blood by density gradient centrifugation provided from Pharmacia)
9. Easy step direct human T cell isolation kit (Miltenyi Biotec)
10. EasyEights magnets (Catalog#18103) or the Big Easy magnet (Calalog#18001)
11. Formaldehyde for fixation
12. 0.1% saponine for permeabilization
13. Monoclonal antibodies (mAbs):
    I. Phycoerythrin(PE)-Cy7-A- conjugated anti TCR Vα7.2 (Miltenyi Biotec)
    II. Fluorescein isothiocyanate(FITC)-A- conjugated anti CD161(R&D systems USA)

III. Peridinin chlorophyll protein complex (PerCP)- conjugated anti CD69 (R&D systems USA)
IV. Pacific blue (PB)- conjugated anti tumor necrosis factor α (TNFα) (R&D systems USA)
V. Allophycocyanin (APC) -A- conjugated anti interleukin 17 (IL-17) (R&D systems USA)
VI. Allophycocyanin(APC) conjugated anti CD38 (R&D systems USA)
VII. Peridinin chlorophyll protein complex (PerCP)- conjugated anti CD45 (R&D systems USA)
VIII. Pacific blue (PB)- conjugated anti CD3 (R&D systems USA)
IX. Allophycocyanin (APC) -A- conjugated anti interleukin 17 (IL-17) (R&D systems USA)
X. Allophycocyanin (APC) conjugated anti insulin receptor (R&D systems USA)
XI. An Isotype immunoglobulin G (IgG) for each antibody labeled with the corresponding fluorochrome used as control (from the same company of each antibody)

14. BD LSR Fortessa (flow cytometer)
15. Aluminium foil
16. Ice
17. Quantibody human cytokine array1 kit (RayBiotech, Inc. Cat#QAH-CYT-1)
18. Distilled water
19. Polypropylene Microcentrifuge tubes

2.2 Methods

To summarize the methodology that was used, figure 2.1 is illustrated the design of the study.

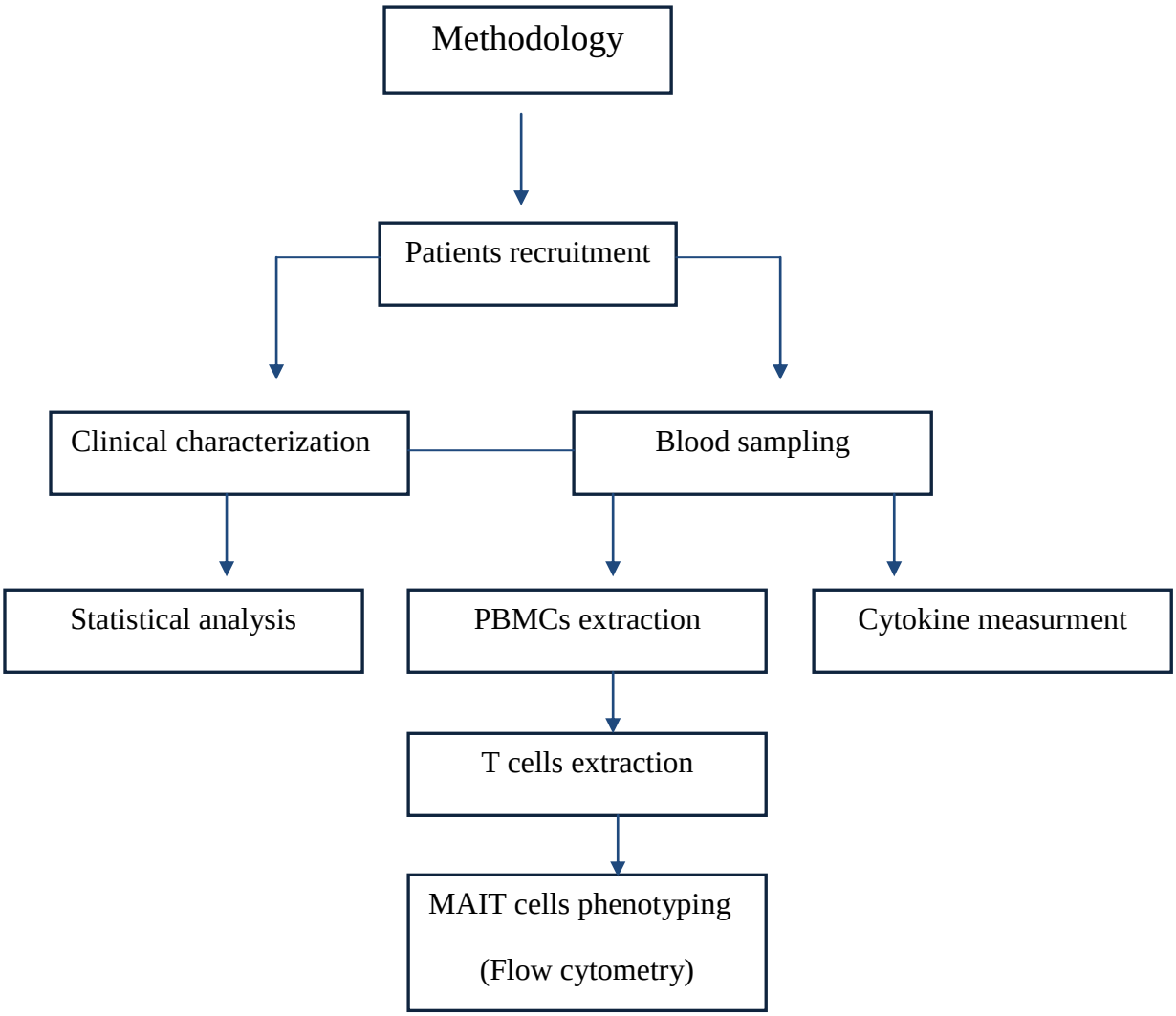

Figure (2.1) Flow chart for the methodology that was used.

2.2.1 Study Population

A stratified convenient sample of 25 histologically documented NAFLD cases with mean of 39.9±9.8 years and 5 healthy controls subjects with maen of 31±1.4 years were collected at Hadassah hospital between November2016 to July 2017. Additionally, three patients with cirrhosis and three healthy donors were recruited in January 2018. All the participiant were men to avoid the gender bias.

Only NAFLD-patients were off therapy at the time of liver biopsy are included to avoid their phenotypic and functional effects on lymphocytes. Patients on steroid administration, recent use of warfarin, metformin, thiaglitazones and insulin for more than one month or any use in last 2 months, or daily alcohol intake >20 gm/day were excluded. Also cases with other competed etiology of liver disease, including viral hepatitis, autoimmune disease, hemochromatosis, Wilson's disease, alpha-1-antitrypsin disease, alcohol or any toxicity were excluded.

2.2.2 Ethical considerations

All subjects who agreed to precipitate were informed briefly about the subject and all signed approval consent (appendix 1) in conformity with the declaration of Helsinki.

2.2.3 Clinical characterization

Body mass index (BMI), serum cholesterol (LDL and HDL) and triglycerides (TG), serum levels of alanine aminotransferase (ALT), hemoglobin-A1c (HbA1c) levels, insulin resistance (calculated according to Homeostasis Model Assessment HOMA) and serum insulin levels were held from the patients' files in the hospital. All biopsies were assessed for the fibrosis severity using NAFLD activity score (NAS) and fibrosis score (Metavir) by pathologist.

2.2.3.1. Homeostasis Model Assessment –insulin resistance HOMA-IR

HOMA-IR is the most sensitive and specific method for measuring insulin resistance {Barseem, 2015 #11}. HOMA-IR was calculated using the formula: HOMA-IR = fasting glucose (nmol/L) × fasting insulin (μU/mL)/22.5. HOMA-IR values greater than or equal to 2.0 or 2.5 is the value used to distinguish between NAFLD patients and healthy controls (Tang *et al.*, 2015).

2.2.3.2.NAFLD activity score (NAS)

NAS calculated by the aggregate of scores of steatosis (0-3), lobular inflammation (0-3) and hepatocyte ballooning (0-2) with final score ranging from 0 to 8 (Goodman, 2007).

2.2.3.3.fibrosis score (Metavir)

The Metavir score grades the level of fibrosis on a 5-point scale from 0 to 4 and the activity, which is the amount of inflammation, is scored on a 4-point scale from A0 to A3. Fibrosis score staged as: F0 = no fibrosis, F1 = perisinusoidal or periportal, F2 = perisinusoidal and periportal, F3 = bridging fibrosis, F4 = cirrhosis. The Activity score: A0 = no activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity (Goodman, 2007).

2.2.4 Sample Collection and Preparation

Five heparin or sodium citrate tubes and one plain tube were collected from each participant at the time of liver biopsy. Plain tube was applied for measurement of certain cytokines in serum, whereas all heparin or citrate tubes were used for isolation of mononuclear cells by density gradient centrifugation. For more purity, peripheral blood mononuclear cells (PBMCs) were used. PBMCs were purified from heparinized or citrated blood by transferring all the five tubes to large tube and then a dilution with phosphate buffer solution (PBS) in one to one ratio was prepared. After that the diluted cell suspension was carefully added in 90° degree over a Ficoll-paque in one to one ratio. After centrifugation at 1600 rpm for 20 minutes without brake and accelerate, the mononuclear layer cell layer was carefully transferred to a new conical tube by pipette. The mononuclear cell layer was then centrifuged at 1600 rpm for 10 minutes at 4C° with brake and accelerates. After centrifugation the cell pellet was resuspended in 1ml of PBS for either immediate processing for the next step or for freezing at-20C°until used.

2.2.5 Isolation of T cells using direct human T-cell isolation kit

For more purity, direct human T-cell isolation kit was used in purifying T cell. The principal of the kit is to isolate T-cell from human blood samples by immunomagnetic negative selection. PBMCs that were purified by centrifugation over a Ficoll-paque gradient were applied to the kit according to the manufacturer's instructions (appendix 2). Following the kit instructions whole blood samples should be used, however, PBMCs samples were found to be more accurate to use.

2.2.6 Flow cytometric analysis

The isolated cells that were obtained from direct human T cell isolation kit were centrifuged at 1600 rpm for 10 minutes. The pellet was then fixed by adding 20µl 4%formaldehyde /100µl PBS for 1 minute on ice. Then 300µl of PBS were added and Centrifugation at 1600 rpm for 10 minutes in 4C° was done to stop the action of formaldehyde. The pellet then was resuspended in 300µl of PBS and stored in the refrigerator until the mixture of monoclonal antibodies was prepared. The monoclonal antibodies (mAbs) of anti TCR Vα7.2 (PE-Cy7), anti CD161 (FITC), anti IL-17 (APC) were used for all samples, whereas anti CD69 (PerCP), anti TNF-α (PB) and were used for all samples except of three F1 patients. Anti CD38 (APC) was used for some samples. The mixture of mAbs was prepared with ice by adding 2-5µl of each antibody diluted in PBS medium. For the intracellular staining of TNF-α and IL-17, 100µl of 0.1% saponine was added to the antibody mixture. Then the antibody mixture was added to the cells and incubated for 20-45 minutes on ice. Washing were performed to remove the unbound antibody was done three times by centrifugations at 1600rpm for 10 minutes at 4C° and adding 300 µl PBS for each time. Finally, the samples were analyzed by a flow cytometer (BD LSR Fortessa). Data were analyzed by FACS express3 software.

Each sample was tested in triplicate. The mAbs are sensitive to light so they must be covered with aluminium foil. Unstained cells were used as negative control and to remove the possible effect of autofluorescence. Single staining cells were applied to determine the level of compensation. IgG isotypes control for each antibody labeled with the same fluorochrome of their corresponding were also used to remove the background staining. The variabilty in the antibodies that were used was due to the availabilty of these antibodies.

2.2.7 Insulin receptor detection by flow cytometeric analysis

Three F4 samples and three healthy samples were used for this experiment. Peripheral blood mononuclear cells (PBMCs) were pulled from the six samples by density gradient centrifugation as described in section 2.2.3. For flow cytometric analysis, the samples stained by anti CD45(pan leukocyte marker), anti CD3(pan T cell marker), anti CD161, anti TCR Vα7.2 and anti-insulin receptor as described in the previous section.

2.2.8 Cytokine measurement

Cytokine levels were measured using quantibody human cytokine array 1 kit. Serum for five serum samples from each fibrosis stage except of F3 and five healthy samples were evaluated for their cytokine levels according to the manufacturer's instructions (appendix 3). Multiplex Sandwich ELISA-based quantitative array is the precept of the kit. Data analysis of the multiple cytokines was performed using the Quantibody Q-Analyzer, an Excel-based program. Results are presented in pg/ml. The exception of F3 patients was due to that the wells were provided in the kit were not enough for all samples and F3 is histologically similar to F4.

2.2.9 Statistical analysis

Statistical analyses were performed using Microsoft Excel or Graph Pad Prism. Data were subjected to Kolmogorov-Smirnov normality test as well as to Shapiro-Wilk normality test and all data were normally distributed so parametric tests were used. All clinical, serum and histology parameters were compared between groups using the unpaired t-test. The flow cytometry results of patients and healthy donors were compared using un-paired t-test. Pearson’s r coefficient for correlation test was used. Statistical significance was determined at p value ≤ 0.05.

Chapter Three

Result

3.1 HOMA-IR scores are associated with increased liver fibrosis

Twenty-five NAFLD patients fulfilled the inclusion/exclusion criteria, all are men with mean age of 39.3±9.8 years and BMI mean of 29.19±9. The patients were stratified according to their fibrosis score as shown in Table (3.1).

We found that LDL levels in NAFLD patients were within the borderline range (130-159 mg\dl). Whereas, the HDL levels and the TG levels in the patients were within the normal range (40-50 mg\dl in men, 150-500mg\dl, respectively). The cholesterol profile findings indicate that this profile can not be used in the prediction of NAFLD. We also observed that there was no significant differences in age, ALT, Triglyceride, High density lipoprotein and Low density lipoprotein were noted between fibrosis score categories.

BMI was not significantly correlated with the increased fibrosis score in our population with p value equal 0.052. As expected HbA1c was within the normal ranges since the selected patient population presented mild metabolic complications and were free of medications. Increased fibrosis score significantly correlated with histologically detected necro-inflammatory activity and the serum level of C-reactive protein. The patients were share high CRP levels as the normal range of CRP is less than 1. This high level of CRP was correlated with the inflammation process of NAFLD.

HOMA scores levels were increased in F2,F3 and F4 patients with scores higher than the normal range (more than 2 is consider IR). In addition, serum insulin were also higher than the normal range (less than 25mIU\ml) in advanced fibrosis stages. Moreover, the increased in the HOMA scores and serum insulin was significantly correlated with fibrosis score progressions and was prominent in patients with advanced fibrosis (p=0.001 and p=0.05, respectively).

Table3.1: Characteristics of NAFLD patients according to Fibrosis score

Characteristics	**Healthy n =5**	**Fibrosis (Metavir Score) (n=25)**				**P-value**
		F1(1.2±0.8) n =6	**F2(1.9±0.5) n =6**	**F3(3.1±0.4) n =6**	**F4(3.5±0.4) n =7**	
Age [years]	31±14	36.6±9.9	38.9±8.7	40.7±11	41.1±9.4	NS
BMI	27.1±3.8	28.35±7.9	29.15±7.9	29.4±10.1	29.85±9.9	0.052
LDL cholesterol [mg/dl]	121±17.5	131.7±26.6	124.7±21.4	134.7±29.1	143.7±29.1	NS
HDL cholesterol [mg/dl]	44±6.7	40.8±8.4	37.6±9.6	37.3±6.5	38.5±3.5	NS
Triglycerides [mg/dl]	144±20.6	147.8±39.5	146.7±31.6	150.2±27.7	151.2±29.3	NS
ALT [IU/L]	77.1±13.4	79.5±47.7	81.3±35.5	78.9±35.0	89±35.7	NS
Inflammatory activity [A]	NA	1.4±0.7	1.6±0.6	2.0±0.0	2.2±0.5	0.02
CRP (mg/dl)	2.7±1.1	3.1±1.7	3.3±1.5	4.1±1.7	4.5±2.3	0.005
HbA1c [%]	3.5±0.45	3.3±0.8	4.9±0.4	3.9±0.3	5.6±0.3	0.0006
Insulin levels (mIU/ml)	NA	20.1±3.8	24.7±4.1	25.7±2.1	27.8±5.1	0.05
HOMA-IR	NA	1.8±0.2	3.0±1.1	3.3±1.0	5.2±0.2	0.001

NAFLD: Non Alcoholic Fatty Liver Disease, HOMA: homeostasis model assessment, LDL: low-density lipoproteins, HDL: high-density lipoproteins, TG: triglycerides, ALT: alanine aminotransferase, HbA1c: Hemoglobin A1C. CRP: c reactive protein. HOMA-IR (homeostasis model assessment –insulin resistance)

3.2 Depletion in the frequency of MAIT cells in F4 NAFLD patients

Human MAIT cells are defined as $CD161^{++}$ $V\alpha7.2^{+}$. We determined the frequency of MAIT cells in our samples by flow cytometry using anti-Vα7.2 and anti-CD161 after isolation of CD3+ cells by human T cell isolation kit (Figure 2.1A, 2.1B).

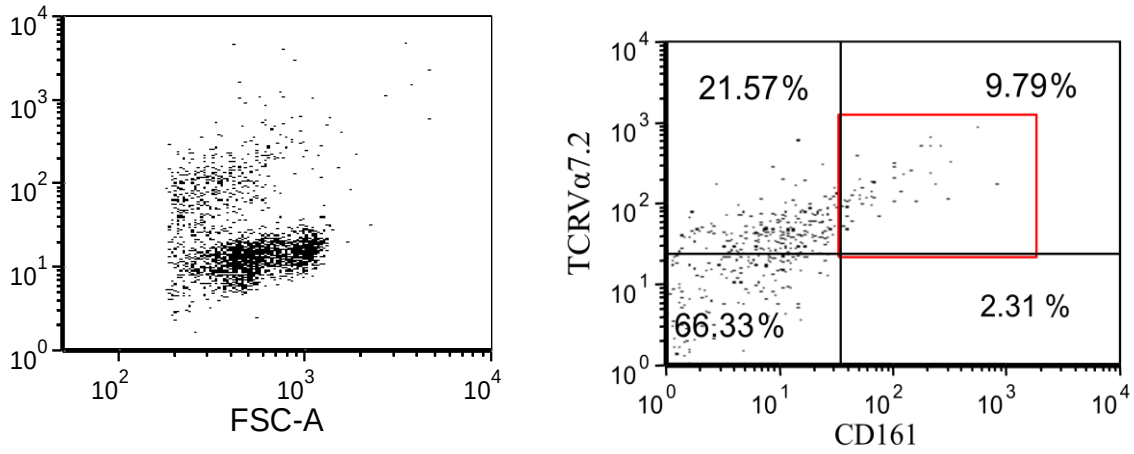

Figure (3.1): .A) Representative dot plot of flow cytometry analysis of the $CD3^+$ cells. B) Shows the gate drawn on our potential population, identified as$V\alpha7.2^+CD161^+$.

A significant depletion in the frequency of circulating MAIT cells was observed in F3-F4 patients with a mean frequency of 1.66%, 1.4% of T cells, respectively. Whereas the mean frequency of MAIT cells in F2 was 3.97% of T cells and F1 patients were 10.62% of T cells. Healthy MAIT cell frequency range was 11.2%-14.2% of T cells (Figure 3.1.C, $p<0.0001$). We also found that the reduction in the frequency of MAIT cells in NAFLD patients can predict severity of disease, as we found a significant difference among stages. The p values of F1-F2, F2-F3 and F3-F4 were found to be far less than 0.05 (not shown in the figure).

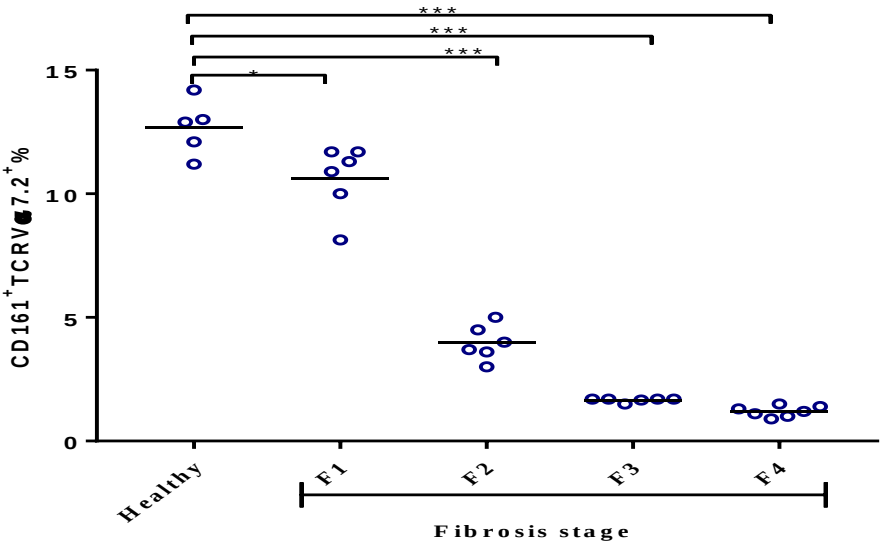

Figure (3.1.C) Percentages of MAIT cells among T cells in patients with NAFLD stages. Each symbol represents the value of one individual. The middle line indicates the mean. * *p=0.025*, ***p<0.001 assessed by t test.

3.3 Activation of MAIT cells is associated with increased liver fibrosis

Activation of Mucosal associated invariant T (MAIT) cells was determined by studying the early activation marker CD69 and CD38. After the determination of our potential population as double positive staining cells of CD161 and TCRVα7.2 in flow cytometry, the percentages of cells express CD69 and CD38 was detected by flow cytometry using anti-CD69 (Figure 3.2.A) and anti-CD38.

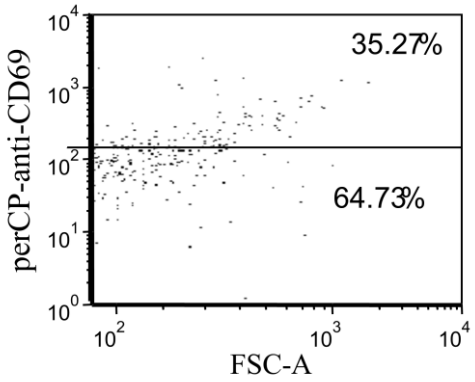

Figure (3.2.A) Flow cytometry dot plot represents the percentages of CD69 expressing cells.

An increase in the expression of CD69 was observed to be proportional to the increasing of fibrosis score, as well in CD38. The mean of the percentages of $CD38^+$ among MAIT cells was higher in advanced stages of fibrosis in which F3 and F4 patients $CD38^+$ cells was 20.1% and 32.7%, respectively, as compared with 8.5% in healthy subjects (Figure 3.2.B). Whereas the mean of the percentages of $CD69^+$ among MAIT cells was 42.3,70.8%, 73.7% and 78.4% in F1, F2, F3 and F4 patients, respectively, which is higher than the mean percentages of $CD69^+$ cells among MAIT cells in healthy that 9.6% (Figure 3.2.C).

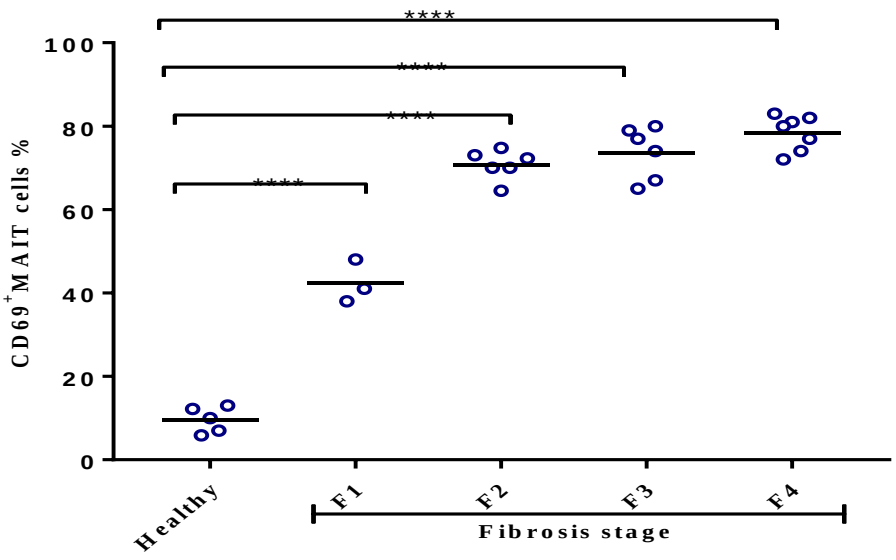

Figure (3.2.B) Percentages of $CD69^+$ cells among MAIT cells in patients with NAFLD stages. Each symbol represents the value of one individual. The middle line indicates the mean. ****p<0.0001 assessed by *t* test.

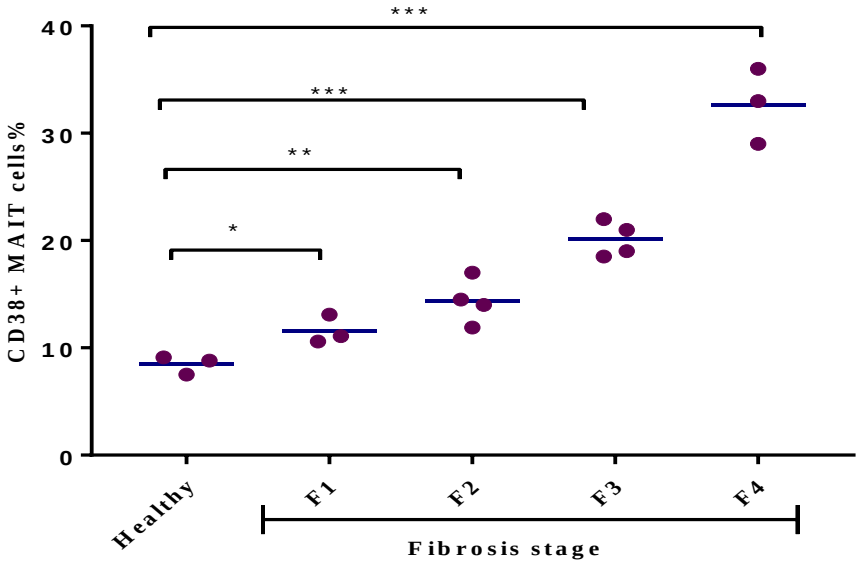

Figure (3.2.C) Percentages of $CD38^+$ cells among MAIT cells in patients with NAFLD stages. Each symbol represents the value of one individual. The middle line indicates the mean. * *p=0.026*, ***p<0.001 assessed by *t* test.

We also found that the increased in the expression of CD38 was significantly difference among stages. The *p* values of F1-F2, F2-F3 and F3-F4 were 0.043, 0.002, 0.0007, respectively. This significantly augmented expression was not found with CD69 among F2-F3 and F3-F4. However, there was a significant increased in the expression of CD69 between F1-F2 patients with *p* value of 0.00001.

The activation of MAIT cells as was determined by the expression of CD69 was inversely correlated with MAIT cell frequency (r= -0.95, p=0.011) as shown in figure (3.2.D).

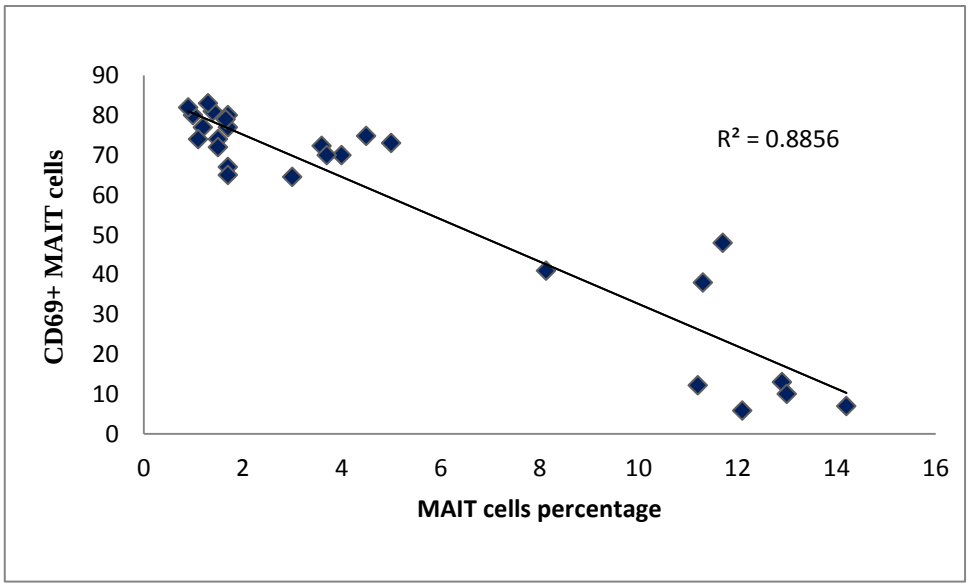

Figure (3.2.D) The correlation between MAIT cells frequency and CD69 expression.

3.4 Increased the frequency of IL-17+ MAIT cells is correlated with increased liver fibrosis

As MAIT cells produce IL-17 upon activation, a pro-fibrogenic cytokine that were found to be involved in the hepatic fibrosis development in autoimmune liver fibrosis and non-autoimmune liver fibrosis (Bottcher *et al.*, 2018; Toubal & Lehuen, 2016). To assess the frequency of IL-17^{+} MAIT cells, the cells were stained with anti-IL-17 and analyzed by flow cytometry (Figure 3.3.A)

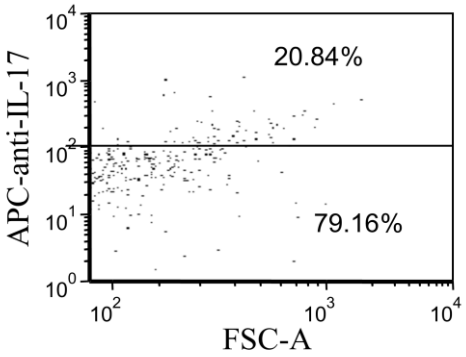

Figure (3.3.A) A flow cytometry dot plot represents the percentages of IL-17^{+} MAIT cells

There was a significantly higher IL-17^{+} MAIT cells in liver fibrosis patients when compared to healthy subjects as seen in figure (3.3.B). Moreover, this elevation was significantly different among stages. The *p* values were less than 0.05 among F1-F2 and F3-F4, but not between F2-F3 with p value of 0.03.

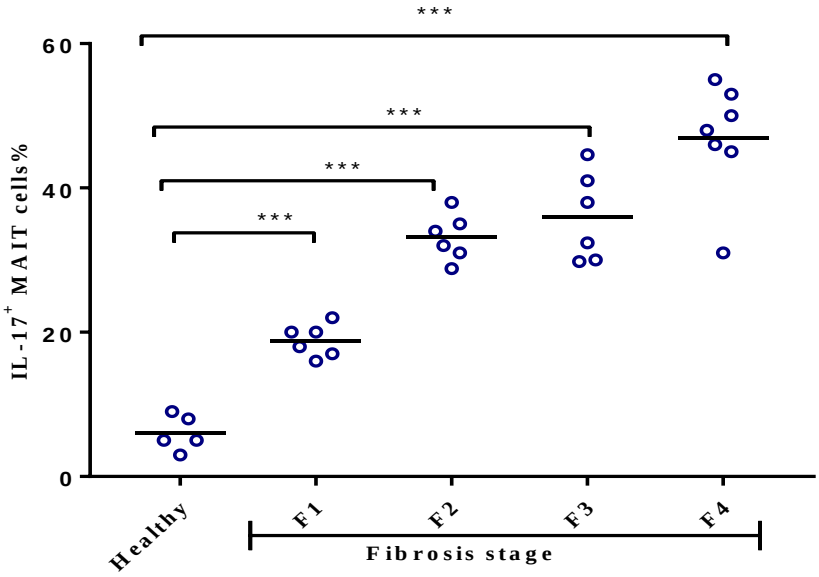

Figure (3.3.B) Percentages of IL-17^{+} cells among MAIT cells in patients with NAFLD stages. Each symbol represents the value of one individual. The middle line indicates the mean. ***p<0.001 assessed by *t* test.

IL-17 positive cells were found to be inversely correlated with the frequency of MAIT cells, this illustrated in figure (3.3.C).

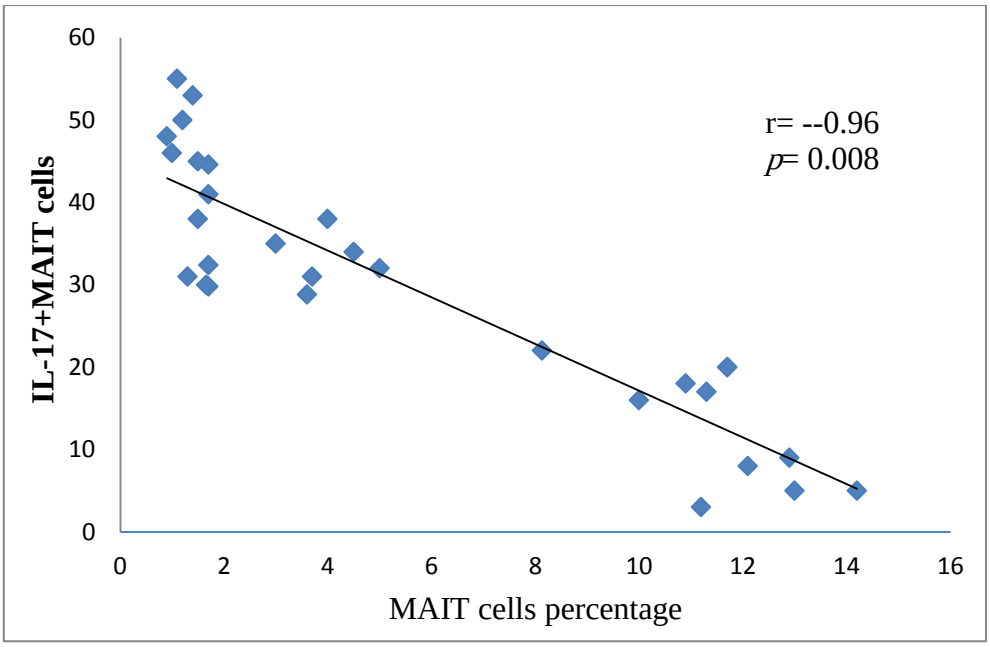

Figure (3.3.C) The correlation between MAIT cells frequency and IL-17^{+} MAIT cells.

3.5 Increased TNF-α expression in F4 NAFLD patients

We further detected other immunological marker, the tumor necrosis factor α (TNF-α), a pro-inflammatory cytokine contributes to the hepatic inflammation, apoptosis of liver cells and induces the insulin resistance (Seo et al., 2013). The frequency of TNF-α^{+} cell among MAIT cells was determined by flow cytometer using anti-TNF-α as seen in figure (3.4.A)

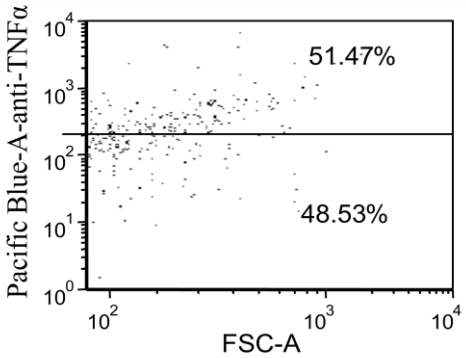

Figure (3.4.A) A flow cytometry dot plot represents the percentages of TNF-α^{+} MAIT cells

Figure (3.4.B) shows a significant increase in the expression of TNF-α in F1, F2, F3, and F4 when compared with healthy subjects. However, this elevation was found to be elevated

mostly in F1 patients followed by a significant depletion in F2 (p<0.0005) then return to be significantly augmented in F3 (p=0.01) after that a non significant elevation found in F4.

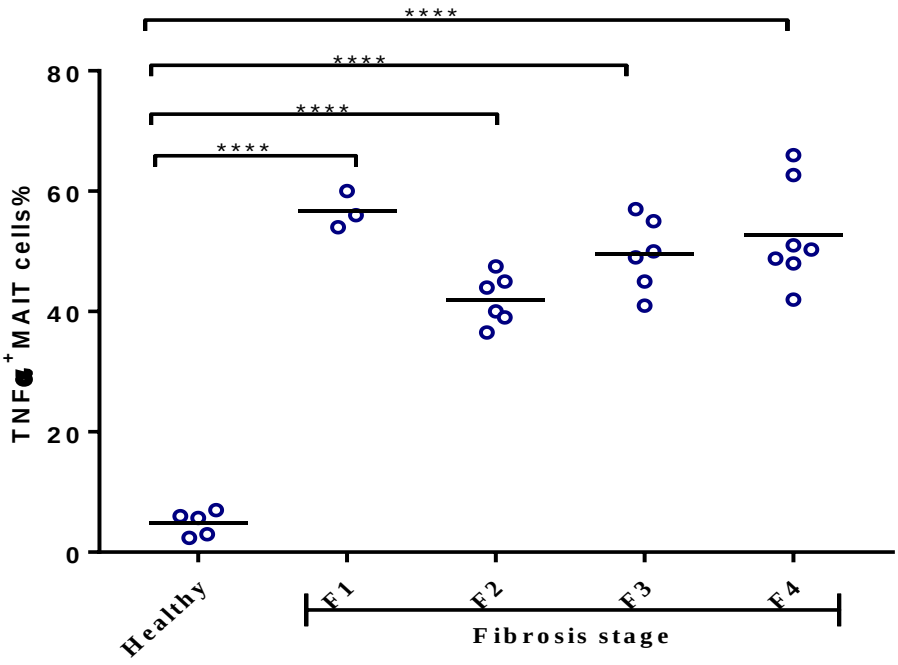

Figure (3.4.B) Percentages of TNF-α^{+} cell among MAIT cells in patients with NAFLD stages. Each symbol represents the value of one individual. The middle line indicates the mean. ****p<0.0001 assessed by *t* test.

3.6 Alterations in the cytokine levels in the serum of NAFLD patients

To get an indication about the inflammatory cytokines that could mediate the stimulation of MAIT cells, the patients' sera tested to multiple cytokines using quantibody human cytokine array 1. From the twenty cytokines tested, nine of them underwent a significant alteration which are IL-1a, IL-1b, IL-2, IL-6, IL-12p70, IL-13, GM-CSF, TNF-α and MMP-9 (Figure 3.5, p<0.05).

The inflammatory cytokine IL-1, TNFα, GM-CSF and IL-12 were significantly increased during the progression of the disease that may reflect a chronic activation of MAIT cells.

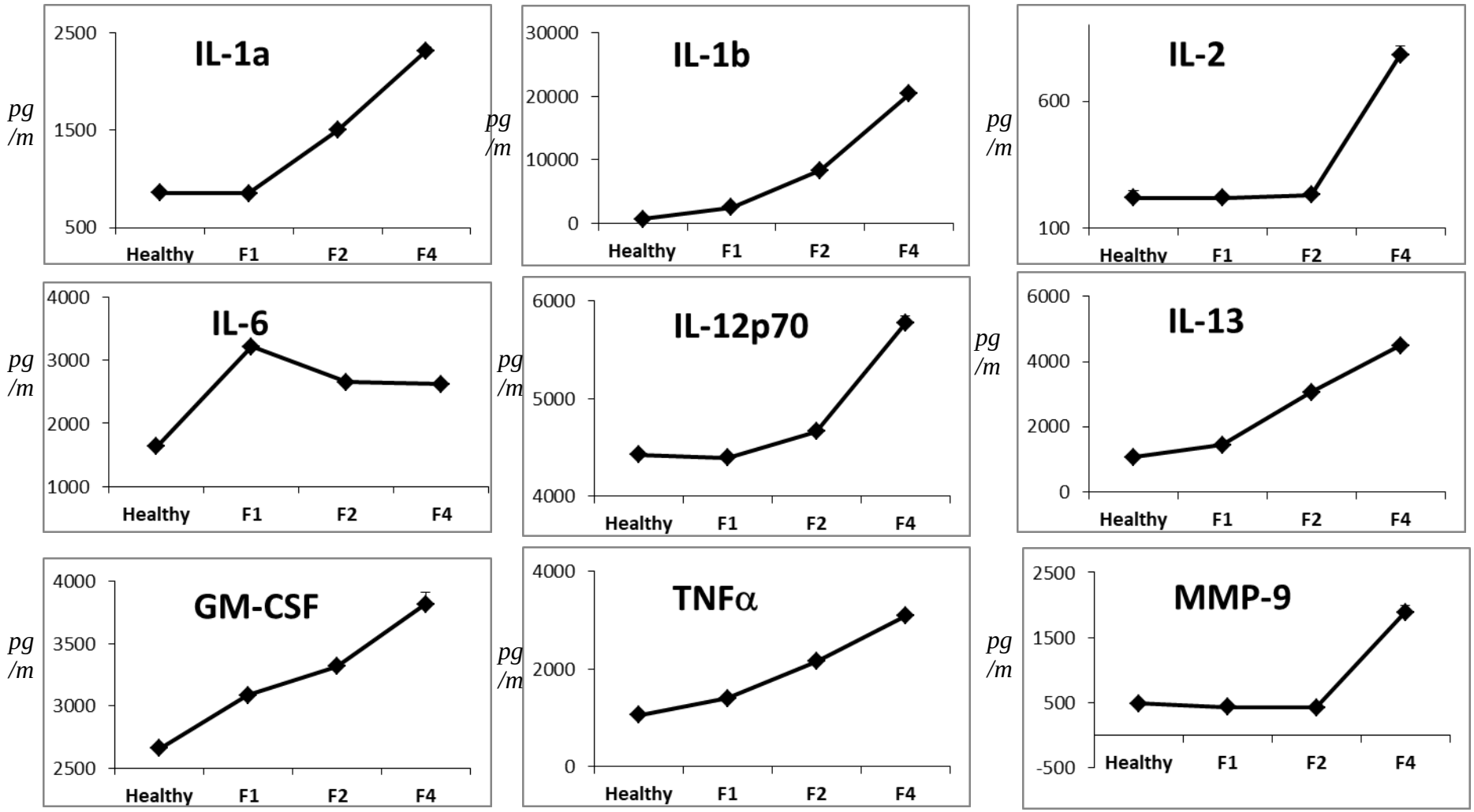

Figure (3.5) Means of the cytokine level detected in the sera of 5 patients from each fibrosis stage F1, F2 and F4 and 5 healthy donors. N=5 in each group. $P<0.05$ between F1 and F4 groups.

3.7 Decreased expression of insulin receptor in F4 NAFLD patients

For this experiment, we utilized six PBMCs samples, three from healthy individuals and three from F4 NAFLD patients to detect their insulin receptor expression. CD45 a pan leukocyte marker and CD3 pan T cell marker were used to determine T lymphocyte population. Then double positive cells for CD161 and Vα7.2 were determined as MAIT cell population. After that, insulin receptor expression in MAIT cells was assessed. As shown in figure (3.6), the insulin receptor was significantly reduced in F4 patients with mean insulin receptor positive cells of 2.4%, as compared with 6.03% a mean insulin receptor positive cells expressed on MAIT cells of healthy donors (p=0.021).

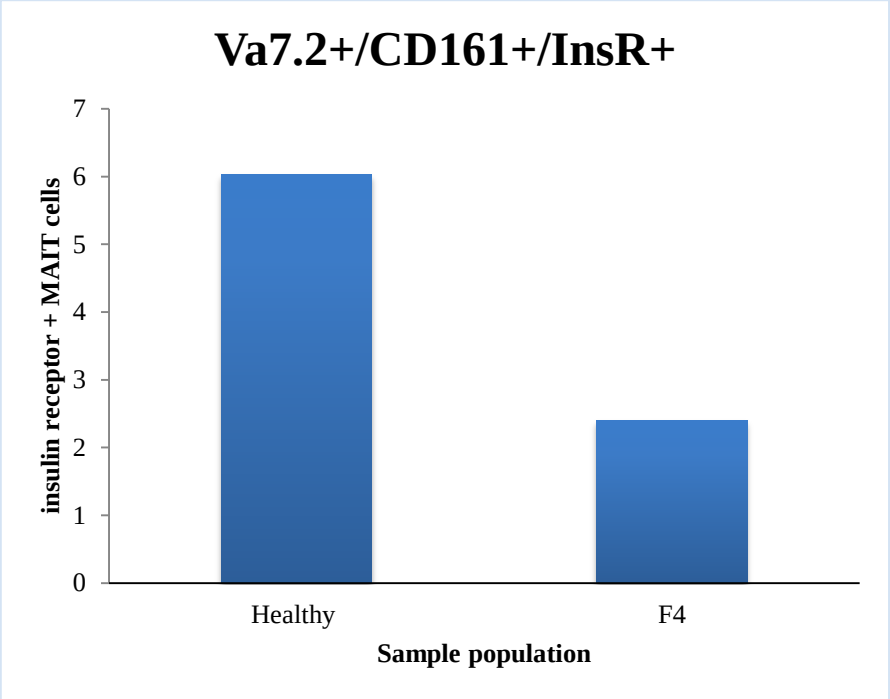

Figure (3.6) Percentages of insulin receptor^{+} cell among MAIT cells in patients with F4 stage. P=0.021 assessed by *t* test.

Chapter Four

Discussion

4.1 Discussion

In the present study, we examined the frequency and the function of MAIT cells in 25 NAFLD patients with different stage of fibrosis compared to five healthy donors. Seven of the NAFLD patients developed cirrhosis accompanied by hyperinsulinemia and insulin resistance. Mean age at biopsy was 39.3±9.8 and BMI mean was 29.19±9. Insulin resistance (calculated by HOMA scores) and insulin serum levels in our patient population increased during fibrosis progression which correlated with the consideration that NAFLD is the hepatic manifestation of the metabolic syndrome (Bolte & Rehermann, 2018). We also found that the body mass index (BMI) was increased with increased liver fibrosis score as reported with other studies (Zelber-Sagi *et al.*, 2017). However, this elevation was not significantly which might be attributed to the small sample size. Similary, we found that the necro-inflammatory activity and the serum level of C-reactive protein was significantly increased with fibrosis progression and was associated with increased the serum level of inflammatory cytokines (figure 3.5). We found an insignificant differences in age, ALT, Triglyceride, High density lipoprotein and Low density lipoprotein between fibrosis score categories. In contrast with a study by Hossain et al. on 432 patients with histologically proven NAFLD in which they revealed that ALT and AST can be used as predictor for fibrosis as they increased with advanced fibrosis stage (Hossain *et al.*, 2009). However, a recent longitudinal study revealed that liver steatosis is the best predictor for fibrosis progression rather than the clinical and biochemical parameters (Lallukka *et al.*,

2017). In our study, insulin resistance calculated by HOMA score can be used as a metabolic predictor for liver fibrosis.

Non-alcoholic fatty liver disease (NAFLD) is a spectrum disease that includes a simple steatosis to NASH; a progressive form of steatosis accompanied with inflammation and injury and with or without fibrosis. 20% of NASH patients might further progress to cirrhosis which increase the risk of hepatocellular carcinoma and liver failure. The prevalence of disease is increasing with an estimation of 1 billion people worldwide have NAFLD. The disease is asymptomatic and the reliable diagnostic procedure is the liver biopsy. The prevalence is thought to be higher than estimated. Thus, there is a need to non-invasive method for diagnosis. Moreover, there is no effective treatment for NAFLD which increases the demand on focusing more on the disease management (Zhang *et al.*, 2018). The liver is enriched with immune cells. One of the largest T cells population in the liver is MAIT cells, a novel innate like T cells that have the ability to recognize riboflavin metabolite derived from microbes and also can be stimulated by cytokines that suggest the important role of MAIT cells in the immunity and homeostasis of the liver (Kurioka *et al.*, 2016).

We investigated the frequency of MAIT cells present in our study population which are defined as cells with double positive for CD161 and Vα7.2. We demonstrated depletion in the circulating MAIT cells in patients with NAFLD. A study by Jeffery at al. found a similar depletion in the circulating MAIT cells in patients with chronic liver diseases including alcoholic steatohepatitis, primary sclerosing cholangitis and primary biliary cirrhosis (Jeffery *et al.*, 2016). Despite the reduction in the circulating MAIT cells, we observed an increase in the activation of MAIT cells as detected by the expression of the activation marker CD69 and CD38.

The depletion and the activation of MAIT cells was reported in other chronic liver diseases with different etiology (Bolte *et al.*, 2017; Bottcher *et al.*, 2018; Riva *et al.*, 2018). We also demonstrated that the depletion and the activation of MAIT cell levels in the peripheral blood of NAFLD patients in correlation with the severity of fibrosis.

In addition, we found an elevation in the serum levels of inflammatory cytokine IL-1α, IL-1β, IL-6, TNF-α, GM-CSF and IL-12 (figure3.5). This elevation was associated with fibrosis progression as in advanced fibrosis stages, the level of these cytokines was higher. This finding was correlated with increased the activation level of MAIT cells, which may elicit the independent activation manner mediated by cytokine.

Gut dysbiosis is found to be correlated with NAFLD (Pappachan *et al.*, 2017). That might give an additional possible way of MAIT cell activation by bacteria translocation from the gut to the liver.

The dramatic reduction in MAIT cell levels could be as a result of their recruitment to the site of infection which is expected. MAIT cells known to own a variety of chemokine receptor enable them to migrate to different tissues or might be due to activation induced cell death mechanism that MAIT cells underwent as a result to the chronic exposure to the inflammatory cytokine. These phenomena was suggested by others in many inflammatory diseases including ankylosing spondylitis, systemic lupus erythematosus and alcoholic liver diseases (Chiba *et al.*, 2017; Gracey *et al.*, 2016; Riva *et al.*, 2018).

Furthermore, we investigated the immune phenotype of MAIT cells through the intracellular staining of IL-17 and TNF-α. IL-17 has the profibrogenic effect on hepatic stellate cells that suggest the possible role of MAIT cells in the fibrosis development (Bottcher *et al.*, 2018). We found that IL-17^{+} MAIT cells were elevated through the fibrosis progression.

In addition, we found an elevation in TNF-α^{+} MAIT cells in NAFLD patient as compared with healthy donors. However, the upregulation of TNF-α was mostly in F1 that might indicate an acute response. TNF-α is an inflammatory cytokine which contributes to the hepatic inflammation, apoptosis of liver cells and induces the insulin resistance. The activation of proinflammatory pathways after exposure to TNF-α induces a state of insulin resistance in which TNF-α impair insulin signalling at the level of the insulin receptor substrate (IRS) proteins (Seo *et al.*, 2013).

Down regulation of insulin renders effector T cells to be less proliferated and cytotoxic and to produce less proinflammatory cytokine which means loss of function condition (Fischer *et al.*, 2017). So for future, we will focus on detecting the functional alteration of MAIT cells in NAFLD.

4.2 Conclusions

The current study demonstrates that the clinical and biochemical markers failed to predict the liver fibrosis in patients with NAFLD. However, insulin resitance calculated with HOMA score might be used as predictor marker for NAFLD.

Our study also revealed that the circulating MAIT cells are reduced and activated when compared to healthy donors as well, when compared among stages. Thus, reflecting the severity of fibrosis. In addition, MAIT cells was found to express more IL-17 and TNF-α than the healthy individual. We also noticed that insulin receptor was down regulated in cirrhotic patients than healthy donors. Thus, suggesting a possible less proliferative and less functional MAIT cells might be present in advanced fibrosis NAFLD patients.

These finding suggested the possible role of MAIT cells in the pathogenesis of non-alcoholic liver disease (NAFLD). That nominates the MAIT cells to be used as a predictor for liver fibrosis in NAFLD diagnosis and prognosis approaches.

4.3 Weaknesses or limitations in the current study plan

The first limitation was the sample size. It was difficult to collect samples fulfill the inclusion and exclusion criteria in this short period of time. The other limitation was not to perform the functional assay for MAIT cells to assess the functional diversity in NAFLD disease.

4.4 Recommendations

MAIT cell is considered a new T cell population that seems to have a promising role in many diseases. For future, functional assay for circulating MAIT cells in NAFLD patients will decipher more about the role of MAIT cell in the progression of NAFLD.

4.5 Funding

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Chapter Five

Supplementary materials

Appendix 1

טופס 2 ה	
0605-16-HMO	מספר:0
טופס הסכמה מדעת להשתתפות בניסוי רפואי	

אני החתום[1] מטה:

שם פרטי:	שם משפחה:
מס' תעודת זהות:	
כתובת:	מיקוד:

1) מצהיר בזה כי אני מסכים להשתתף בניסוי רפואי, כמפורט במסמך זה.

2) מצהיר בזה כי איני משתתף בזמן חתימת מסמך זה, בניסוי רפואי אחר הכרוך בשימוש במוצר מחקר כלשהו, וכי אני מתחייב לא להשתתף בכל ניסוי רפואי אחר הכרוך בשימוש במוצר מחקר במשך כל תקופת ניסוי זה.

3) מצהיר בזה כי הוסבר לי על-ידי:

שם החוקר המסביר:

3.1) כי החוקר הראשי (שם הרופא): **פרופ ריפעת ספדי** קיבל ממנהל המוסד הרפואי, אישור לביצוע הניסוי, כמשמעותו בתקנות בריאות העם (ניסויים רפואיים בבני-אדם תשמ"א-1980), להלן הניסוי הרפואי.

3.2) כי לחוקר הראשי ולחוקרי המשנה **יש זיקה**[2] ליזם הניסוי[3]. אם יש, פרט:

החוקר הראשי והמשנה הם יזמי המחקר.

3.3) כי הניסוי הרפואי נערך במטרה: **התשובות של החלבון Neuroligin-4 על תפקודם של תאי ה-NKבמחלת הלייפות הכבדית**

3.4) כי אני חופשי לבחור שלא להשתתף בניסוי הרפואי, וכי אני חופשי להפסיק בכל עת את השתתפותי בניסוי, כל זאת מבלי לפגוע בזכותי לקבל את הטיפול המקובל.

3.5) כי במקרה של מילוי שאלון – אני רשאי שלא לענות על כל השאלות שבשאלון או על חלק מהן.

3.6) כי מובטח לי שזהותי האישית תשמר סודית על-ידי כל העוסקים והמעורבים במחקר ולא תפורסם בכל פרסום, כולל בפרסומים מדעיים.

3.7) כי המוסד הרפואי פעל להסדרת כיסוי ביטוחי הולם של החוקרים, הרופאים והצוות הרפואי העוסקים בניסוי הקליני מפני תביעות שיוגשו ע"י משתתפים בניסוי הקליני ו/או תביעות צד ג' הקשורות

[1] הטופס נכתב בלשון זכר מטעמי נוחות בלבד ומיועד לשני המינים.

[2] קשר של העסקה בשכר, או קשר מסחרי או עסקי, או קשר משפחתי או אישי, וכל קשר אחר, לרבות קשר של כפיפות בעבודה, שיש בו כדי לעורר חשש לקיום ניגוד עניינים או תלות.

[3] אם החוקר הראשי הוא גם יזם הניסוי, יש לציין זאת במפורש.

3	16/05/2017	טופס 2 ה עברית	
Version	Version Date תאריך גרסה		גרסה

עם הניסוי הקליני בין בתקופת ביצוע הניסוי ובין לאחריו. אין באמור כדי לפגוע בזכויותיי על פי כל דין.

3.8) כי מובטחת לי נכונות לענות לשאלות שיועלו על-ידי זכן האפשרות להיוועץ בגורם נוסף (לדוגמא רופא-משפחה, בני משפחה וכו'), באשר לקבלת החלטה להשתתף בניסוי הרפואי ו/או להמשיך בו.

3.9) כי בכל בעיה הקשורה לניסוי הרפואי אוכל לפנות ל-**פרופ' ריפעת ספדי** בטלפון/נייד: **02-6777721** **050-8573574**, בכל שעות היממה. עלי לדווח מיד לרופא שפרטיו לעיל על כל בעיה רפואית, פציעה או אירוע בריאותי אחר העשוי להיות קשור למחקר. אם אפגע כתוצאה מהשתתפותי במחקר, עלי לפנות אל רופא המחקר על מנת לקבל טיפול רפואי מתאים וכן פרטים נוספים על זכויותיי בהקשר זה. חתימה על טופס זה אינה גורעת מזכויותיי לפי החוק.

4) מצהיר כי נמסר לי מידע מפורט על הניסוי הרפואי, על פי המשאים המפורטים להלן:

4.1) **רקע כללי וחשיבות הניסוי.** במעבדה של פרופ' ספדי התגלה חלבון חדש שמעורב בדיכוי הרגם של תאי הצלקת על ידי תאי המערכת האימונית מהם תאי ,NK תאים שאמורים למנוע התפתחות המחלה. מטרת המחקר הינה לבודד תאי ה NK -מהדם ההיקפי ושאריות ביופסיות הנלקחות וללמוד את התערבותם של הגן.

4.2) **מטרת הניסוי.** בדיקת מעורבות המפלוֹל של החלבון Neuroligin-4 בתאי ה NK -במהלך התפתחות השחמת הכבדית במחלת הכבד שומני ומודלים של מחלת כבד בעכברים.

4.3) **מספר המשתתפים בניסוי.** 20

4.4) **התקופה הצפוייה למשך ההשתתפות בניסוי.** בדיקה חד פעמית

4.5) **שיטות- תיאור מוצר המחקר, תיאור בקצרה של ההליכים השונים במשך תקופת הניסוי (טיפול זמעקב), תוך הבחנה ברורה בין ההליכים המחקריים לבין ההליכים המקובלים ברפואה.**

לצורך בדיקת רמת ביטויו של החלבון NLG4 בתאי המערכת האימונית כולל תאי ה Natural Killing - Cellsבדמם ו כבדם של אוכלוסיית המחקר צריכים הבא:

איסוף הדם ההיקפי מהמשתתף במחקר על ידי לקיחת חמש מבחנות כחולות במעבדה על ידי האח.

איסוף שאריות הביופסיות מהמשתתף במחקר .

מהדם והביופסיות הנאספים יופקו תאי מערכת החיסונית הנחקרים במעבדה ספיציפית תאי ה - Natural Killing Cellsעל ידי השקעתם במכשיר הנקרא צינטרפוגה והפקתם על ידי השימוש בקיטים ספיציפים להפקת התאים..

על ידי השימוש בשיטה הנקראת Fluorescent-Activated Cell Sorting Staining (FACS) שזו שיטה שצובעת את חלבוני התא הספיציפים והרצויים לם נוכל לבדוק את רמת ביטוין של החלבון.

4.6) **היתרונות הצפויים למשתתף או לאחרים, כתוצאה מהניסוי.**

אנו לא בטוחים שמחקר זה יעזור לך באופן ישיר אבל כך נוכל ללמוד הרבה על החלבון NLG4 ולעזור בעתיד לאחרים.

4.7) **הסיכונים הידועים ו/או אי-הנוחות שניתן לחזותם למשתתף במחקר. במידה שיש בניסוי הרפואי**

סיכון למשתתף - הסבר על הטיפול הרפואי שיקבל במקרה של פגיעה בבריאותו והאחריות לנתינתו.

מהחולה יילקחו חמישה מבחנות כחולות של דם פריפרי, בדיקה זו הינה בדיקת דם רגילה שאין בה שום סיכון לחולה שתילקחנה על ידי אח מוסמך.

בקשר לביופסיות, הן יילקחו משאריות הביופסיות שנלקחו לחולה שנדרש לכך ולא ספיציפית למחקר שלנו, מקבלים כ- 2 ס"מ משאריות הביופסיה הנלקחה.

לקיחת הביופסיה תהיה תחת השגחה מצוות רפואי מקצועי. כי מי שתזרם את הביופסיה לא יצטרך לעשות את זה ספציפית בשבילנו, אלא אם בדרש לעשות את זה מסיבות רפואיות כל שהן שמחייבות אותו. לכן התורם יהיה תחת השגחה מסודרת המורכבת מצוות הכירורגים או הרופא המטפל שלוקחים ממנו את הביופסיה. תרומת הביופסיה אינה מכאיבה, אם מדובר בחולה מנותח שהכירורג מצא שיש לו הבחנה של כבד שומני והחליט לקחת לו ביופסיה שממנה נקבל שאריות, החולה לא ירגיש את זה כי הינו מורדם ומושגח מצוות כירורגי מקצועי. אם מדובר בחולה שמתבקש לעשות ביופסיה רגילה לצורך רפואי שממנה נקבל שאריות גם כן התהליך אינו מכאיב בכלל כי ישנה הרדמה מקומית על ידי השימוש בחומר הנקרא לידוקאיין. חוץ מזה ומושגח מצוות רפואי מקצועי.

שני בדיקות אלו לא דווח בהם על שום סיבוכים או כאבים מקודם. במקרה ויש, החולה יידרש להיות עם מעקב רפואי מסודר בבית החולים.

4.8) **המחקר כולל איסוף רקמות / דגימות:**

טופס 2 ה	
0605-16-HMO	מספר:0
טופס הסכמה מדעת להשתתפות בניסוי רפואי	

מקור הדגימות: ביופסיה של כבד וכן דגימת דם
אזפן שמירת הדגימות:לא מזוהות
אם הדגימות נשמרות כמזוהות, המשתתף רשאי בכל עת לבקש שהדגימות יושמדו.
מקום שמירת הדגימות:במקפיאי המעבדה של פרופ' ספדי.
משך שמירת הדגימות:שנתיים
מחק את המיותר: המשתתף מסכים / לא מסכים לכך שישתמשו בדגימות שנלקחו למחקרים עתידיים שיאושרו על פי כל דין.

תתימת המשתתף

4.9) מידע רלוונטי אחר (כפי שנמסר על-ידי יזם הניסוי).

5) מצהיר בזה כי את הסכמתי הנ"ל נתתי מרצוני החופשי וכי הבינותי את כל האמור לעיל. כמו-כן, קיבלתי עותק של טופס הסכמה מדעת זה, נושא תאריך וחתום כדין.

6) עם חתימתי על טופס הסכמה זה, אני מתיר ליזם הניסוי הרפואי, לוועדת הלסינקי המוסדית, לגוף המבקר במוסד הרפואי ולמשרד הבריאות גישה ישירה לתיקי הרפואי, לשם אימות שיטות הניסוי הרפואי והנתונים הקליניים. גישה זו למידע הרפואי שלי תבוצע תוך שמירת סודיות, בהתאם לחוקים ולנהלים של שמירת סודיות.

שם המשתתף בניסוי הרפואי	חתימת המשתתף בניסוי	תאריך

במקרה הצורך[4]

שם העד הבלתי תלוי	מספר תעודת זהות	חתימת העד	תאריך

הצהרת החוקר / חוקר המשנה:

ההסכמה הנ"ל נתקבלה על-ידי, וזאת לאחר שהסברתי למשתתף בניסוי הרפואי כל האמור לעיל וכן וידאתי

[4] במקרה שהמשתתף בניסוי, או נציגו החוקי, אינם מסוגל לקרוא את טופס ההסכמה מדעת, עד בלתי תלוי חייב להיות נוכח במשך ההסבר על מהות הניסוי הרפואי. לאחר שהמשתתף או נציגו החוקי, הביע את הסכמתו בעל-פה להשתתפות בניסוי, העד יחתום על טופס ההסכמה, תוך ציון תאריך החתימה.

טופס 2 ה	
0605-16-HMO	מספר:0
טופס הסכמה מדעת להשתתפות בניסוי רפואי	

שכל הסבריי הובנו על-ידו.

שם החוקר המסביר	חתימה, חותמת ומס' רשיון	תאריך

Appendix 2

This document is available at www.stemcell.com/PIS

EasySep™

EasySep™ Direct Human T Cell Isolation Kit

Negative Selection

Catalog #19661

For processing 100 mL whole blood

STEMCELL™ TECHNOLOGIES

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Document #28074 | Version 1_1_3

Description

Isolate highly purified T cells directly from human whole blood by immunomagnetic negative selection.

The benefits of this kit include:

- > 99.9% RBC depletion without the need for density gradient centrifugation, sedimentation, or lysis
- Up to 97% purity of isolated cells
- Fast, easy-to-use and column-free
- Isolated cells are untouched

This kit targets non-T cells for removal with antibodies recognizing specific cell surface markers. Unwanted cells are labeled with antibodies and EasySep™ Direct RapidSpheres™, and separated using an EasySep™ magnet. Desired cells are simply collected into a new tube and are immediately available for downstream applications such as flow cytometry, culture, or DNA/RNA extraction.

Component Descriptions

COMPONENT NAME	COMPONENT #	QUANTITY	STORAGE	SHELF LIFE	FORMAT
EasySep™ Direct Human T Cell Isolation Cocktail	19661C	2 x 2.5 mL	Store at 2 - 8°C. Do not freeze.	Stable until expiry date (EXP) on label.	A combination of monoclonal antibodies in PBS.
EasySep™ Direct RapidSpheres™ 50300	50300	4 x 2.5 mL	Store at 2 - 8°C. Do not freeze.	Stable until expiry date (EXP) on label.	A suspension of magnetic particles and monoclonal antibodies in PBS.

PBS - phosphate-buffered saline

Components may be shipped at room temperature (15 - 25°C) and should be refrigerated upon receipt.

Precipitate may be observed in the cocktail vial but will not affect performance.

Sample Preparation

For optimal RBC depletion, collect blood using heparin or acid citrate dextrose (ACD) as an anticoagulant. The use of K2EDTA or K3EDTA as an anticoagulant is not recommended.

For best recovery, use unprocessed human whole blood. Recovery of the desired isolated cells decreases with samples that are older than 24 hours.

The volume of blood that can be processed depends on the EasySep™ magnet used for the isolation procedure. Blood samples must be placed in the required tube to properly fit into the appropriate EasySep™ magnet (see Tables 1 and 2).

Recommended Medium

PBS (Catalog #37350) that is free of Ca++ and Mg++.

Directions for Use – Manual EasySep™ Protocols

See page 1 for Sample Preparation and Recommended Medium. Refer to Tables 1 and 2 for detailed instructions regarding the EasySep™ procedure for each magnet.

Table 1. EasySep™ Direct Human T Cell Isolation Kit Protocol

		EASYSEP™ MAGNETS	
STEP	INSTRUCTIONS	 EasySep™ (Catalog #18000)	"The Big Easy" (Catalog #18001) 
1	Collect sample within the volume range.	0.5 - 1.5 mL	1.5 - 7.0 mL
	Add whole blood sample to required tube.	5 mL (12 x 75 mm) polystyrene round-bottom tube (e.g. Catalog #38007)	14 mL (17 x 95 mm) polystyrene round-bottom tube (e.g. Catalog #38008)
2	Vortex RapidSpheres™. NOTE: Particles should appear evenly dispersed.	30 seconds	30 seconds
3	Add Isolation Cocktail to sample.	50 µL/mL of sample	50 µL/mL of sample
4	Add RapidSpheres™ to sample.	50 µL/mL of sample	50 µL/mL of sample
5	Mix and incubate.	RT for 5 minutes	RT for 5 minutes
6	Add recommended medium to top up the sample to the indicated volume. Mix by gently pipetting up and down 2 - 3 times.	Top up to 2.5 mL	• Top up to double the volume for samples ≤ 5 mL • Top up to 10 mL for samples > 5 mL
7	Place the tube (without lid) into the magnet and incubate.	RT for 5 minutes	RT for 5 minutes
8	Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring the enriched cell suspension* into a new tube.	Use a new 5 mL tube	Use a new 14 mL tube
9	Add RapidSpheres™ to the new tube containing the enriched cells.	Use same volume as in step 4	Use same volume as in step 4
	Mix and incubate.	RT for 5 minutes	RT for 5 minutes
10	Remove the tube from the magnet and place the tube from step 9 (without lid) into the magnet and incubate for a second separation.	RT for 5 minutes	RT for 5 minutes
11	Pick up the magnet, and in one continuous motion invert the magnet and tube**, pouring the enriched cell suspension into a new tube.	Isolated cells are ready for use	Use a new 14 mL tube
12	Remove the tube from the magnet and place the new tube (without lid) into the magnet and incubate for a third separation.	---	RT for 5 minutes
13	Pick up the magnet, and in one continuous motion invert the magnet and tube**, pouring the enriched cell suspension into a new tube.	---	Isolated cells are ready for use

RT; room temperature (15 - 25°C)

* Following the first magnetic separation the collected cells may contain a significant amount of RBCs and may look similar to the original unprocessed human whole blood sample.

** To minimize RBC contamination in the isolated cells, pour off the sample along a clean area of the tube (i.e. the opposite side to where the sample was poured in).

Table 2. EasySep™ Direct Human T Cell Isolation Kit Protocol

		EASYSEP™ MAGNETS		
STEP	INSTRUCTIONS	EasyEights™ (Catalog #18103) 5 mL tube	EasyEights™ (Catalog #18103) 14 mL tube	Easy 50 (Catalog #18002)
1	Collect sample within the volume range.	0.5 - 1.5 mL	1.5 - 7.0 mL	7 - 30 mL
	Add whole blood sample to required tube.	5 mL (12 x 75 mm) polystyrene round-bottom tube (e.g. Catalog #38007)	14 mL (17 x 95 mm) polystyrene round-bottom tube (e.g. Catalog #38008)	50 mL (30 x 115 mm) conical tube (e.g. Catalog #38010)
2	Vortex RapidSpheres™. NOTE: Particles should appear evenly dispersed.	30 seconds	30 seconds	30 seconds
3	Add Isolation Cocktail to sample.	50 µL/mL of sample	50 µL/mL of sample	50 µL/mL of sample
4	Add RapidSpheres™ to sample.	50 µL/mL of sample	50 µL/mL of sample	50 µL/mL of sample
5	Mix and incubate.	RT for 5 minutes	RT for 5 minutes	RT for 5 minutes
6	Add recommended medium to top up the sample to the indicated volume. Mix by gently pipetting up and down 2 - 3 times.	Top up to 2.5 mL	• Top up to double the volume for samples ≤ 5 mL • Top up to 10 mL for samples > 5 mL	• Top up to double the volume for samples ≤ 25 mL • Top up to 50 mL for samples > 25 mL
7	Place the tube (without lid) into the magnet and incubate.	RT for 5 minutes	RT for 5 minutes	RT for 10 minutes
8	Carefully pipette*** (do not pour) the enriched cell suspension into a new tube. NOTE: Collect the entire clear fraction from top to bottom. For optimal recovery, also collect a small volume of RBCs (up to 10% of the starting sample volume).	Use a new 5 mL tube	Use a new 14 mL tube	Use a new 50 mL tube
9	Add RapidSpheres™ to the new tube containing the enriched cells.	Use same volume as in step 4	Use same volume as in step 4	Use same volume as in step 4
	Mix and incubate.	RT for 5 minutes	RT for 5 minutes	RT for 5 minutes
10	Remove the tube from the magnet and place the tube from step 9 (without lid) into the magnet and incubate for a second separation.	RT for 5 minutes	RT for 5 minutes	RT for 5 minutes
11	Carefully pipette*** (do not pour) the enriched cell suspension into a new tube. NOTE: Collect only the clear fraction.	Use a new 5 mL tube	Use a new 14 mL tube	Use a new 50 mL tube
12	Remove the tube from the magnet and place the new tube (without lid) containing the enriched cells into the magnet and incubate for a third separation.	RT for 5 minutes	RT for 5 minutes	RT for 5 minutes
13	Carefully pipette*** (do not pour) the enriched cell suspension into a new tube. NOTE: Collect only the clear fraction.	Isolated cells are ready for use	Isolated cells are ready for use	Isolated cells are ready for use

RT; room temperature (15 - 25°C)
*** Collect the entire enriched cell suspension, all at once, into a single pipette (e.g. for EasyEights™ 5 mL tube use a 2 mL serological pipette [Catalog #38002]; for EasyEights™ 14 mL tube use a 10 mL serological pipette [Catalog #38004]).

Notes and Tips

REMOVAL OF RESIDUAL RBCs IN THE ISOLATED CELLS

Typically, further RBC depletion is not required following cell isolation. If residual RBCs are visible in the isolated cell pellet following centrifugation after the end of the protocol, resuspend in a small volume (0.2 - 2.5 mL) of recommended medium or desired culture medium and place in a smaller EasySep™ magnet for an additional 5-minute separation. Collect the supernatant; the isolated cells are ready for use in downstream applications. Residual RBCs may also be lysed using Ammonium Chloride Solution (Catalog #07800).

ASSESSING PURITY

For purity assessment of T cells by flow cytometry use the following fluorochrome-conjugated antibody clones:

- Anti-Human CD3 Antibody, Clone UCHT1 (Catalog #60011), and
- Anti-Human CD45 Antibody, Clone HI30 (Catalog #60018)

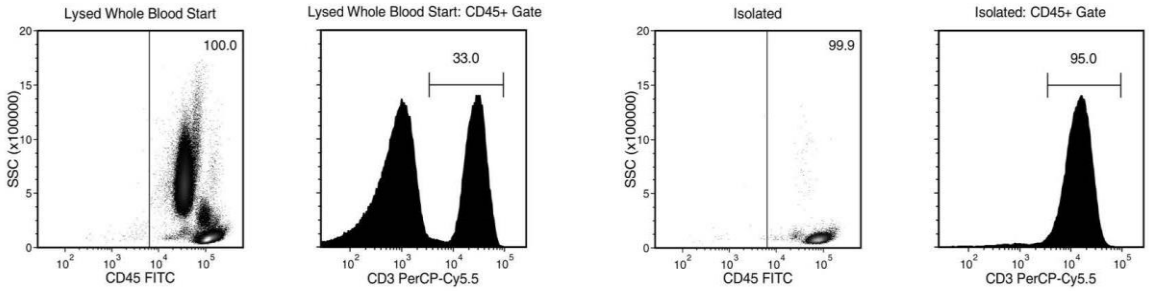
Purity may also be assessed using alternative fluorochrome-conjugated markers such as:

- Anti-Human CD4 Antibody, Clone OKT4 (Catalog #60016), and
- Anti-Human CD8 Antibody, Clone RPA-T4 (Catalog #60022), and
- Anti-Human CD45 Antibody, Clone HI30

NOTE: It is recommended to assess purity on the CD45-positive cells to exclude debris, platelets, and RBCs.

Data

Starting with human whole blood from normal healthy donors, the typical T cell (CD3+) content of the non-lysed final isolated fraction is 95.3 ± 1.4% (gated on CD45) or 94.9 ± 1.5% (not gated on CD45).



In the above example, the T cell (CD3+) content of the lysed whole blood start sample and non-lysed final isolated fraction is 33.0% and 95.0% (gated on CD45), respectively, or 33.0% and 94.9% (not gated on CD45), respectively. The starting frequency of T cells in the non-lysed whole blood start sample above is 0.059% (data not shown).

Appendix 2

Quantibody® Human Cytokine Array 1

Quantitative measurement of 20 human cytokines

Catalog #: QAH-CYT-1

User Manual
Last revised November 18, 2016

Caution:
Extraordinarily useful information enclosed

RayBiotech
Empowering your proteomics
ISO 13485 Certified

3607 Parkway Lane, Suite 100
Norcross, GA 30092
Tel: 1-888-494-8555 (Toll Free) or 770-729-2992, Fax:770-206-2393
Web: www.RayBiotech.com, Email: info@raybiotech.com

Table of Contents

Please read the entire manual carefully before starting your experiment

I. Overview

Cytokines Detected (20)	GM-CSF, GRO alpha/beta/gamma, IFN-gamma, IL-1 alpha (IL-1 F1), IL-1 beta (IL-1 F2), IL-10, IL-12 p70, IL-13, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), MCP-1 (CCL2), MIP-1 alpha (CCL3), MIP-1 beta (CCL4), MMP-9, RANTES (CCL5), TNF alpha, VEGF-A *See Section IX for Array Map*
Format	One standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody is arrayed in quadruplicate.
Detection Method	Fluorescence. Go to www.RayBiotech.com/Scanners for a list of compatible laser scanners.
Sample Volume	50 - 100 µl per array
Reproducibility	CV <20%
Assay Duration	6 hours

II. Introduction

Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation. They are involved in interactions between different cell types, cellular responses to environmental conditions, and maintenance of homeostasis. In addition, cytokines are also involved in most disease processes, including cancer and cardiac diseases.

The traditional method for cytokine detection and quantification is through the use of an enzyme-linked immunosorbent assay (ELISA). In this method, target protein is immobilized to a solid support. The immobilized protein is then complexed with an antibody that is linked to an enzyme. Detection of the enzyme complex can then be visualized through the use of a substrate that produces a detectable signal. While this traditional method works well for a single protein, the overall procedure is time consuming and requires a relatively high volume of sample. Thus, conservation of precious small sample quantities becomes a challenging task. Innovations in microarray technology over the last decade have addressed this problem. A long-standing leader in the field, Raybiotech, has pioneered the development of cytokine antibody arrays, which have now been widely applied in the research community with hundreds of peer reviewed publications, including top-tier journals such as *Cell* and *Nature*.

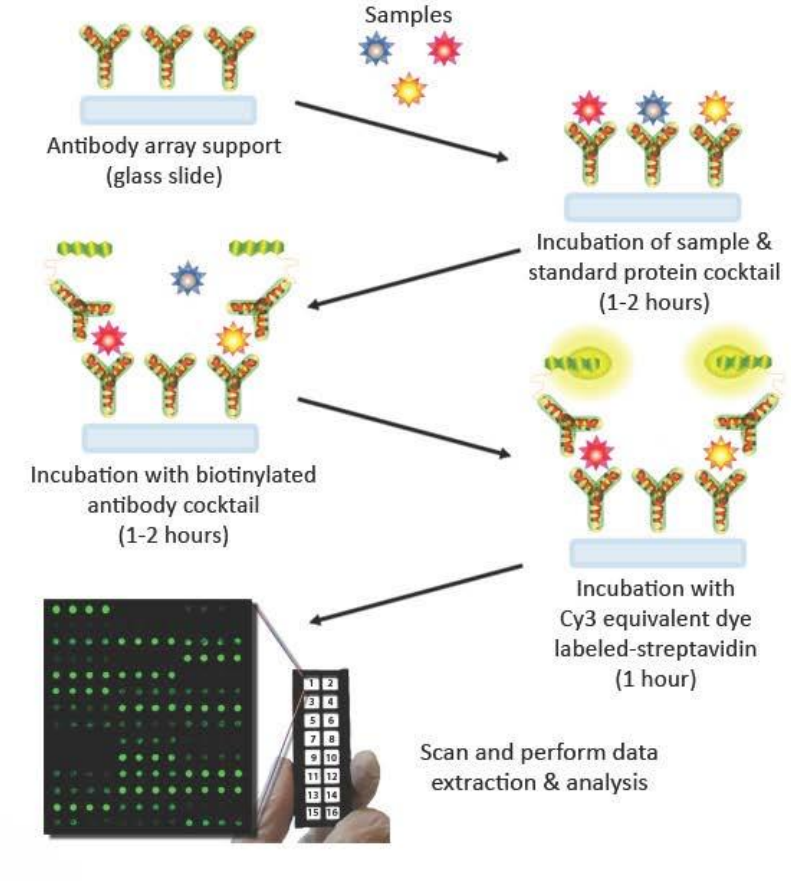
The Quantibody® array, our multiplexed sandwich ELISA-based quantitative array platform, enables

researchers to accurately determine the concentration of multiple cytokines simultaneously. It combines the advantages of the high detection sensitivity & specificity of ELISA and the high throughput of arrays. Like a traditional sandwich-based ELISA, it uses a pair of cytokine specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different epitope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-conjugated Cy3 equivalent dye, using a laser scanner. Unlike the traditional ELISA, Quantibody products use an array format. By arraying multiple cytokine specific capture antibodies onto a glass support, quantitative, multiplex detection of cytokines in one experiment is made possible.

In detail, one standard glass slide is divided into 16 wells of identical cytokine antibody arrays. Each antibody, together with the positive controls is arrayed in quadruplicate. The slide comes with a 16-well removable gasket which allows for the process of 16 samples on one slide. Four slides can be nested into a tray, which matches a standard microplate footprint and allows for automated robotic high throughput process of 64 arrays simultaneously. For cytokine quantification, the array specific cytokine standards, whose concentration has been predetermined, are provided to generate a standard curve for each cytokine. In a real experiment, standard cytokines and samples will be assayed in each array simultaneously through a sandwich ELISA procedure. By comparing signals from unknown samples to the standard curve, the cytokine concentration in the samples will be determined.

Quantibody® array kits have been confirmed to have similar detection sensitivity as traditional ELISA. Our current high density Quantibody kits allow scientists to quantitatively determine the concentration of 1000 human, 200 mouse, and 67 rat cytokines in a single experiment. This is not only one of the most efficient products on the market for cytokine quantification, but makes it more affordable for quantification of large number of proteins. Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool for drug and biomarker discovery.

III. How It Works



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IV. Materials Provided

	Catalog #	Component Name	1 Slide Box	2 Slide Box*
1	QAH-CYT-1S	Human Cytokine Array 1 Glass Slide	1	2
2	QA-SDB	Quantibody® Sample Diluent	15 ml	
3	AA-WB1-30ML	20X Wash Buffer I	2 x 30 ml	3 x 30 ml
4	AA-WB2-30ML	20X Wash Buffer II	30 ml	
5	QAH-CYT-1-STD	Human Cytokine Array 1 Lyophilized Standard Mix**	1 Vial	
6	QAH-CYT-1B	Human Cytokine Array 1 Biotinylated Antibody Cocktail	1-25 µl	2 x 1-25 µl
7	QA-CY3E	Cy3 equivalent dye-conjugated Streptavidin	5 µl	2 x 5 µl
8	QA-SWD	Slide Washer/Dryer	1 x 30 ml Tube	
9	QA-ADH	Adhesive Film	1	2

* 4 slide kits are comprised of 2 separate 2 slide kits.
** See Section X for detailed cytokine concentrations after reconstitution.

V. Storage

Upon receipt, all components should be stored at -20°C. The kit will retain activity for up to 6 months. Once thawed, the glass slide, standard mix, antibody cocktail and dye-conjugated Streptavidin should be kept at -20°C. All other components may be stored at 4°C. The entire kit should be used within 6 months of purchase.

VI. Additional Materials Required

- Benchtop rocker or orbital rocker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- 1.5 ml Polypropylene microcentrifuge tubes

VII. General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing conditioned media is required, it is highly recommended that complete medium be used as a control since many types of sera contains cytokines.
- We recommend the following parameters for your samples: 50 to 100 µl of original or diluted serum, plasma, cell culture media, or other body fluid, or 250 µg/ml-1 mg/ml (after a 5-fold to 10-fold dilution to minimize the effects of any detergent(s)) of protein for cell and tissue lysates.
  If you experience high background or if the fluorescent signal intensities exceed the detection range, further dilution of your sample is recommended.

B. Handling Glass Slides

- Do not touch the surface of the slides, as the microarray slides are very sensitive. Hold the slides by the edges only.
- Handle all buffers and slides with powder free gloves.
- Handle glass slide/s in clean environment.
- The Quantibody slides do not have bar codes. To help distinguish one slide from another, transcribe the slide serial number from the slide bag to the back of the slide with an ultra-fine point permanent marker. **Please Note:** Red and black permanent markers can significantly interfere with fluorescent signal detection. We recommend marking your slides with a green or blue ultra-fine point permanent marker. Please write the number on the very bottom edge of the slide. Do not write on the arrayed well areas.

C. Incubation

- Completely cover array area with sample or buffer during incubation.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rocking or rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or <70 µl of sample or reagent is used.
- Several incubation steps such as step 6 (blocking), step 7 (sample incubation), step 10 (detection antibody incubation), or step 13 (Cy3 equivalent dye-streptavidin incubation) may be done overnight at 4°C. Please make sure to cover the incubation chamber tightly to prevent evaporation.

VIII. Protocol

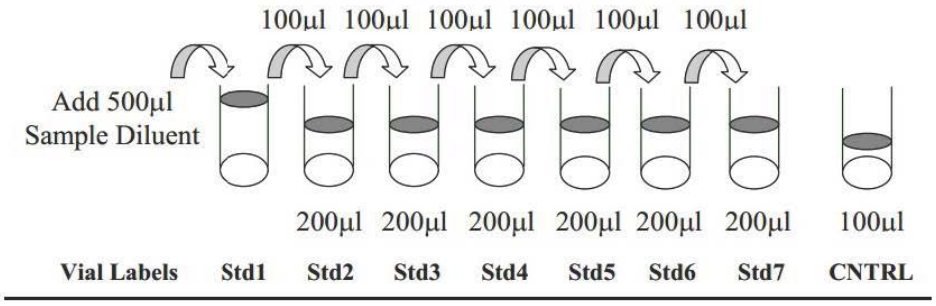
A. Completely Air Dry The Glass Slide

1. Take out the glass slide from the box, and let it equilibrate to room temperature inside the sealed plastic bag for 20-30 minutes. Remove slide from the plastic bag, peel off the cover film, and let it air dry for another 1-2 hours.

Incomplete drying of slides before use may cause the formation of "comet tails," thin directional smearing of antibody spots.

B. Prepare Cytokine Standard Dilutions

There is only one vial of standard provided in the two-slide kit, which is enough for making two standard curves. Reconstitute the lyophilized standard within one hour of usage. If you must use the standard for two different days, store only the Std1 dilution at -80°C.



2. Reconstitute the Cytokine Standard Mix (lyophilized) by adding 500 µl Sample Diluent to the tube. For best recovery, always quick-spin vial prior to opening. Dissolve the powder thoroughly by a gentle mix. Labeled the tube as Std1.

3. Label 6 clean microcentrifuge tubes as Std2 to Std7. Add 200 µl Sample Diluent to each of the tubes.

4. Pipette 100 µl Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 100 µl Std2 to tube Std3 and so on.

5. Add 100 µl Sample Diluent to another tube labeled as CNTRL. Do not add standard cytokines or samples to the CNTRL tube, which will be used as negative control. For best results, include a set of standards in each slide.

Since the starting concentration of each cytokine is different, the serial concentrations from Std1 to Std7 for each cytokine are varied which can be found in Section X.

C. Blocking & Incubation

6. Add 100 µl Sample Diluent into each well and incubate at room temperature for 30 minutes to block slides.

7. Decant buffer from each well. Add 100 µl standard cytokines or samples to each well. Incubate arrays at room temperature for 1-2 hour.

Longer incubation time is preferable for higher signals. This step may be done overnight at 4°C.

We recommend using 50 to 100 µl of original or diluted serum, plasma, conditioned media, or other body fluid, or 250 µg/ml-1 mg/ml of protein for cell and tissue lysates. Cover the incubation chamber with adhesive film during incubation, especially if less than 70 ul of sample or reagent is used.

8. Wash:

- Decant the samples from each well, and wash 5 times (5 min each) with 150 µl of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step. Dilute 20x Wash Buffer I with H2O.

- *(Optional for Cell and Tissue Lysates)* Put the glass slide with frame into a box with 1X Wash Buffer I (cover the whole glass slide and frame with Wash Buffer I), and wash at room temperature with gentle rocking for 20 min.

- Decant the 1x Wash Buffer I from each well, wash 2 times (5 min each) with 150 µl of 1X Wash Buffer II at room temperature with gentle rocking. Completely remove wash buffer in each wash step. Dilute 20X Wash Buffer II with H2O.

Incomplete removal of the wash buffer in each wash step may cause "dark spots," the background signals higher than the spots.

D. Incubation with Biotinylated Antibody Cocktail & Wash

9. Reconstitute the detection antibody by adding 1.4 ml of Sample Diluent to the tube. Spin briefly.

10. Add 80 µl of the detection antibody cocktail to each well. Incubate at room temperature for 1-2 hour.

Longer incubation time is preferable for higher signals and backgrounds

11. Decant the samples from each well, and wash 5 times (5 mins each) with 150 µl of 1X Wash Buffer I and then 2 times with 150 µl of 1x Wash Buffer II at room temperature with gentle rocking. Completely remove wash buffer in each wash step.

E. Incubation with Cy3 Equivalent Dye-Streptavidin & Wash

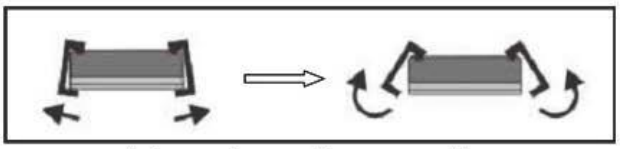
12. After briefly spinning down, add 1.4 ml of Sample Diluent to Cy3 equivalent dye-conjugated streptavidin tube. Mix gently.

13. Add 80 µl of Cy3 equivalent dye-conjugated streptavidin to each well. Cover the device with aluminum foil to avoid exposure to light or incubate in dark room. Incubate at room temperature for 1 hour.

14. Decant the samples from each well, and wash 5 times (5 mins each) with 150 µl of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step.

F. Fluorescence Detection

15. Disassemble the device by pushing clips outward from the slide side. Carefully remove the slide from the gasket.



Be careful not to touch the surface of the array side.

16. Place the slide in the Slide Washer/Dryer (a 4-slide holder/centrifuge tube), add enough 1x Wash Buffer I (about 30 ml) to cover the whole slide, and then gently shake at room temperature for 15 minutes. Decant Wash Buffer I. Wash with 1x Wash Buffer II (about 30 ml) and gently shake at room temperature for 5 minutes.

17. Remove water droplets completely by gently applying suction with a pipette to remove water droplets. Do not touch the array, only the sides.

    You may also dry the glass slide by a compressed N_2 stream.

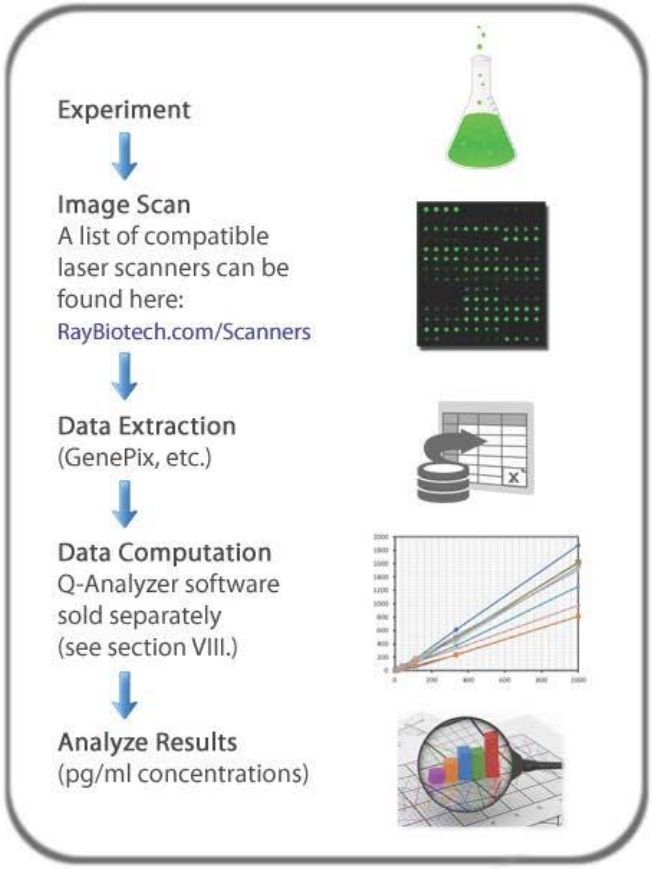
18. Imaging: The signals can be visualized through use of a laser scanner equipped with a Cy3 wavelength (green channel) such as Axon GenePix, or Innopsys Innoscan. Make sure that the signal from the well containing the highest standard concentration (Std1) receives the highest possible reading, yet remains unsaturated.

In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans, with a higher PMT for low signal cytokines, and a low PMT for high signal cytokines.

G. Data Analysis

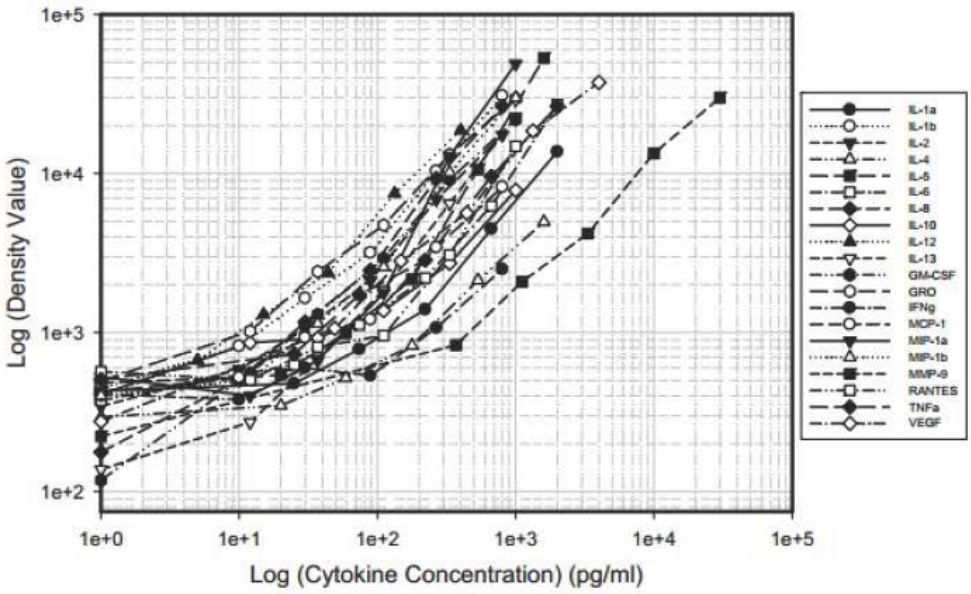
19. Data extraction can be done using the GAL file that is specific for this array along with the microarray analysis software (GenePix, ScanArray Express, ArrayVision, MicroVigene, etc.). GAL files can be found here: www.RayBiotech.com/Gal-Files.html.

    Need help analyzing all that data? Copy and paste your data into the Q-Analyzer Tool specific for this array, catalog number: **QAH-CYT-1-SW**. More information can be found in Section XII.



IX. Array Map & Standard Curves

Each antibody is printed in quadruplicate horizontally								
	1	2	3	4	1	2	3	4
A	POS1				POS2			
B	IL-1 alpha				IL-1 beta			
C	IL-2				IL-4			
D	IL-5				IL-6			
E	IL-8 (CXCL8)				IL-10			
F	IL-12 p70				IL-13			
G	GM-CSF				GRO			
H	IFN gamma				MCP-1 (CCL2)			
I	MIP-1 alpha (CCL3)				MIP-1 beta (CCL4)			
J	MMP-9				RANTES (CCL5)			
K	TNF-alpha				VEGF			



X. Standard Concentrations

After reconstitution, the lyophilized cytokine standard mix contains the following concentrations for each antigen included.

Serial standard concentration (pg/ml)

(pg/ml)	Cntrl	Std7	Std6	Std5	Std4	Std3	Std2	Std1
IL-1α	0	3	8	25	74	222	667	2,000
IL-1β	0	3	8	25	74	222	667	2,000
IL-2	0	5	16	49	148	444	1,333	4,000
IL-4	0	3	8	25	74	222	667	2,000
IL-5	0	3	8	25	74	222	667	2,000
IL-6	0	3	8	25	74	222	667	2,000
IL-8	0	1	2	5	15	44	133	400
IL-10	0	3	8	25	74	222	667	2,000
IL-12p70	0	1	4	12	37	111	333	1,000
IL-13	0	1	4	12	37	111	333	1,000
GM-CSF	0	3	8	25	74	222	667	2,000
GRO	0	3	8	25	74	222	667	2,000
IFNγ	0	14	41	123	370	1,111	3,333	10,000
MCP-1	0	3	8	25	74	222	667	2,000
MIP-1α	0	14	41	123	370	1,111	3,333	10,000
MIP-1β	0	1	4	12	37	111	333	1,000
MMP-9	0	14	41	123	370	1,111	3,333	10,000
RANTES	0	3	8	25	74	222	667	2,000
TNFα	0	3	8	25	74	222	667	2,000
VEGF	0	5	16	49	148	444	1,333	4,000

XI. Spiking & Recovery

The antibody pairs used in the kit have been tested to recognize their specific antigen. The spiking and recovery rates of each cytokine in 2x diluted serum (SR), plasma EDTA (PLE), plasma citrate (PLC), plasma heparin (PLH), and culture media (CM) are listed in the following tables.

The spiking recovery rate for human culture media and serum

Cytokine	Spiking	CM	CM+Ag	CM%	Serum	Serum+Ag	Serum%
IL-1α	1000	6	817	81%	0	1103	110%
IL-1β	400	1	324	81%	0	437	109%
IL-2	400	0	348	87%	2	324	81%
IL-4	800	1	948	118%	1	824	103%
IL-5	800	0	571	71%	2	667	83%
IL-6	1000	178	1102	92%	1	830	83%
IL-8	400	over	over	-	8	437	107%
IL-10	500	14	555	108%	33	410	75%
IL-12p70	200	0	227	114%	1	158	78%
IL-13	500	2	451	90%	0	459	92%
GM-CSF	500	56	587	106%	0	511	102%
GRO	500	5	607	120%	49	604	111%
IFNγ	400	1	468	117%	20	422	100%
MCP-1	400	over	over	-	851	1325	119%
MIP-1α	800	0	1014	127%	0	761	95%
MIP-1β	500	0	569	114%	48	643	119%
MMP-9	15000	1	10888	73%	15279	27964	85%
RANTES	1000	0	918	92%	3258	4288	103%
TNFα	1000	4	840	84%	0	1035	103%
VEGF	2000	2651	4596	97%	3	2424	121%

XII. Quantibody® Q-Analyzer

The Q-Analyzer is an array specific, Excel-based program. It is much more than a simple calculation macro; it performs sophisticated data analysis (see below for description).

The Q-Analyzer Tool specific for this array is catalog number: **QAH-CYT-1-SW**.

Key features:

- Simplicity: Easy to operate and requires no professional training. With a simple copy and paste process, the cytokine concentration is determined.
- Outlier Marking & Removing: The software can automatically mark and remove the outlier spots for more accurate data analysis
- Normalization: The program allows for intra- and inter-slide normalization for large numbers of samples.
- Two Positive Controls: The program utilizes the two positive controls in each array for normalization.
- Two Analytical Algorithms: Users can choose either linear regression or log-log algorithms to meet their analytical needs.
- Two Data Outputs: standard curves and digital concentration.
- User Intervention: The program allows for user manual handling of outliers and other analytical data.
- Lower and Upper Limits Determination: The program automatically marks out the values below or above the detection range.
- Standard Deviation: The program outputs the standard deviations of the quadruplicate spots for data accuracy.
- Analytical Tips: Q-Analyzer analysis tips are included in the program.

XIII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Increase incubation time or change sample incubation step to overnight
	Too low protein concentration in sample	Lessen dilution or do not dilute sample. Concentrate sample if necessary.
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven signal	Bubble formed during incubation	Decrease amount of rocking during incubations. check for bubble formation and remove bubbles.
	Arrays are not completed covered by reagent	Completely cover arrays with solution for all required steps.
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
Poor standard curve	Cross-contamination from neighboring wells	Avoid overflowing wash buffer and other solutions into neighboring wells.
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate standard reconstitution or Improper dilution	Reconstitute the lyophilized standard well at the room temperature before making serial dilutions. Check pipettes and ensure proper serial dilutions.
	Inadequate detection	Increase laser power so the highest standard concentration for each cytokine receives the highest possible reading yet remains unsaturated.
	Use freeze-thawed cytokine standards	Always use new cytokine standard vial for new set of experiment. Discard any leftover.
High background	Overexposure	Lower the PMT or signal gain.
	Dark spots	Completely remove wash buffer in each wash step.
	Insufficient wash	Increase wash time and use more wash buffer
	Dust	Work in clean environment
	Slide is allowed to dry out	Don't dry out slides during experiment.

XIV. Publications Citing This Product

1. Sharma M., Anderson S., Schoop R., Hudson J., Induction of multiple pro-inflammatory cytokines by respiratory viruses andreversal by standardized Echinacea, a potent antiviral herbal extract. Antiviral Research 83 (2009) 165-170
   Species: Human
   Sample Type: Conditioned Media

2. Jiang, Weidong, Ruo-Pan Huang, and Ruochun Huang. "Protein Expression Profiling by Antibody Array Analysis with Use of Dried Blood Spot Samples on Filter Paper." Journal of Immunological Methods. ScienceDirect, 25 Nov. 2013. Web.
   Species: Human
   Sample Type: Serum

3. Zhan Y., Zou S., Hua F., Li F., Ji L., Wang W., Ye Y., Sun L., Chen H., Cheng Y. High-dose dexamethasone modulates serum cytokine profile in patients with primary immune thrombocytopenia. Immunology Letters Volume 160, Issue 1, July 2014, Pages 33-38
   Species: Human
   Sample Type: Serum

4. Wu H., et al. Upregulation of innate immune responses in a T cell/histiocyte-rich large B cell lymphoma patient with significant autoimmune disorders mimicking systemic lupus erythematosus. Annals of Hematology (2014) 93:353-354
   Species: Human
   Sample Type: Plasma

More citations for this product may be available.
Contact techsupport@raybiotech.com.

XV. Experiment Record Form

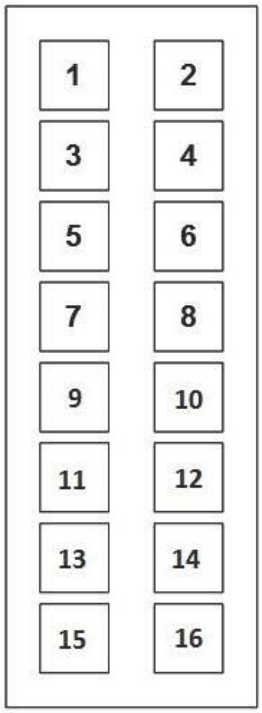
Date:_____

File Name:_____

Laser Power:_____

PMT:_____

Well No.	Sample Name	Dilution factor
1	CNTRL	
2	Std7	
3	Std6	
4	Std5	
5	Std4	
6	Std3	
7	Std2	
8	Std1	
9		
10		
11		
12		
13		
14		
15		
16		



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XVI. How to Choose a Quantibody® Array?

Species-based selection:

Human (QAH-)	Mouse (QAM-)	Rat (QAR-)	Bovine (QAB-)	Canine (QAC-)
Equine (QAE-)	Feline (QAF-)	Primates (QAN-)	Porcine (QAP-)	Rabbit (QAL-)

Function-based selection:

Adhesion Molecule Arrays	Angiogenesis Arrays	Bone Metabolism Arrays	Chemokine Arrays
Custom Arrays	Cytokine Arrays	Growth Factor Arrays	IGF Signaling Arrays
IL-1 Family Arrays	Immune Response Arrays	Inflammation Arrays	Interleukin Arrays
Isotyping Arrays	MMP Arrays	Obesity Arrays	Ophthalmic Arrays
Periodontal Disease Arrays	Receptor Arrays	Th1/Th2/Th17 Arrays	

Cytokine Number-based selection:
Arrays are available in the Quantibody® platform to detect 1000 human, 200 mouse, or 67 rat proteins. GLP-Compliant testing services are also available.

To learn more about the Quantibody® Antibody Array, visit
www.RayBiotech.com/Quantibody-Multiplex-Elisa-Array.html

تتوقع شدة تليف الكبد في المرضى الذين يعانون من (MAIT)خلايا التائية المحيطة بالأغشية (NAFLD) مرض الكبد الدهني غير الكحولي

اعداد: دانيه عيسى عبد الفتاح البكري

إشراف: د.جوني عامر

ملخص:

مقدمة : خلايا (MAIT) تتكون من مجموعة فرعية من الخلايا التائية التي يمكن تنشيطها بالمنتجات البكتيرية والسيتوكينات لإنتاج TNF-α و IL-17 و granzymes. لا يعرف سوى القليل عن دور خلايا MAIT في تطور التليف في مرض الكبد الدهني غير الكحولي.

الأهداف : تقييم عدد خلايا MAIT والعلامات الظاهرية لها ومدى ارتباط هذه الظواهر مع شدة تليف الكبد في مرضى NAFLD.

الطرق والمواد : تم مشاركة 25 مريضا بالكبد الدهني غير الكحولي العمر، و 5 اصحاء في هذه الدراسة. مؤشر كتلة الجسم، ناقلة الامين الألانين(ALT)، ومستويات الانسولين في الصيام ،و مستوى تخزين السكر ،و مقاومة الانسولين (HOMA-IR) ،و الدهون الثلاثية،و البروتين الدهني مرتفع الكثافة،و البروتين الدهني منخفض الكثافة و البروتين المتفاعلC (CRP) تم ربطها مع درجات التليف في حالات NAFLD في البالغين الذين يفتقرون إلى متلازمة الأيض أو غيرها من مسببات الكبد. في المختبر ، تم الحصول على عينات الدم من المتبرعين الأصحاء والمرضى NAFLD. و باستخدام direct human T cell isolation kit، حصلنا على خلايا تائية نقية. ثم باستخدام قياس التدفق الخلوي(Flow cytometry)، حددنا خلايا MAIT التي تحتوي على CD161 و TCR

Vα7.2. كما حددنا علامات مثل TNFα و CD69 و CD38 وIL17 لفحص العلامات المناعية للخلايا. و باستخدام quantibody human cytokine array 1، اكتشفنا مستويات السيتوكين في الدم. باستخدام قياس التدفق الخلويFlow cytometry ، حددنا وجود مستقبلات الأنسولين على سطح خلايا MAIT في ثلاثة مرضى من الدرجة الرابعة.

النتائج : 25 حالة استوفت معايير التضمين / الاستبعاد للعينات، جميع العينات كانت لذكور ، متوسط العمر عند الخزعة 39.3 ± 9.8سنة، ومتوسط مؤشر الجسم كان 9 ± 29.19. سبعة يعانون من تشمع الكبد حيث كانوا يعانون من التليف من الدرجة الرابعة. (HOMA-IR) وجد انه أهم مؤشر لخطورة التليف. كان لدى المتبرعين الأصحاء 12.68٪ ± 1.11 خلية MAIT وتم ملاحظة نقص ملحوظ في خلايا MAIT للمرضى الذين يعانون من NAFLD إلى 10.62٪ ± 1.37 و 3.9٪ ± 0.71 و 1.66٪ ± 0.08 و 1.2٪ ± 0.21 مع درجات التليف F1وF2 و F3 و F4،على التوالي. علاوة على ذلك ، أظهرت خلايا MAIT من المرضى ارتفاع في علامات التنشيط مثل CD69 و CD38،مقارنة مع المانحين الاصحاء. كانت خلايا MAIT تظهر أيضا ارتفاع في IL-17، وهو سيتوكين مؤيد للألياف ، فضلا عن TNFα ، وهو سيتوكين مؤيد للالتهاب. كانت هذه النتائج مترابطة خطيًا مع شدة التليف. كما لاحظنا ارتفاع في السيتوكينات الالتهابية بما في ذلك IL-12 الذي يعرف بتنشيط خلايا MAIT. لوحظ ايضا انخفاض في مستقبلات الأنسولين في مرضى F4 مقارنة مع المتبرعين الأصحاء.

الاستنتاج : انخفاض خلايا MAIT في مرضى NAFLD ترتبط ارتباطا عكسيا مع مرحلة التليف. تشير بياناتنا إلى أن خلايا MAIT قد تساهم في تطور التليف في مرضى NAFLD وقد تمثل هدفاً تشخيصياً جديدًا.