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THE ROLE OF INNATE IMMUNITY IN OBESITY-INDUCED TISSUE INFLAMMATION

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To my family

POPULAR SCIENCE SUMMARY OF THE THESIS

The immune system is the body's defense against external and internal threats, including everything from bacteria and viruses that cause infections to the transformation of our own cells into cancer cells. The immune system consists of a diverse set of cells, which need to function fully for our bodies to stay protected. However, several previous studies have shown that obesity, which is becoming an increasingly common condition, can sometimes disturb our immune system, making us more susceptible to viruses or cancer development. However, exactly how obesity affects the immune system is not fully known. Therefore, the studies included in this thesis attempts to improve our knowledge of obesity-induced tissue inflammation. This knowledge is needed in order to improve our treatment of obesity-associated diseases and in our understanding of how obesity can coexist with viral infections or malignancies.

Obesity is often accompanied with a low-grade inflammation which, unknowingly to the individual, may develop and progress for years thus affecting several different organs. In this thesis, obesity-induced inflammation in the adipose tissue and liver is studied in the context of obesity, as well as in the obesity induced liver disease non-alcoholic fatty liver disease (NAFLD). Our studies focus on two important parts of the immune system. The first, the macrophages, are important cells that can sense incoming threats, engulf them and then present proteins of the threats to other parts of the immune system, e.g., T cells, that can then be activated. The second, NK cells are cells that can recognize cells of the body that are going through different kind of stress, e.g., have turned in to malignant cells or have been infected by viruses.

The studies included in this thesis thus demonstrate how these cells were affected in liver and adipose tissue (**study I** and **study II**), as well as the surrounding environment in the circulation (**study III**). We identify possible novel subsets (**study I**), novel surface markers (**study II**) and potential biomarkers of obesity-induced liver disease (**study III**).

While future studies are needed to corroborate, validate and further expand on our results, the data presented in the study adds new knowledge to the role of innate immunity in obesity-induced tissue inflammation.

ABSTRACT

The prevalence of obesity is rapidly increasing globally, which causes an increase in associated diseases such as type 2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD). In addition to the detrimental effect of obesity on several organs such as the liver and adipose tissue, obesity also has a profound effect on the immune system. The immune system consists of a complex interplay of different immune cells and other factors interacting with each other and the surrounding tissue in order to protect the body from both external (e.g., infectious) and internal (e.g., cancer) threats. As part of the innate immune system, natural killer (NK) cells play an important part in the defense against viral infections as well as malignancies. Another innate immune cell population, macrophages, are common immune cells that can perform a multitude of different functions, ranging from initiating immune responses, act as scavengers and contribute to wound healing. Recent studies have suggested that obesity, and the chronic low-grade inflammation that accompanies it, disturbs the innate immune system which could lead to impairments in its protective functions and thereby contribute to disease development. However, the complexity of the heterogeneous functions and forms of innate immunity are only partially understood. The overall aim of this thesis is to add knowledge on how innate immunity is affected by obesity in order to improve our understanding and, in the future, treatment of obesity-related diseases.

In **study I**, the NK cell population in patients with the hepatic presentation of the metabolic syndrome nonalcoholic fatty liver disease (NAFLD) was examined. The receptor repertoire and function of circulating NK cells from patients with non-alcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH) and healthy controls were compared. In addition, tissue-derived NK cells derived from adipose tissue and liver were assessed. The results show that while some receptors, such as NKG2D, had increased on NK cells in NASH compared to healthy controls, the NK cell population remains mainly unaltered by NAFLD. This is of interest as NK cells are believed to possess anti-fibrotic properties, whilst they are also important mediators of innate immunity in other liver diseases, such as hepatitis C. In addition, analysis of adipose tissue derived NK cells demonstrated a population of cells with tissue residency markers such as CD49 and CD69. While further explored since then, this was at the time one of the first reports of tissue resident NK cells in adipose tissue. However, future studies are warranted to increase the knowledge of these cells and how they are related to human disease and obesity development.

In **study II** the surface proteome of adipose tissue macrophages (ATMs) was examined to increase the knowledge of this heterogeneous population of immune cells. Adipose tissue macrophages are key players in adipose tissue inflammation. Since the composition and localization of adipose tissue depots in humans affect the risk of metabolic complications, macrophages derived from different anatomical niches such as visceral and subcutaneous adipose tissue were studied. The results include novel surface proteins associated with pro- and anti-inflammatory phenotypes of adipose tissue macrophages, as well as considerable differences between macrophages derived from subcutaneous adipose tissue (SAT) and

visceral adipose tissue (VAT). Three novel surface markers (CD85a, CD48 and CD371) were identified on adipose tissue derived M1-like macrophages and CD51 and α9β1 on M2-macrophages. Finally, the presence of insulin resistance was linked to the relative increase in pro-inflammatory macrophages in both SAT and VAT, supporting an association between adipose tissue inflammation and insulin resistance.

In the final project, **study III**, the immune proteome in serum of NASH patients was measured to find a signature that separates NASH patients from those without ongoing liver inflammation (NAFL) in order to improve diagnostics. Thus, serum was collected from NAFL-patients, NASH-patients as well as from healthy, lean controls. In total, 92 inflammatory serum proteins were measured using a proximity extension assay (PEA). The results revealed a NASH-specific inflammatory imprint with 13 serum proteins which, independently of comorbidities and fibrosis stage, differed between NAFL and NASH patients. Out of these proteins, sulfotransferase A1 (ST1A1) and stem cell factor (SCF) were found in higher levels in NASH-patients compared to NAFL and healthy controls. Pathway analysis of deregulated proteins revealed dysregulation in several pathways in NASH, including NFκβ and IL-18 signaling. Analysis of IL-18, EN-RAGE and ST1A1 in a pre-existing single cell transcription data set derived from liver cells suggested involvement of hepatic macrophages and periportal hepatocytes in NASH pathogenesis. Finally, the differentially expressed proteins could also be used to subgroup NASH patients into different clusters. Taken together, **study III** increases our understanding of the circulating serum proteome in NASH, suggesting a way to distinguish NASH-patients from NAFL and provides insight into possible subgroups and pathogenesis. However, these results need further validation in larger cohorts as well as further analysis both over time and regarding disease progression.

In conclusion, the results presented in this thesis provide novel data on the effect obesity has on key players of innate immunity. While there is an inflammatory imprint in the serum proteome of NASH-patients, circulating NK cells retain their phenotype compared to controls. While tissue-resident NK cells previously have been identified in the liver, we provide one of the early reports of NK cells in visceral adipose tissue that display tissue residency markers. In addition, we demonstrate significant heterogeneity within the macrophage population in adipose tissue and we compare ATMs from subcutaneous and visceral adipose tissue, linking this to the development of insulin resistance. In conclusion, we have added a couple of pieces to the jigsaw puzzle that is human immunology, but still future studies are warranted to further elucidate and validate our results and to in-depth investigate their effect on human disease development.

LIST OF SCIENTIFIC PAPERS

I. Stiglund N, Strand K, Cornillet M, Stål P, Thorell A, Zimmer CL, Näslund E, Karlgren S, Nilsson H, Mellgren M, Fernø J, Hagström H, Björkström NK. Retained NK Cell Phenotype and Functionality in Non-alcoholic Fatty Liver Disease. Front Immunol. 2019 Jun 4;10:1255.

II. Strand, K., Stiglund, N., Eimstad Haugstoyl, M., Kamyab, Z., Langhelle, V, Dyer, L., Busch, C., Cornillet, M., Drange Hjellestad, I., Nielsen, HJ., Njolstad, PR., Mellgren, G., Björkström, NK., Ferno, J. Subtype-Specific Surface Proteins on Adipose Tissue Macrophages and Their Association to Obesity-Induced Insulin Resistance. Front Endocrinol. 2022 Apr 11;13:856530.

III. Stiglund N, Hagström H, Stål P, Cornillet M, Björkström NK. Deregulated Peripheral Proteome Reveals NASH-specific Signature Identifying Patient Subgroups With Distinct Liver Biology. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ADA	Adenosine deaminase
ADCC	Antibody-dependent cellular cytotoxicity
AT	Adipose tissue
ATM	Adipose tissue macrophage
AUROC	Area under Reciever operating curve
BMI	Body mass index
BLIMP1	B lymphocyte-induced maturation protein-1
CAR	Chimeric antigen
CCL2	C-C motif chemokine ligand 2
CCR2	C-C motif chemokine receptor 2
CD	Class of differentiation
CLS	Crown-like structure
CNS	Central nervous system
CMV	Cytomegalovirus
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
ELISA	Enzyme-Linked ImmunoSorbent Assay
EN-RAGE	Extracellular Newly identified Receptor for Advanced Glycation End Products
ER	Endoplasmic reticulum
FASL	FAS ligand
FFA	Free fatty acid
FIB-4	Fibrosis-4
FLt3L	FMS-like tyrsoine kinase 3 ligand
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HFD	High fat diet
HOMA-IR	Homeostatic model assessment of insulin resistance
HSC	Hepatic stellate cell
IBD	Inflammatory bowel disease

IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cell
KC	Kupffer cell
KIR	Killer cell immunoglobulin-like receptor
LAM	Lipid-associated macrophage
MAFLD	Metabolic dysfunction-associated fatty liver disease
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MICA/B	MHC I chain-related protien A/B
MME	Metabolically activated macrophage
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Nonalcoholic steatohepatitis
NFκB	Nuclear factor κB
NK	Natural killer
NKG2D	Natural killer group 2D
NLR	Node like receptor
NLRP3	Node like receptor protein 3
PAI	Plasminogen activator inhibitor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PEA	Proximity extension assay
PNPLA3	Patatin-like phospolipase domain-containing protein 3
SAT	Subcutaneous adipose tissue
SCF	Stem cell factor
SLAMF	signaling lymphocytic activation molecule family
ST1A1	Sulfotransferase 1A1
SVF	Stromal vascular fraction

T2DM	Type 2 diabetes mellitus
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TRAIL	TNF-related apoptosis-induced ligand
Tregs	Regulatory T cells
ULBP1	UL16 binding protein
UMAP	Uniform manifold appoximation and projection
VAT	Visceral adipose tissue

INTRODUCTION

1.1 GENERAL INTRODUCTION

The prevalence of obesity has risen exponentially during the last decades, which has led to a rapid increase in associated diseases such as type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and nonalcoholic fatty liver disease (NAFLD)[1–3]. Obesity has been linked to the development of several cancers, e.g., esophageal adenocarcinoma, endometrial, colon and renal cancers[4–6]. In addition to the substantial morbidity and decreased quality of life for patients, including a loss of up to 20 years in life-expectancy, the obesity pandemic puts a strain on health care systems globally[3,7]. The need to further understand the processes that give rise to complications such as type 2 diabetes and obesity-induced liver inflammation (NAFLD) is therefore necessary to improve current treatment strategies. Several studies have reported that obesity has a negative impact on the immune system and its ability to combat cancer as well as several infections. The most recent example where obesity is a risk factor for severe infections is the COVID-19 pandemic[8–10]. Thus, it is important to increase the knowledge of how obesity affect the immune system in order to improve our treatment strategies. To understand obesity-induced inflammation it is important to first get to know the immune system and its key players.

1.1.1 The immune system

Ever since Edward Jenner created the first vaccine in 1798, generations of scientists have spent their lives studying the immune system. As the main defense against pathogens, a functional immune system is an absolute requirement for life. The past couple of years, the increased knowledge of the immune system has given rise to several new revolutionary cancer treatments, such as the chimeric antigen receptor (CAR) T cells[11,12] and the immune checkpoint inhibitors[13,14].These have drastically improved the prognosis of certain subgroups of cancer patients and show much promise[13,14]. In several autoimmune and chronic inflammatory diseases, e.g., inflammatory bowel disease (IBD), many new treatments target mediators of inflammation, such as tumor necrosis factor (TNF) and interleukin (IL)-23. During the COVID-19 pandemic, immune-modulatory treatments, such as corticosteroids and IL-6 inhibitors has been an integral part of the treatment associated with increased survival rate. Thus, many of our clinical treatments today utilize our knowledge of the immune system. However, the understanding of the immune system is still rudimental. In recent decades, there has been an increased understanding of more specialized immunological niches in different organs, such as the uterus or liver. The immune system seems to have adapted to the different requirements of various organs. Two examples of unique situations to which the immune system must adapt are the tolerant environment in a liver continuously exposed to antigens from the gut, or the uterus when two individuals coexist during pregnancy. NK cells derived from the uterus have a unique phenotype that differ from the NK cells that are normally found circulating in blood. Also, in T cell biology, an increased understanding has arisen regarding tissue resident cells in contrast to circulating T cells[15–17].

However, exactly how immune cells have adapted in many organs, such as adipose tissue, remains largely unknown. In addition to the concept of tissue residency, recent years have highlighted more functions of the immune system than previously known. While the pivotal role of the immune system in inflammatory processes for long has been recognized, such as wound healing or autoimmunity, only in recent years have the role of immunity in other important pathogenic processes such as fibrosis development in the liver or adipose tissue inflammation been appreciated.

1.1.2 Innate immunity

The immune system has traditionally been divided into innate and adaptive immunity. The innate immune system contains the initial, rapid response to pathogens and other stimuli that occurs within minutes to hours, in contrast to adaptive immunity that takes days to weeks to initiate. In general, innate immunity is more unspecific and encompasses recognition of classes of molecules, e.g., damage-associated molecular patterns (DAMPs), or pathogen-associated molecular patterns (PAMPs), which detects features like cell death or infection. The adaptive immune system, on the other hand, develops slower and tends to be more specific towards pathogens. It mainly features lymphocytes that generate cellular immunity, such as T cells, or humoral immunity, including B cells and antibodies, and is important for generation of the long-term immunological memory. However, the lines between adaptive and innate immunity are sometimes blurred, such as in the case of invariant T cells that display both innate and adaptive features.

The innate immune system is the first to respond to immunological triggers, such as infections. The initial immune response involves many diverse features, from epithelial barriers, the complement system and antimicrobial peptides to a range of immune cells with a plethora of different functions. Innate immunity has an important role in the initial response not only against pathogens, but also in other important processes such as wound healing or other sterile inflammations. The innate response often includes sentinel cells, such as dendritic cells or macrophages. These can sense stimuli and then initiate immune responses and effector functions, e.g., from cells such as NK cells that can rapidly respond to virus infected cells. Recently, the innate/adaptive paradigm has become partially blurred with several cell types and immunological features that exist in the borderland between innate and adaptive immunity. One such example is the nonconventional T cells, that display both innate and adaptive features. Another example is NK cells, previously firmly categorized within the innate immune system that has recently been appreciated to have both memory-like features as well as showing specific responses, towards e.g., cytomegalovirus (CMV)[18,19]. Both are properties previously recognized as confined within the concept of adaptive immunity.

1.1.3 Natural killer (NK) cells

NK cells are important effectors of the innate immune system. In peripheral blood, NK cells comprise approximately 15 % of the circulating lymphocytes. As part of the group 1 innate lymphoid cells (ILCs), the main role of NK cells has been considered to be defense against

viral infections and malignancies[20,21]. NK cells arise from progenitors in the bone marrow and then either circulate in the blood or infiltrate tissues, such as liver, adipose tissue or the uterus[22]. The action of NK cells is determined through signaling via an array of activating and inhibitory receptors on the cell surface, e.g., FASL and TRAIL-receptor[23–26]. One model of NK cell activation is by "missing self" where NK cells are activated in response to cells that lack or have downregulated major histocompatibility complex (MHC) class I expression, which is a common mechanism for viruses or tumors to evade cytotoxic T cell responses. Inflammatory conditions can upregulate stress ligands for activating receptors on NK cells on different cell types in response to a variety of stress signals. This enables NK cells to act against stressed cells even in the presence of MHC class I, if a certain activating threshold is reached[25]. Recent studies have also emphasized more specific responses by activating receptors, e.g., against different viruses, such as CMV. When NK cells are activated, they release granules that contain cytolytic enzymes, e.g., perforin, granzymes and granulysin. NK cells can also kill virus infected cells using antibody-dependent cellular cytotoxicity (ADCC) or by inducing cell death through receptor-ligand interaction. In addition, they can produce and release a range of cytokines and chemokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF)[27,28]. These cytokines then act on neighboring cells to initiate and direct inflammation.

Traditionally, NK cells have been divided into two subsets, the cytotoxic CD56^{dim} NK cells and the cytokine producing CD56^{bright} NK cells[21,29]. Most NK cells, approximately 90 %, in peripheral blood, are CD56^{dim} NK cells, whereas CD56^{bright} NK cells can be found in higher frequencies in tissues[30]. For long, circulating NK cells, as a part of the innate immune system, were believed to retain a static phenotype during their life span without further differentiation from the more immature CD56^{bright} to mature CD56^{dim} NK cells. However, this view has been revised in the past decade and it is now clear that NK cells can differentiate further[31] and even acquire memory- or adaptive like features[31].

NK cells do not only patrol the body in the circulation but can also be found in many peripheral tissues[22]. NK cells are found in high proportions in the liver and the uterus where they comprise up to 30 and 45%, respectively, of all lymphocytes[32,33]. Due to the easy accessibility, still much more is known of circulating NK cells compared to NK cells that are derived from tissues. During the years when this thesis was composed, a role for NK cells in obesity-induced inflammation has been highlighted and the area is undergoing intense scientific investigation.

1.1.4   Macrophages

Another important innate immune cell is the macrophage. Macrophages play a key role in many immune responses, both against external (e.g., pathogens) as well as internal stimuli (e.g., cell death). Macrophages exist in large numbers in many tissues. There are many different subtypes and macrophages display a considerable phenotypical and functional heterogeneity. Macrophages are part of a larger family of myeloid cells, including dendritic

cells and monocytes, with sometimes overlapping terminology. In general, monocytes exist in peripheral blood where they patrol the body, only infiltrating tissue when they encounter inflammatory signals such as increased integrin expression in vessels that are upregulated in response to tissue inflammation. Dendritic cells are the main antigen presenting cells and provide an important link between sensing pathogens and activating the adaptive immune cells, including T and B cells. In a way, macrophages provide both of these functions. They can on one hand sense antigens, often encountered through phagocytosis, and then present those to other cells through MHC class II. On the other hand, they can also directly mechanically engulf and kill bacteria as well as other foreign bodies e.g., deposited in wounds. Macrophages are also important cells in orchestrating immune responses by producing several cytokines and chemokines that influence and direct other cell types. However, the macrophage population is diverse, and their heterogeneity is undergoing intense scientific investigation, utilizing many of the novel research tools that have been developed during recent years.

1.1.5 Tissue immunology

In order to protect the body from infections, it is important to have an immune system ready to respond at the site of origin. Conventionally, immune cells have often been studied in peripheral blood. Even though it has long been known that e.g., T cells recirculate through tissues and lymph nodes on their quest to patrol the body for harmful pathogens. Therefore, what was seen in blood was often taken as a model for what exists in tissues. This has been especially true in the study of disease manifestations. One exception is macrophages, where tissue-specific macrophages was identified early in many different tissues, e.g., the microglia in the central nervous system (CNS), the Kupffer cells in the liver and the Langerhans cells in the skin[34]. Tissue derived myeloid cells can have different origins, with some infiltrating tissue in response to inflammatory stimuli while others have developed from progenitors in fetal livers that then permanently resides in their homing tissue[34]. Thus, there is a large heterogeneity within the macrophage subset, that at this point is only partially understood.

In other cell types, this kind of tissue-residency profile have only recently been described[15]. Since the recognition of T cells displaying a more tissue resident phenotype, NK cells in several different tissues have been found to express tissue residency markers such as CD69, CD103 and CD49a[16,17,33,35–38], which are all molecules involved in retaining cells in the tissue[20].Tissue-resident NK cells are found in many tissues. In general, terminally differentiated, highly cytotoxic NK cells predominate in blood, bone marrow, spleen and lungs, whereas tissue resident NK cells with a more immature and pre-cursor phenotype are found in mucosal and lymphoid sites[39]. This seems stable over time. Tissue resident lymphocytes also express distinct transcription factors such as Hobit and BLIMP[15,40]. However, the knowledge of the phenotype, function and role of tissue resident NK cells is still very limited, and at the time of study I very little was known of NK cells in adipose tissue.

1.2   NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide with a global prevalence estimated to a staggering 25 % [41]. As the name suggest, it is caused by fat accumulation (steatosis) in the liver and is closely associated to obesity, even though NAFLD also exist in lean individuals[42]. Some NAFLD patients develop non-alcoholic steatohepatitis (NASH) which is characterized by a chronic low-grade liver inflammation that can persist over years[43]. Patients with NASH have an increased risk of developing hepatocellular carcinoma (HCC), liver cirrhosis and subsequent liver failure[44–46]. The prevalence of NAFLD has increased alongside the global obesity pandemic and NAFLD is now considered the most common liver disease worldwide[47–49]. Due to the increased prevalence, complications associated to NAFLD puts strain on health care systems worldwide, where NAFLD-induced cirrhosis is estimated to become the most common cause of liver transplantation[48]. Thus, improved understanding and knowledge could result in improved treatment options.

1.2.1   Diagnostic challenges

As of today, there are no non-invasive tests that can separate NASH patients from individuals with simple steatosis (non-alcoholic fatty liver, NAFL). Therefore, the diagnosis still depends on a liver biopsy, which is then histologically assessed for the disease hallmarks steatosis, inflammation and hepatocyte ballooning (**Figure 1**)[50–52]. The "NAFLD activity score" (NAS) is a histological scoring system established by the Pathology Committee of the NASH Clinical Research Network to standardize clinical trials and has been used to diagnose NASH[50].
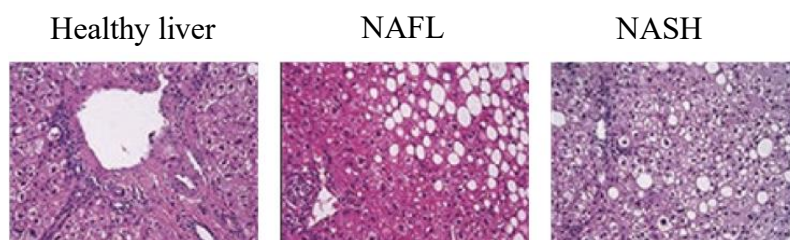

Figure 1. Liver histology sections from healthy liver, NAFL and NASH. Healthy liver with a central vein (left), steatotic hepatocytes (middle) and characteristic ballooning hepatocytes (right). Adapted from Stiglund et al, *Front. Immunol.* 2019[53].

The scoring system takes three histological features into account: steatosis, inflammation and ballooning hepatocytes. In addition, degree of fibrosis is often assessed since NASH while advanced fibrosis stage 3 and 4 (bridging fibrosis and cirrhosis) is associated with worse outcomes[54]. Steatosis is graded on a 0-3 scale where 0p = <5% of steatosis, 1p = 5-33 %, 2p = 33-66 % and 3p = >66%[51]. The level of lobular inflammation evaluates the number of lymphocyte infiltrates per microscopic field in 20x magnitude. This is graded on a 0-3 scale

(0p represents no lobular lymphocyte infiltrates, 1p represents one lobular infiltrate, 2p represents 2-4 lymphocyte infiltrates, and 3p represents >4 lymphocyte infiltrates)[51]. Hepatocellular ballooning, or ballooning hepatocytes, are enlarged, apoptotic hepatocytes with a distinct histological appearance which are characteristic in NASH[51]. The levels of hepatocellular ballooning are graded on a 0-2 scale where 0p represents no ballooning hepatocytes present, 1p represents the presence of a few ballooning hepatocytes and 2p represents the presence of many ballooning hepatocytes. The NAS is the un-weighted sum of the scores and a NASH diagnosis is consistent with a score of 5 or higher[50].

Recently, there is a discussion about a change of terminology from the previous NAFLD that is focused on the absence of alcohol consumption, (due to the histological similarities to alcohol induced steatosis), towards metabolic dysfunction-associated fatty liver disease (MAFLD)[55]. The discussion reflects the need to move away from the somewhat simplified NAFL/NASH dichotomy to increase focus on disease activity and the levels of fibrosis. This in order to appreciate how the disease exist as a continuum of pathogenic features with substantial heterogeneity. However, in this thesis the terminology current at the time of the initiation of study is used.

Obtaining a liver biopsy is an invasive measure associated with considerable risk e.g., risk of serious bleeding, as well as substantial patient discomfort[56,57]. Due to this, repeated sampling over time to monitor disease activity and/or treatment responses is often not feasible. Thus, there is a need for non-invasive diagnostic test, preferentially through peripheral blood tests, which can identify those patients who would benefit from further diagnostics through a liver biopsy. This is the context of study III, where circulating serum proteins are measured in NAFL and NASH patients with the intention to identify disease-specific signatures, in order to aid in the development of future diagnostic tools.

1.2.2 NAFLD pathogenesis

The prognosis of NAFLD patients is varying, with some patients who develop liver inflammation and fibrosis while others remain in a more benign state with only simple steatosis. The cause for this is currently not known. Current consensus is that NAFLD is a multifactorial disease, and that the disease develops due to the interaction of several factors, both intrahepatic and extrahepatic, e.g., genetic defects in lipid storage, dietary habits, composition of microbiota, insulin resistance as well as aberrant immune activation[43,58]. The heterogeneous disease development and prognosis suggest that subgroups exist among the NAFLD patients which is hinted at by some studies[59], but future studies are warranted. The "multiple hits model" is currently the most widely accepted theory of NASH pathogenesis[57,60]. This model emphasizes the importance of multiple parallel hits, such as endoplasmic reticulum (ER) stress[61] and lipotoxicity, which act synergistically to cause liver inflammation[60]. At the center of NAFLD pathogenesis is an inability to store lipids, which is indicative of the risk allele PNPLA3 that contributes to defective lipid storage. During obesity development and insulin resistance free fatty acids (FFAs) are released from the

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adipose tissue and ectopic fat deposition in the liver is promoted. The types of lipids are important, creating lipotoxicity and ER stress which cause different types of cell death, including the apoptosis seen in the characteristic ballooning hepatocytes[62]. In addition, an increased intestinal permeability seen in obesity increases the exposure of the liver to bacterial products[44].

Taken together, these proinflammatory stimuli creates an intrahepatic environment that triggers inflammation, that is considered a major contributor to NAFLD.

1.2.3   Inflammation in NAFLD

While it is known that some patients develop inflammation in addition to the hepatic steatosis, many questions about the nature of the inflammation still remain. Approximately 10-25 % of NAFLD patients develop nonalcoholic steatohepatitis. Usually, patients with NASH have both portal as well as lobular inflammation[54]. IL-1 family cytokines are important in NASH, triggered by NLRP3 inflammasome activation, e.g., IL-1b that can promote liver steatosis, inflammation and fibrosis development[63]. Initially NASH was believed to be driven by type 1 mediated immunity, generated in the adipose tissue. However, recent studies have also emphasized the role of type 2 immunity in addition to release of pro-inflammatory TGF-β[64]. While the immune response in the liver on one hand can be detrimental during acute inflammation, it is also pivotal in wound healing responses and in the clearance of inflammation. Thus, the role of immune cells in NASH have been rather difficult to assess due to the complexity of pathogenesis and disease process. Several studies have implied a role for CD44, a pro-inflammatory receptor expressed on many leukocytes, in the development and progression of NASH, where it is associated to macrophage activation in both liver and adipose tissue[65–69]. In addition, the ligands of CD44, osteopontin (OPN), E-selectin and hyaluronic acid, are all upregulated in NASH and/or fibrosis. While several recent studies have revealed a role for adaptive immunity[70–72], innate immunity is still considered a driver of the inflammation. In addition, it is known that obesity and a high-calorie, low-fiber fast food diet itself creates inflammation, both in adipose tissue as well as in inflammatory changes in the microbiome in the gut[73]. In responses to lipotoxicity, proinflammatory cytokines as well as gut-derived bacterial products activates liver-resident macrophages, Kupffer cells (KCs), as well as triggers infiltration of pro-inflammatory cells, including monocyte-derived macrophages, from circulation[74]. This leads to a pro-inflammatory state in the liver which further activates hepatic stellate cells (HSCs)[75]. These cells promote fibrosis development and can further aggravate the intrahepatic inflammation, creating a vicious circle between fibrosis development and increased immune activation.

1.2.4   Inflammatory mediators in serum

As previously mentioned, there is a lack of biomarkers that distinguish NASH patients[76]. The most assessed biomarker is circulating keratin (CK)-18, which is cleaved by caspase-3 in hepatocytes that leaks into circulation during NASH-development[77]. However, due to its modest accuracy, CK-18 is not used in clinical diagnostics. Many studies have assessed the

levels of single cytokines in the serum of NAFLD patients[58,78,79]. However, several recent studies have attempted to make broader serum proteome analysis. Ajmera et al measured 32 serum proteins in 648 NAFLD patients and found increases of several inflammatory cytokines such as IL-1, IL-18 and TNF-α in NASH patients with significant fibrosis but only increased levels of plasminogen activator inhibitor (PAI)-1 in NASH-patients compared to non-NASH patients[80]. A recent study measured 1305 serum proteins in 113 NASH-patients with low (stage 0-2) or high (stage 3-4) levels of fibrosis. 97 proteins were differentially between the low and high fibrosis group and a 4-protein model differentiated the patients with a level of AUROC 0.74. In a validation cohort this division was similar or better to (fibrosis) FIB-4, aspartate to platelet ratio and NAFLD fibrosis score[81]. However, these scoring systems measures degree of fibrosis and not level of inflammation per se. Thus, in study III, part of the aim was to provide a diagnostic tool to identify NASH compared to NAFL-patients without hepatic inflammation.

1.2.5 NK cells in the liver

Since the liver is continuously exposed to antigens from the gut, which could potentially trigger inflammation, it is pivotal that it remains in a tolerogenic homeostatic state. Through innate sensors, such as toll-like receptors on hepatic stellate cells and macrophages, the livers thus exist in a state of "endotoxin tolerance", preventing excessive inflammatory responses. The liver also contains high levels of lymphocytes. NK cells comprise approximately 30-50 % of the intrahepatic lymphocytes, which is higher than found in circulation. In addition, compared to peripheral blood, there is also an increased proportion of CD56^{bright} NK cells and within this population, a certain subgroup of NK cells expressing tissue residency markers (such as CD49a, CD69, CD103, CXCR6) can be found[30,33,38].

As part of the hepatic immune system, NK cells are involved in the pathogenesis of several liver diseases. Several studies in recent years have shown the importance of NK cells in the pathogenesis and clearance of chronic viral hepatitis infections in humans[82,83]. The pro-inflammatory environment, as well as NK cell ligands up regulated on stressed cells, is believed to activate NK cells in the context of liver disease. One receptor through which NK cells can sense cells undergoing stress is through the activating NK cell recptor Natural killer group 2D (NKG2D). NKG2D binds to ligands such as MICA, MICB and ULBP that are upregulated on the cell surface in response to cellular stressors, e.g., including DNA damage responses, viral infections or oxidative stress. NK cells then respond by e.g., IFNγ-production, which has been reported as a contributing factor in several pathogenic processes, such as viral infections, fibrosis development, tumor development and as a part of liver regeneration[84]. Interestingly, several recent articles have demonstrated how NK cells can limit fibrosis development in mice, by diminishing the action of hepatic stellate cells (HSCs)[85–87]. This is of great interest, since fibrosis development is a negative prognostic factor for patients with NAFLD[88,89], providing a possible target for future anti-fibrotic

8

therapies. However, the role of NK cells in fibrosis development in humans is still unknown and future studies are warranted.

1.2.6   NK cells in NAFLD

Several mouse models of NASH have highlighted the significance of the innate immune system, in particular NK cells, during disease development[84]. Increased numbers of NK cells and a higher level of their ligands can be found in NASH-livers[90,91]. NK cells can sense cellular stress by different receptors, including the NK cell receptor NKG2D (**Figure 2**). Activated NK cells can then produce pro-inflammatory cytokines, such as Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL)[90]. On the other hand, more recent studies connect TRAIL deletion to worse metabolic outcomes in mice and describes lower levels of TRAIL in humans with NASH[92]. In addition, NK cell activation in response to IL-15 promotes NASH-development in mice [93] and NK cells are also thought to play an important role in regulating fibrosis development in NASH[85,87]. Further, osteopontin-producing hepatic NK cells can induce obesity-induced hepatic ER stress and insulin resistance[94].
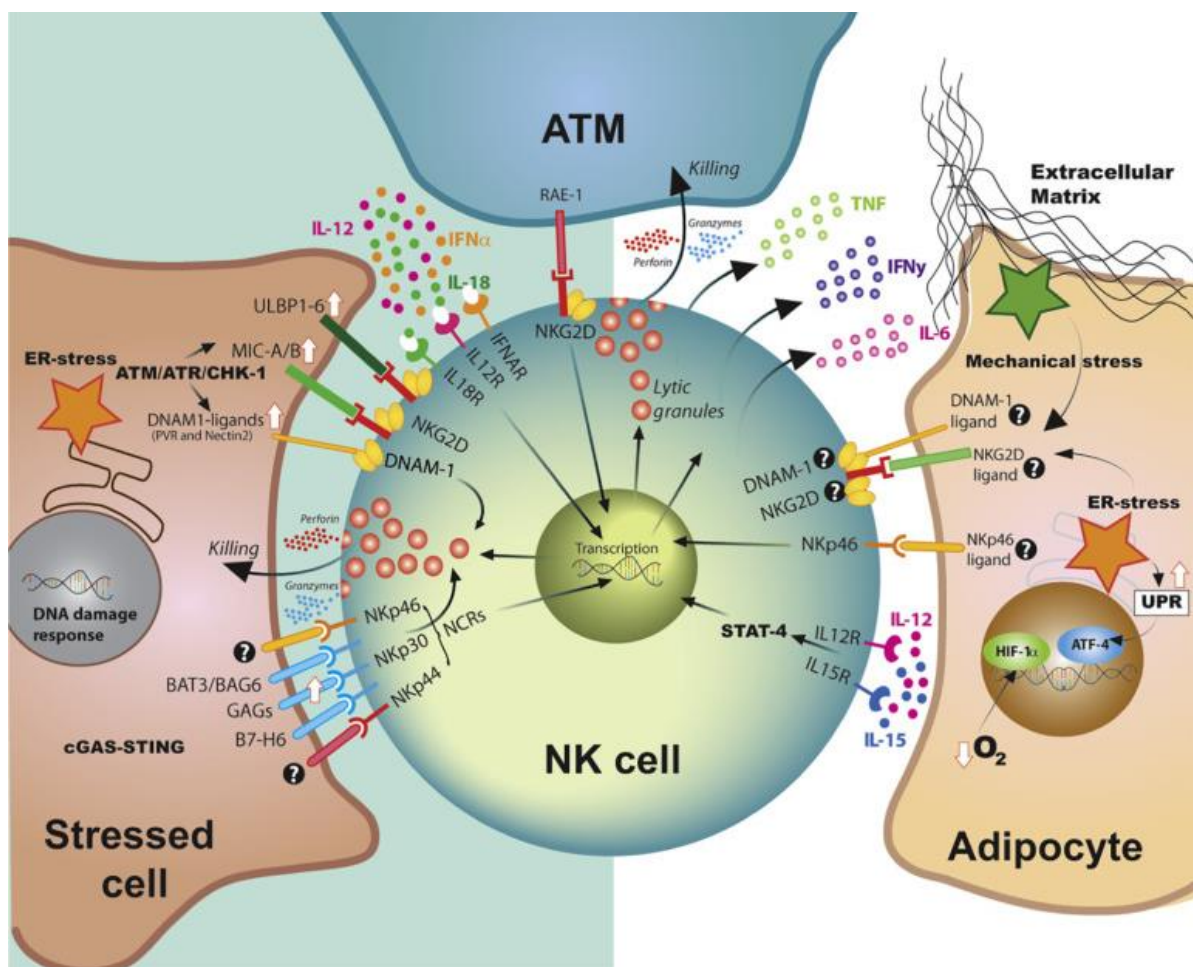

Figure 2. Mechanisms by which NK cells can sense cellular stress during obesity. ATM: Adipose tissue macrophage. Reproduced from Ferno et al. *Trends Endocrinol Metab*. 2019[95].

Obesity, which often co-exist with NAFLD, can itself alter NK cell phenotype, metabolism, and function[96–99]. NK cells also express receptors for adipokines, and e.g., leptin, can influence NK cell function, migration and proliferation[100–104]. While previous studies interestingly report that decreased NK cell function in obesity can be restored by weight loss and exercise, the results need to be further validated and explored due to some methodical shortcomings[105,106]. Several recent studies in mice have suggested NK cells to be important for development of insulin resistance[107–109], a pathological process with a high comorbidity with NASH. However, many studies have been performed in murine models, there is still a lack of knowledge on if and how NK cells, and in particular tissue resident NK cells, contribute to disease in humans with NASH. Thus, **study I** involves an in-depth study of NK cells in patients with NAFLD.

1.3 ADIPOSE TISSUE INFLAMMATION

Obesity does not only affect the liver but a whole range of other organs, including the adipose tissue. With the upsurge of the obesity pandemic, associated metabolic diseases, such as type 2, rapidly increase[7]. Adipose tissue exists for the human body to be able to store excessive energy in a time of excess, in order to utilize during periods of fasting. Lean adipose tissue exists in a tolerogenic immune state, characterized by a Th2 response driven by interleukin (IL)-5, IL-4 and IL-13 derived from mainly innate type 2 lymphoid cells, eosinophils, regulatory T cells (Tregs) and alternatively activated macrophages[110,111]. The adipose tissue, especially the visceral adipose tissue (VAT), responds to obesity with adipocyte hyperplasia and hypertrophy in response to an influx of lipids[112]. This can lead to a dysregulated response with adipocyte hypoxia, fatty acid flux dysregulation, extracellular matrix protein deposition, and adipocyte cell death[113,114]. These stimuli cause a stress-response in adipocyte and stromal cells, leading to production of proinflammatory cytokine production and upregulation of stress-induced ligands which in turn recruits and activates immune cells[113,115,116]. During the subsequent Th1 pro-inflammatory response, cells from both the adaptive and the innate immune system are activated and accumulate in the adipose tissue. They then produce cytokines such as IL-1β, TNF, IL-6, IL-12 and interferon-γ, that further contribute to the pro-inflammatory environment, contributing to insulin-resistance, development of type 2 diabetes as well as systemic inflammation[117,118].

The adipose tissue is a secretory organ that secretes a range of endocrines, in turn affecting other tissues, including adipokines such as leptin, adiponectin, visfatin and resistin[119]. Through systemic signaling, the adipose tissue can direct systemic metabolism and energy homeostasis, but it can also initiate and direct inflammatory responses and tissue damage. Many of the adipokines are pro-inflammatory e.g., leptin and, in addition, especially visceral adipose tissue release pro-inflammatory cytokines such as IL-1B, IL-6, IL-8, IL-12 and TNF in obesity[120–122]. Visceral adipose tissue produces more inflammatory mediators than subcutaneous adipose tissue. Thus, the adipose tissue displays a low grade, chronic inflammation in obesity that is linked to insulin resistance.

1.3.1 Macrophages as key players

Adipose tissue is home to large numbers of adipose tissue macrophages (ATMs). In response to obesity, they contribute to the inflammatory environment. The macrophage population is very heterogeneous. In mice, macrophages have traditionally been divided into pro-inflammatory (M1 macrophages) and anti-inflammatory (M2 or alternatively activated macrophages). In lean adipose tissue, alternatively activated macrophages contribute to an anti-inflammatory homeostatic state. However, macrophages accumulate in the early stages of adipose tissue inflammation. When activated, the ATMs undergo a shift from anti-inflammatory M2-like cells towards more proinflammatory M1-like cells[117,123]. They then proceed to produce large quantities of pro-inflammatory IL-1β and TNF that further contributes to the adipose tissue inflammation. These cytokines are also important for the development of obesity-induced insulin resistance. What triggers this polarization of ATMs toward a more pro-inflammatory phenotype is not known, but several factors, such as hypoxia, cell stress and lipotoxicity, has been proposed.

Obesity is associated with an increased number of ATMs in adipose tissue[124]. The increase is in part due to an infiltration of circulating monocytes, recruited in response to chemokines secreted from adipocytes (adipokines), such as CCR2 (or MCP-1). These infiltrating monocytes then polarize toward pro-inflammatory or M1 macrophages[123], which then produce proinflammatory cytokines who recruit even more immune cells, contributing to an inflammatory milieu[124]. Within adipose tissue, M1 macrophages can gather around necrotic adipocytes in aggregates known as crown-like structures (CLS). The pro-inflammatory macrophages in these CLSs express CD11c, which is the integrin alpha X chain protein. In contrast, M2 macrophages express CD206 and are usually found dispersed in the adipose tissue[123,125]. M1 and M2-macrophages also show different bioenergetics, where M1-macrophages utilize glycolysis whereas M2-macrophages use oxidative phosphorylation *in vitro*[126]. However, many macrophage subtypes that do not allow for a classification within the M1-and M2-dichomoty, do not follow this but use mixed bioenergetics.

The M1/M2 paradigm, initially identified in mice, has traditionally been used to describe ATMs but does not capture the complexity of the heterogeneous ATM population. For example, ATMs from obese humans do not express some of the classical M1 markers that have been described in mice. In contrast, they have been shown to express markers of M2 macrophages and produce anti-inflammatory as well as pro-inflammatory cytokines. Of note, not only do murine and human immune system differ greatly, but studies on adipose tissue can be performed in different adipose tissue depots, e.g., epidydimal fat, that is not easily translatable to human conditions. This is important to bear in mind when comparing studies in humans and mice.

Recent studies have identified several novel populations of ATMs within obese adipose tissue, including metabolically activated macrophages, neuron-associated macrophages (NAMs)[128–132]. In CLS, proinflammatory, lipid-laden CD9+ ATMs expand in response to

obesity[127], whereas the lipid-associated macrophages (LAMs) found in the same structure acts protectively. Many of these recently described macrophage subtypes respond to different metabolic mediators that are found locally in the adipose tissue, such as lipids such as palmitate, glucose, oxidized lipids etc[128,130].. These macrophages are distinct from both M1 and M2 macrophages and can perform both beneficial and detrimental functions[128,129]. However, how ATMs differ between different adipose tissue depots, such as subcutaneous and visceral, have not been fully elucidated. Thus, the complete heterogeneity of macrophages in adipose tissue has not yet been fully appreciated and much work remain to characterize the phenotype and function of ATMs in detail.

1.3.2   NK cells as mediators of insulin-resistance by polarization of macrophages

As mentioned in the previous section, it is not clear what can initiate macrophage polarization. In mice, group 1 innate lymphoid cells (ILCs) can respond to obesity by proliferation and cytokine production, such as IFN-y[108,109]. INF-γ a cytokine known to drive macrophage polarization during infections[134] and INF-γ depletion and/or neutralization has been shown to reduce M1-macrophage accumulation as well as insulin resistance in obese mice[135]. NK cells accumulate in the adipose tissue in response to obesity[108,136]. The IFN-γ produced by ILC1s can trigger polarization of pro-inflammatory M1-like ATMs from anti-inflammatory M1-like macrophages, which in turn contributes to the development of insulin resistance in mice[107,108]. NK cell depletion also leads to decreasing numbers of both total and M1 macrophages along with improved insulin resistance, with no effect on body weight[107,108]. One study report that, though macrophage activation is still present in obesity in mice lacking NK cell and other lymphocytes, the obese mice remain insulin sensitive[137]. This suggests an important role for NK cells as regulators of macrophage polarization and insulin resistance in obesity.

On the other hand, adipose tissue derived ILCs has been shown to kill adipose tissue macrophages in steady state, in particular M2-like macrophages that are thought to be important for maintaining the homeostasis of the adipose tissue. By killing M2-like macrophages these might be prevented to develop into pro-inflammatory M1-like macrophages. However, since ILCs display less cytotoxicity [138] this ability seems hampered in obesity, leading to increased frequency of macrophages in the adipose tissue (AT). ILCs have been found to increase during development of obesity, but only during the first few days of starting mice on a high fat diet (HFD). During sustained obesity, however, the frequency of AT ILCs decreases in both humans and mice[139]. Increased levels of ratio of M1/M2 macrophages in human visceral adipose tissue correlate to the levels of NK cells. However, if these NK cells represent tissue resident subsets or if this represent causality is not yet known[140].
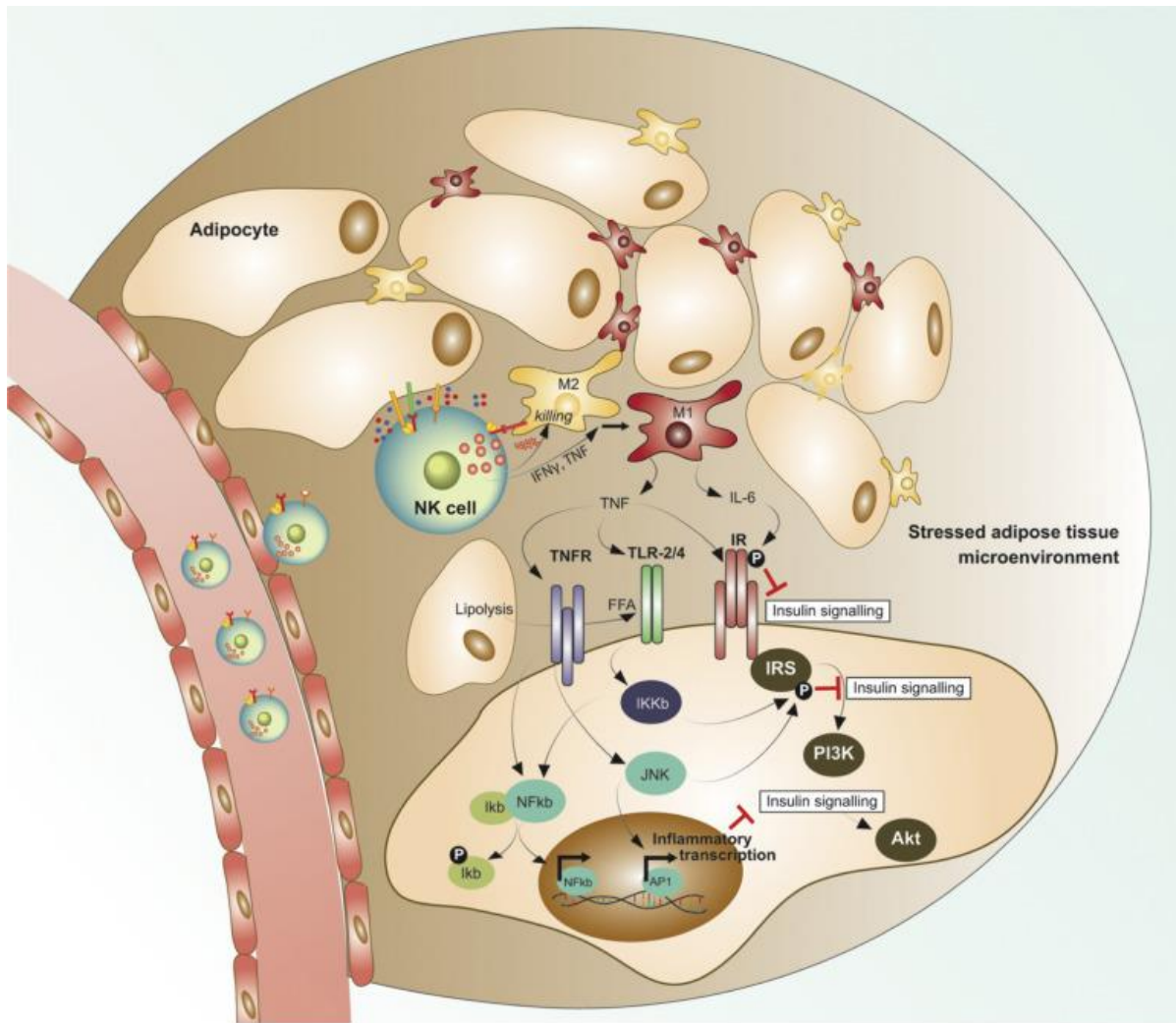
Figure 3: NK cells as initiators of macrophage polarization, and subsequent insulin resistance, in adipose tissue during obesity. Reproduced from Ferno et al. *Trends Endocrinol Metab*, 2019[95].

Thus, the literature is divided in their theories of the nature of NK cell-macrophage interactions in adipose tissue. Since many of the studies mainly have been performed in mice, this emphasizes the importance of studies of human adipose tissue, to further increase our knowledge of these important pathological processes.

2 RESEARCH AIMS

The overall aim of this thesis is to examine the role of innate immunity in obesity-induced inflammation.

Specific aims:

- **Project I**: To explore the presence of disease-specific changes within circulating and tissue-resident NK cell populations in patients with liver biopsy-confirmed NAFLD.
- **Project II:** To perform a surface proteome screen of adipose tissue macrophages and investigate their relationship to the presence of insulin resistance in obese individuals.
- **Project III:** To characterize the inflammatory signature in peripheral blood of NASH-patients and use this to 1) distinguish them from patients with simple steatosis, and 2) increase the knowledge of NASH-pathogenesis and the prescence of subgroups with distinct biology.

3   MATERIALS AND METHODS

3.1   ETHICAL CONSIDERATIONS

Ethical permits have been obtained for all studies included in this thesis. During the design of the study and throughout the application process careful ethical considerations have been applied. In all studies, study participants provide written consent, after receiving oral as well as written information regarding the study. All participants have been informed that they may withdraw their participation at any given timepoint, and that participation in the study will not in any way affect the health care that they are receiving. All patient identities and personal data have been handled with utmost care and coding systems have been applied to keep the data anonymous. Protocols have been in place in case the studies would have encountered previously unknown disease findings. Our protocols follow the ethical guidelines of the 1975 Declaration of Helsinki and we have received ethical permits from the Stockholm Ethical Review board or Regional Etisk komité for forskningsetik Vest (REK Vest) covering all our projects, as per the following numbers: Dnr 2010/678-31/3, 2006/971-31/1, 2006/229-31/3, and 2014/979-31/1 (**study I**), REK 2015/2343 and REK 2010/502 (**study II**) and dnr 2013/2285-31/3 and Dnr 2006/229-31/3 (**study III**). In all studies, the possible benefit of the patients has been considered to be greater than the risks endured by the study participants. The aim of the studies has been to study obesity-induced inflammation in the hope of gaining new knowledge that in the future could benefit the patients, e.g., by improving the treatment of these patients.

3.2   STUDY POPULATIONS

Three different patient cohorts have been recruited for the studies included in this thesis. The first cohort, used in **study I**, consist of 26 patients with NAFLD/NASH recruited from the Gastroenterology Clinic at Karolinska University Hospital. From these patients, peripheral blood samples were collected. In addition, 26 patients undergoing bariatric surgery was recruited, from Ersta hospital (n=20) or Danderyd's hospital (n=6): paired peripheral blood samples and liver and adipose tissue biopsies was collected during surgery. **Study II** included two patient cohorts that consist of in total 80 obese patients who have undergone bariatric surgery at Voss Hospital, Norway. The first cohort consisted of 57 patients and was used for the characterization of ATMs from subcutaneous and visceral adipose tissue, as well as measurement of inflammatory gene expression and immunohistochemistry (IHC) analysis of crown-like structures (CLS). The second cohort consisted of 23 obese individuals and were used for the verification of surface protein expression. Subcutaneous adipose tissue derived from liposuctions were used for the surface proteome screen. Buffy coats derived from healthy blood donors at the Blood bank services at Haukeland University Hospital was used as healthy controls.

In **study III**, sera from 35 patients with NAFL and 35 patients with NASH was collected from the Gastroenterology clinic at Karolinska University Hospital. In addition to these,

blood samples from healthy donors from the blood donor bank at Skanstull, Stockholm, was included as healthy controls in both **study I** and **study III**. These have been normal weight (BMI< 25), had normal liver enzymes and no presence of type 2 diabetes.

All patients with NAFLD underwent a diagnostic liver biopsy during routine clinical practice, which was then acquired and graded according to NAFLD activity score (NAS). NAS > 5 has been used as a definition of NASH. The presence of fibrosis has been classified separately and recorded. This focus on disease activity and active inflammation has been chosen since the focus in all the studies is on the inflammatory aspect of the disease, rather than e.g., fibrosis development. There are several classification systems for diagnosis/classification of NAFLD. The diagnosis system used in **study I** and **study II** are in line with the latest recommendations of 2020, which stresses the importance of viewing the disease as a continuum of symptoms with a larger focus on disease activity (as represented by NAS) as well as an appreciation of degree of fibrosis. Thus, the cohort might not always correlate to those prioritized in the hepatology clinic, since some patients with high levels of fibrosis have no inflammation. But these patients are of course at a high risk of adverse events and should be identified and followed up by a hepatologist. However, the study design focusing on disease activity, has been chosen to best assess the effect the disease has on the immune system.

3.3 COLLECTION AND PROCESSING OF SAMPLES

All immunological studies included in this thesis have been performed on human materials. Peripheral blood of patients as well as healthy controls was collected in heparin-coated vacuum tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation and either analysed directly or cryopreserved for later analysis. Liver biopsies were processed through mechanical as well as enzymatic digestion. In addition, liver biopsies were stored for 24 hours in 70 % ethanol before transferred to Histocon and formalin paraffin-embedded for later sectioning, staining and histological assessment. Adipose tissue, both subcutaneous as well as visceral adipose tissue from omentum major, was mechanically and enzymatically digested, centrifuged and the subsequent stromal vascular fraction (SVF) was collected for analysis. While the samples in **study I** and **III** was frozen after collection and then thawed for analysis, the samples in **study II** were processed and analysed directly, as macrophages are more sensitive to freezing than NK cells.

3.4 FLOW CYTOMETRY

In the immunological studies included in this thesis, flow cytometry is a central method to study immune cells. Fluorochrome-conjugated antibodies with different specificities are mixed with cells and then bind to targets on the cell-surface or intra-cellular depending on the protocol. The cells can then be run through a flow cytometer, in a single-cell fashion, where the light emitted from the fluorochromes when hit by different lasers can be recorded. Thus, information of the expression of different epitopes on one singular cell can be measured,

providing a detailed image of the cell in question. In all studies, several antibodies were chosen to identify the cell through their lineage, and then other markers of interest were identified on the same cell. One example is NK cells systematically identified as $CD56^+CD3^-$ cells. In addition, dead cell markers have been used to exclude dead cells which could cluster together and thus provide false positive signals. Some markers are expressed on the cell surface whereas others are produced and stored intracellularly. Thus, requiring additional protocol steps where the cell surface is permeabilized to allow access for the fluorochrome-conjugated antibodies. In **study I** and **study II**, some cells are stained directly ex vivo, while in other experiments, the cells are pre-stimulated with e.g., inflammatory cytokines to measure functional responses. Flow cytometry is a powerful tool that enables analysis of millions of single immune cells derived from tissues or circulation. However, it only measures a limited number of proteins, up to 30 intracellular or surface proteins. While this is many targets in the context of advanced flow cytometry, where the possibility to measure markers have grown during the last decade from approximately 10 markers to 30, the method itself, based on wavelengths is limited compared to for example single cell sequencing where the entire transcriptome of a cell can be measured. However, while transcriptomics gives an idea of the unbiased transcription it does not show which proteins are translated and expressed on the cell. The surface molecules detected by flow cytometry are biologically important for cell function, interaction with its environment and for signalling. In addition, flow cytometry enables for researchers to pose more direct questions, as is done in the validation cohort of **study II**, where custom made panels of antibodies can be produced depending on the hypothesis.

3.5   SURFACE PROTEOME SCREEN (LEGEND SCREEN)

Since macrophages are a heterogeneous cell population that has been not fully characterized, we wanted to perform a large screen of all the known CD-molecules often used in immunology (**study II**). This was intended to on one hand increase our knowledge of which markers different macrophage subset expressed, hinting at their functional importance. And on the other hand, aid in the rather complicated area of cell identification using surface markers when studying macrophages with flow cytometry. Since macrophages consist of a heterogeneous population there is still a lack of knowledge on how to best identify and define them. As a result of this, a large range of different markers are used within the research area which can make research results difficult to compare between studies and species. To perform this, we used the LEGENDScreen^{TM} Human PE Kit platform from Biolegend (cat#700007), which allowed us to assess 361 surface markers simultaneously, with 10 different isotype controls. To be able to reach the cell numbers needed for such a large screen, we isolated cells from subcutaneous adipose tissue from a patient undergoing surgical liposuction. The SVF fraction was then stained with a backbone of flow cytometry markers that identify subpopulations of myeloid cells. In addition, cells were barcoded using different fluorophore-conjugated CD45-antibodies (BV650 and AF700 respectively) to distinguish immune cells derived from blood and adipose tissue. Peripheral blood sample was used as a comparison to the adipose tissue. While the strength of the method is the possibility to

measure a large span of surface molecules in an unbiased way, one limitation is that the surface screen is performed on a single individual and that the possibility to generalize the results to larger cohorts is limited. Thus, we used a validation cohort in order to assess the markers on a group level, since we know that there can be a large heterogeneity in surface marker expression on immune cells derived from different individuals.

3.6 PROXIMITY EXTENSION ASSAYS (PEA)

In **study III**, a proximity extension assay (PEA) developed by Olink was used to measure the levels of inflammatory proteins in the serum of NAFLD patients. Methodologically, 20 μl serum was thawed and transferred to a 96-well plate which was then shipped to for analysis. The panel used consisted of 92 pre-selected inflammatory serum proteins, in their Inflammation panel. The markers that did not pass the internal quality control for the assay or had values below the limit of detection were excluded from the study.

Since these serum proteins were pre-selected, **study III** should not be interpreted as a comprehensive screen of all serum proteins in NAFLD patients. However, at the time of study initiation, **study III** comprised one of the largest screens of inflammatory serum protein performed in NAFLD patients. In short, in the PEA, antibodies conjugated with oligonucleotides with certain specificity bind to serum proteins, hybridize and can then be measured using qPCR. Therefore, it is a very sensitive and specific method, also compared to the traditional Enzyme-Linked ImmunoSorbent Assay (ELISA), since two antibodies need to bind to dual epitopes, located closely together, on the target in order for it to be recognized[141]. This also decreases the risk of cross-reactivity. However, the method is not quantifiable as such, and if quantification is needed, an ELISA can be performed in order to further measure the levels of inflammatory cytokines. In addition, our results in **study III** would benefit from further validation in an independent, external validation cohort. Since **study III** was initiated, the technology has been further developed, where the current panels, e.g., Olink explore, enables the measurement of up to 1532 serum markers. This would be interesting to pursue in future studies, and we note that the pipeline analysis presented in **study III** could possibly be used for this.

4 RESULTS AND DISCUSSION

The three papers included in this thesis explore the role of innate immunity in obesity-induced inflammation in metabolic disease. In **study I**, we investigate how the circulating NK cell population is affected in NASH patients, compared to NAFL and healthy controls. In **study II,** we focus on the macrophages found in adipose tissue and examine their relationship to the development of insulin resistance. However, the role of immune cells is to a large extent determined by their surrounding environment. Thus, to further improve our understanding of the inflammation of NASH, we explore the humoral milieu by measuring the inflammatory serum proteome of these patients in **study III**. Taken together, our studies provide novel insight into how innate immunity is affected in patients with obesity-induced inflammation.

4.1 PROJECT I: RETAINED NK CELL PHENOTYPE AND FUNCTIONALITY IN NON-ALCOHOLIC FATTY LIVED DISEASE

NK cells are important in many liver diseases, e.g., the viral hepatitis infections where they are pivotal for viral clearance. NK cells regulate immune responses, act against fibrosis producing cells as well as eliminate hepatocytes that display stress signals. Thus, there is a potential importance of NK cells in the pathogenesis of NASH since apoptotic hepatocytes as well as fibrosis development are important pathogenic features. Since there was a lack of knowledge of how the NK cell compartment was affected in NAFLD, the aim of **study I** was to investigate NK cell phenotype and function in NAFLD.

While the frequency and number of myeloid cells, such as pDCs, were decreased in patients with NASH, the NK cell frequency and numbers in NASH patients were similar to those in NAFL patients as well in healthy controls. Previous studies have reported a decreased numbers of NK cells in obesity, which has been linked to the increased risk at developing malignancies and infections during obesity[97,142–144]. However, the reports on the NK cell frequency are contradictory. Viel et al reported an increased NK cell frequency[97]which is due to an overall increase in absolute numbers of lymphocytes in individuals with increasing levels of BMI, rather than on a change in frequency[97]. On the contrary, Tobin et al report decreased NK cell frequency in obese adolescents[98]. When only NK cell frequency of total leukocytes or lymphocytes is measured, a possible expansion of other immune cells would be reflected in a relative decrease of NK cells. Thus, these results should be interpreted with caution. While few studies have examined NK cell in NASH, many studies have reported a retained NK cell frequency in obesity, both total and CD56^{dim} and CD56^{bright} ratio which is in line with our results[138,145–150].

While we found increased expression of NKG2C, KIRs and CD57 on CD56^{dim} NK cells compared to CD56^{bright} NK, neither presence of NAFLD nor obesity affected the NK cell receptor repertoire. One exception was the activating NK cell receptor NKG2D, that was found upregulated on NK cells from NASH patients compared to healthy controls (**Figure 4**).

NKG2D expression was also found at higher levels on both CD56^{bright} and CD56^{dim} NK cells derived from obese patients compared to lean controls. While our results should be confirmed in a larger cohort, the fact that NAFL-patients had similar levels to healthy controls could suggest that this represents an effect of NASH, rather than obesity on the NK cell population. T cells from obese individuals or NAFL/NASH-patients did not have a corresponding increase in NKG2D expression, which could imply that the effect was specific to NK cells.
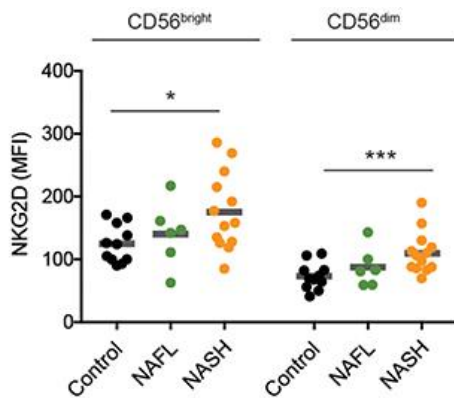

Figure 4. Increased NKG2D expression on NK cells from NASH-patients compared to controls. Adapted from Stiglund et al, *Front. Immunol.* 2019[53].

NKG2D is an activating receptor on NK cells, that binds to the ligands MICA, MICB and ULBP that are all ligands that upregulated on the cell surface in response to cellular stressors, e.g., including DNA damage responses, viral infections or oxidative stress. Triggering of NK cells through NKG2D leads to NK cell mediated cytotoxicity. Previous murine studies have indicated an increased expression of NKG2D ligands in the liver of NASH[91], which is in line with the inflammatory hepatic state during NASH with lipotoxicity, hepatocyte stress and apoptosis. In addition, MICA alleles have been associated with the histological severity of NAFLD, indicating an important role of NKG2D activation[151]. MICA-NKG2D interaction is also important for NK cell mediated defenses in HCC, a feared feature of NAFLD patients, where antibodies against NKG2D-ligands can restore NK cell cytotoxicity[152,153]. Thus, it is plausible that a corresponding increase in soluble NKG2D-ligands could be found in circulation, since the ligand can sometimes be shed from the cell surface[154]. However, in our study, MICA and MICB were not found at higher levels in circulation, nor was it expressed on healthy, primary liver cells. However, these ligands tend to be increased in conditions of cells stress. Therefore, it would be interesting to see how they are expressed on hepatocytes derived from NASH livers. Interestingly, NKG2D has been one of the receptors that has enabled NK cells to kill hepatic stellate cells (HSCs), thus limiting fibrosis development[85–87,155]. Therefore, it would be interesting to further investigate the intrahepatic cell compartment to identify the expression of NKG2D ligands more fully as well as the expression and function of hepatic NK cells. Whether NK cells target HSC's or stressed hepatocytes during NASH through NKG2D should be further investigated.

It is known that some immunological situations create an imprint on the NK cell population in humans where parts of the NK cell population mature. Examples of these situations include different viral infections, such as CMV. However, NK cells from NASH-patients express no difference in the differentiation status compared to controls, nor did they display a decreased NK cell diversity, a feature that has previously been described in other chronic liver inflammations, such as viral infections[83]. Perhaps the inflammatory environment of NASH, characterized by a low grade, asymptomatic, chronic inflammation, is too low to cause a similar imprint on the circulating NK cell compartment, since the NASH-patients display lower levels of systemic inflammation than hepatitis C patients[156].

NK cells are important in the defense against viral infections[157], fibrosis development as well as in the defense against malignancies (e.g., hepatocellular carcinoma)[99,100,158]. While NK cell functionality is hampered in other liver diseases[85–87,155] and during metabolic disease[97–99], not much is known about NK cell function in NAFLD. We therefore performed functional experiments, where NK cells were activated with IL-18/IL-12 and then co-cultured with the cell line K562s. We measured the responses by both cytokine production as well as effector function using CD107a as a marker of degranulation. We could then identify that NK cells did not show any functional defects, nor could we see any functional aberrancies in their ability to perform multiple functions (**Figure 5**).
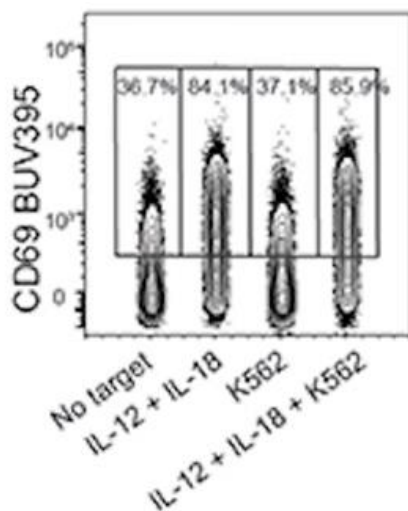

Figure 5. CD56^{dim} NK cells respond to cytokine stimulation with upregulation of CD69. Adapted from Stiglund et al, *Front. Immunol.* 2019[53].

Our data contrasts what was previously found in Viel et al where NK cells from obese individuals had an increased CD69 expression, suggesting a more activated phenotype[97]. In addition, NK cells from obese individuals responded more weakly to target cells with degranulation than NK cells derived from lean individuals. In addition, Tobin et al report decreased NK cell function in obese adolescents[98]. However, none of this analysis did

separate the more cytokine producing CD56^{bright} from the more granular, cytotoxic CD56^{dim} NK cells and a shift in the NK cell population toward CD56bright NK cells that have been seen in some[145,159], but not all studies of NK cells in obese individuals, could explain these results. In addition, several of the individuals did have severe obesity with BMI over 50 and the data suggest a dose-response relationship where the most obese individuals had the least degree of degranulation[97]. The patients in our NAFL/NASH cohort had much less severe obesity in general, which could explain why no obesity-induced functional defect was detected in our study.

Since the circulating NK cells do not always reflect the tissue compartment, NK cells derived from liver and adipose tissue were isolated. Differences were found between liver, adipose tissue, and peripheral blood NK cells. NK cells were found at a higher frequency in the liver than in circulation or in adipose tissue, which is in line with previous knowledge of NK cell enrichment in the liver[33,38,84]. The NK cell compartment in liver and adipose tissue was skewed towards a higher frequency of CD56^{bright} NK cells, where they represented almost half of the NK cell compartment compared to about 10 % in circulation. NK cells in the liver display a more immature phenotype in their expression of the differentiation markers (NKG2A, KIRs and CD57), whereas AT NKs had a differentiation status more reminiscent of that found in peripheral blood. Farber et al recently demonstrated that the levels on CD57 on NK cells are found higher in blood, bone marrow spleen and lungs[39] while NK cells derived from lymph nodes, tonsils and gut display lower levels of CD57, thus finding increased CD57 expression where a higher portion of CD56^{dim} CD16^{+} NK cells are found[39]. This is in line with our results, where more CD56^{dim} CD16^{+} NK cells as well as more CD57 expression are found in adipose tissue compared to liver. Unsupervised SNE-analysis, taking all the measured markers into account, further emphasized the differences between tissue-derived NK cells and circulating NK cells with mainly a considerably higher expression of CD69. We identified that the expression of tissue residency markers where mainly found within the CD56^{bright} NK cell subset, which has previously been seen in other tissues.

While it is well known that tissue resident NK cells are found in liver, much less has been known of NK cells in adipose tissue. Interestingly, in **study I**, a subset of NK cells found in adipose tissue displayed tissue residency markers, such as CD69 and CD49a (**Figure 6**).

Since **study I** was published, several studies have reported the presence of subsets of NK cells in adipose tissue that are important in obesity-induced adipose tissue inflammation. One of those is an IL-6 receptor[+] myeloid like NK cells subset that appears to be involved in adipose tissue inflammation and subsequent insulin resistance[147]. Several studies have demonstrated reduced cytotoxicity and cytokine production from NK cells by IL-6 treatment[160]. However, how these cells display tissue residency markers is not known and would be interesting to pursue in future studies. The NK cells that were identified in adipose tissue of obese individuals displayed high levels of CD49a, which is interesting since studies of immune cell circulation patterns using parabiosis experiment in mice, have identified an innate lymphoid cell expressing CD49a that maintained long term tissue residency in adipose

tissue during obesity[109]. NK cells in adipose tissue have also been implied as initiators of adipose tissue inflammation, contributing to insulin resistance.
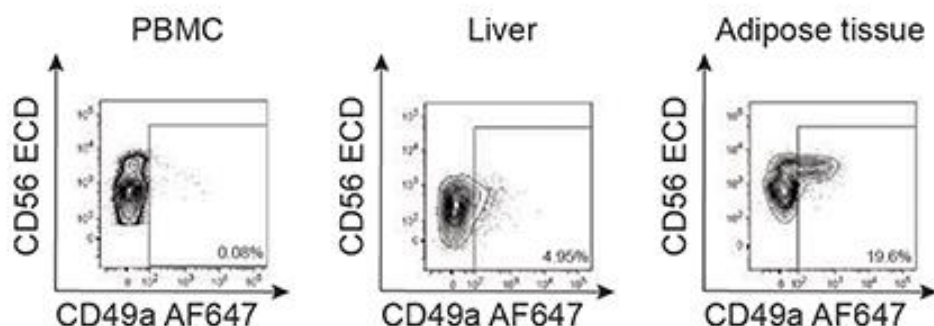

Figure 6. NK cells from adipose tissue display tissue residency markers such as CD49a. Representative flow cytometry plots of total NK cells derived from blood (left), liver (middle) and adipose tissue (right). Adapted from Stiglund et al, *Front. Immunol*. 2019[53].

Since NAFLD is a liver pathology, our hypothesis was that more activated NK cells would be found in the liver of NASH patients due to the ongoing inflammatory state. However, no difference was noted between healthy, NAFL or NASH livers in the frequency of NK cells with tissue residency markers. And no difference was identified when comparing NK cells from patient with high or low fibrosis or insulin resistance. This could be due to the low number of patients, and future studies should aim to increase our knowledge on both the phenotype as well as the function of intrahepatic NK cells in NAFLD.

In conclusion, **study I** provides novel, in-depth analysis of NK cells from patients with NASH. The receptor repertoire remained stable in patients compared to controls except for the receptor NKG2D, which was seen increased in obese patients. NK cells remained the same functional responsiveness *in vitro* as controls. While a possibly tissue resident NK cell population was found in adipose tissue, no differences in NK cell phenotype was found in tissue in relation to disease status. However, much remain to be learned, and future studies are warranted.

4.2 PROJECT II: SUBTYPE-SPECIFIC SURFACE PROTEINS ON ADIPOSE TISSUE MACROPHAGES AND THEIR ASSOCIATION TO OBESITY-INDUCED INSULIN RESISTANCE

In adipose tissue, an abundant heterogeneous macrophage population contribute to the induction and maintenance of adipose tissue inflammation[123]. Adipose tissue inflammation in turn has been associated with the generation of obesity-induced insulin resistance, a key component in metabolic diseases such as type 2 diabetes[117,161–163]. In **study II**, the surface proteome of macrophages was investigated to increase the knowledge of the phenotype of

pro-and anti-inflammatory adipose tissue macrophages. The distribution of adipose tissue into different depots, such as visceral (VAT) and subcutaneous adipose tissue (SAT), is important for the clinical presentations of obesity, with a more detrimental disease progression associated to visceral obesity[164–166]. Thus, we also wanted to carefully characterize the differences in the macrophage population between VAT and SAT to increase the understanding of the disease manifestations as well as investigate the association between macrophage phenotype and degree of insulin resistance.

In **study II**, macrophages derived from SAT and VAT in a sizable cohort of individuals with obesity was analyzed, which allowed us to identify depot specific immunophenotypes in SAT as well as VAT. Even though the highest proportion of myeloid cells was found in SAT, supported by increased relative gene expression of the macrophage marker CD68, our study showed higher levels of pro-inflammatory M1-like CD11c$^+$ CD206$^+$ macrophages in visceral adipose tissue (VAT) compared to subcutaneous adipose tissue (SAT). Previous studies have shown ambiguous results of how the expanding adipose tissue in obesity and the depots are related to inflammatory phenotype of macrophages[125,167]. Obese individuals with large visceral adipose tissue depots tend to develop more severe metabolic complications than individuals with more subcutaneous distribution of fat. Literature also describes a large infiltration of macrophages and higher levels of inflammation[124,168,169]. However, some studies highlight an association between accumulation of macrophages in rather SAT instead of VAT[125,170]. Perhaps the discrepancies reported are due to the heterogenous population of macrophages, including the many novel subsets of metabolically activated ATMs, and the possibility that different populations expand in different contexts of obesity, which ranges from the more "healthy obese"[168] phenotypes to the more severe comorbidities including diseases such as type 2 diabetes. In addition, many studies have been performed in mice and, in addition to the large differences between murine and human immune system, the adipose tissue depots also differ between mice and human. Many murine studies of adipose tissue have been performed on perigonal or epididymal fat which does not correspond to human visceral adipose tissue[171], which is important to keep in mind while comparing these data.

It is well known that the myeloid compartment in an inflamed organ consist of both cells that resided in the tissue before the onset of inflammation, local proliferation as well as cells that have been recruited to the tissue in response to the inflammation. Interestingly, our study further revealed that pro-inflammatory CD11c$^+$ CD206$^+$ macrophages displayed high levels of the chemotactic receptor CCR2, which was found on monocytes in peripheral blood but was not at all present on the M2-like CD11c$^-$ CD206$^+$ macrophages. This might strengthen the hypothesis that these cells have been recruited to the tissue from blood in response to inflammatory signals. Indeed, the ligand of CCR2, C-C motif chemokine ligand 2 (CCL2), also referred to monocyte chemoattractant protein 1 (MCP1), is increased in adipose tissue inflammation in response to obesity and to contribute to tissue infiltration of monocytes as well as local proliferation[115,172,173]. The total lack of CCR2 on the M2-like CD11c$^-$ CD206$^+$ macrophages might suggest that they have resided longer in the tissue, which would be consistent with a more tissue-resident phenotype. These cells also express less of pro-

inflammatory receptors (e.g., lower levels of HLA-DR, CD40 and CD44) which is consistent with a more anti-inflammatory phenotype. With this said, all cells residing in tissue will also modify their behavior and phenotype in response to local inflammation. In this context, we chose to use a terminology that reserves the term pro-inflammatory to cells which show a more activated phenotype, e.g., with higher levels of antigen-presentation as defined by HLA-DR expression. However, while studied in other tissue contexts[174] the exact kinetic and migration patterns of monocytes/macrophages in obesity-induced adipose tissue inflammation remains to be explored further. The surface marker expression within the cell populations also showed a large variation, suggesting a distinct heterogeneity within the cell populations.

Due to the suggested heterogeneity, we performed a large surface proteome screening of M1-like-and M2-like macrophages in SAT and compared to peripheral blood monocytes in order to increase the understanding of which markers can be found expressed on ATMs. The reason why subcutaneous adipose tissue, and not visceral adipose tissue, was used in this instance was due to the large numbers of cells needed to study 361 different surface proteins. By using subcutaneous tissue derived from liposuctions, we were able to yield a high number of tissue-derived cells. However, this represents a limitation of the study where ideally also visceral adipose tissue could have been studied. In order to be able to combine cells from adipose tissue as well as peripheral blood while still being able to distinguish them, we used barcoding with two different anti-CD45-conjugated antibodies. This enabled us to identify immune cells derived from the different tissues in the same sample. While a substantial overlap in protein expression were observed between monocytes and macrophages, the surface proteome screen revealed several novel surface proteins specific to M1-like and M2-like macrophages. Key findings were then validated in a second cohort of obese patients. When analyzing the surface proteome of monocytes, M1-like (CD11c$^+$ CD206$^+$) macrophages and M2-like (CD11c$^-$ CD206$^+$) macrophages respectively, we found several distinct surface proteins. In general, the blood derived monocytes expressed higher levels of surface proteins compared to myeloid cells derived from adipose tissue. Several surface proteins were specifically expressed on blood monocytes, e.g., integrins or other molecules involved in cell-adhesion such as CD99, CD102 (also known as ICAM-2), CD93 and CD61 (integrin β3). Adipose tissue-derived monocytes on the other hand expressed only three distinct markers CD274 (PD-L1), CD83 and CXCL16. A weakness of the surface proteome screen however is that that blood and adipose tissue were not derived from the same individual, which could contribute to these discrepancies. Therefore, a larger validation was performed on a larger cohort. M1-like macrophages expressed higher levels of CD48, CD371, CD85a, CD49d, CX3CR1 and CD52 compared to M2-like macrophages while M2-like macrophages expressed higher levels of CD116, CD51, CD26 and integrin α9β1. AT-derived monocytes expressed a similar receptor repertoire to M1-like macrophages. However, some markers such as CD40, CD74, FcεRα were expressed exclusively on M1-like macrophages, not on AT monocytes. Integrin β5 and CD276 were exclusively expressed on ATMs, both M1-and M2-like, but not on monocytes.

In order to verify the results from the surface proteome screen, subcutaneous and visceral adipose tissue was collected from 23 obese individuals undergoing bariatric surgery. Indeed, M1-like macrophages expressed high levels of CCR2/CD192, CD85a, CD48 and CD371 while M2-like-macrophages expressed high levels of CD26, CD116, CD51 and integrin α9β1. Interestingly, M1-like macrophages did not only express higher levels of CCR2 than M2-like macrophages, but they did express higher levels in VAT than their counterpart isolated from subcutaneous adipose tissue. This could be due to a higher infiltration of inflammatory cells into VAT in response to adipose tissue inflammation. While M1-like macrophages expressed high levels of inflammatory markers such as CD48 and CD371, a large heterogeneity was revealed within the macrophage population, indicating the presence of subgroups within this distinct population. This is in line with previous finding, where several adipose tissue specific macrophage subtypes have been identified, including metabolically activated macrophages, neuron-associated macrophages (NAMs)[132], using single cell transcriptional studies[128–131]. Many of these recently described macrophage subtypes have in common that they respond to different metabolic mediators that are found locally in the adipose tissue, such as lipids such as palmitate, oxidized lipids etc[128,130]. And whereas some have pro-inflammatory properties, others display protective properties in obesity, such as the lipid-associated macrophages (LAMs), which also have been described in liver[133]. How the surface markers that we have identified are expressed on these cells would be interesting to further study.

One of the novel markers that was found increased on M1-like macrophages was the immunomodulatory receptor CD48. CD48 is a member of the signaling lymphocytic activation molecule family (SLAMF) of receptors and acts through adhesion and activation of a range of immune cells[175,176]. CD48 is often found upregulated on activated cells and can also activate other immune cells such as T cells or eosinophils. Interestingly, CD48 is an important ligand for the NK cell receptor, CD244 (or Natural Killer Cell Receptor 2B4) which has previously been shown to activate and modify NK cell responses[177–180]. As mentioned in the introduction, several studies have proposed macrophage NK cell interactions during obesity development. However, even though CD48-CD244 signaling is involved in several other inflammatory diseases[181,182], the role of CD48 in adipose tissue and obesity has been poorly studied. Thus, it would be interesting to further pursue if CD48 has a role in adipose tissue inflammation, during health and obesity-induced inflammation, possibly through NK cell mediated effects.

All in all, several markers showed different results between the surface proteome screen and the validation cohort which probably is due to this large ATM heterogeneity where several markers can be found expressed in both macrophage populations and in widely varying levels. In VAT M1-like macrophages express higher levels of CD51 than M2-like macrophages but the same was not seen in SAT. Though expressed in at low levels, integrin α9β1 was expressed on more M2-macrophages than in their M1-like counterparts in both SAT and VAT.

Due to the patterns of heterogeneous receptor expression suggesting macrophage subpopulations, a UMAP was performed to assess the multivariate relationships of marker expression. Monocytes, M1-like and M2-like macrophages were divided into separate clusters. In addition, myeloid cells derived from circulation separated from myeloid cells originating from adipose tissue. While M1-like macrophages from adipose tissue co-localized, suggesting phenotypical similarities, two distinct clusters of M2-like macrophages can be seen, depending on their origin in VAT or SAT. This might suggest differences in this cell population depending on which adipose tissue depot they exist in. Thus, present and future studies are needed to further understand ATM heterogeneity on a transcriptional level.

We also assessed the relationship between the presence of pro-inflammatory macrophages and the presence of insulin resistance, in the form of HOMA-IR, and found a positive association with a relative increase in pro-inflammatory M1-like macrophages in both SAT and VAT. In addition, ratio of M1-like macrophages/M2-like macrophages also correlated to circulating levels of triglycerides (TGs), indicating worse metabolic health.

Lastly, we explored if the levels of macrophages were related to other immunological components such as the presence of crown-like structures (CLS) and the pro-inflammatory gene expression in SAT and VAT. Interestingly many pro-inflammatory genes were found at higher relative gene expression in SAT compared to VAT, including CD68 and CCL2, the ligand to CCR2. Crown-like structures, CLS, were found in few numbers and in similar levels in SAT and VAT. SAT did contain larger adipocytes than VAT. Previously the CLS were believed to be structures where macrophages surrounded dying hepatocytes. However, recent studies have proposed the hypothesis that metabolically activated macrophages[128] or lipid-associated macrophages (LAMs) accumulates in CLSs to protect adipocytes[133,183]. The metabolically activated macrophages are activated by metabolic mediators such as lipids and glucose and represent a distinct activation profile not defined as pro-or anti-inflammatory, e.g., with expression of transcription factor PPARγ that is involved in lipid metabolism.

In conclusion, we identify novel surface markers on ATMs such as CD85a, CD48 and CD371 on M1-like macrophages and CD51 and integrin a9b1 om M2-like macrophages. In addition, our UMAP analysis identified two distinct populations of M2-like macrophages from SAT and VAT, suggesting that further research might be required to investigate these populations.

4.3 PROJECT III: DEREGULATED PERIPHERAL PROTEOME REVEALS NASH-SPECIFIC SIGNATURE IDENTIFYING PATIENT SUBGROUPS WITH DISTINCT LIVER BIOLOGY

As described in the introduction, while there are several biomarkers, scoring systems and other measuring methods, such as Fibroscan, to identify NAFLD patients with high levels of fibrosis, there are no reliable serum biomarkers for patients with NASH. This not only makes it difficult to identify these patients but makes tracking of treatment effect on the liver inflammation impossible without continuous sampling with liver biopsies. Thus, in **study III,**

we wanted to measure the immune proteome in the serum of NASH patients in order to find a signature that separates NASH patients from those without inflammation (NAFL). We collected serum from NAFL-patients (n=35), NASH-patients (n=35) and healthy, lean controls (n=15) and measured 92 inflammatory serum proteins using a proximity extension assay (PEA). Our analysis revealed a distinct inflammatory imprint in NASH compared to NAFL patients as well as healthy controls. Many of the significantly altered inflammatory serum proteins were present at lower level in NASH compared to the other cohorts, except for ST1A1 and SCF, that were both found at significantly higher levels in NASH. 13 serum proteins were differentially expressed between NASH and NAFL patients and seven serum proteins between NASH compared to healthy controls. Using logistic regression analysis, we could identify six of these proteins (ADA, FLt3L, EN-RAGE, IL-18, IL-6, and ST1A1) as the best combination of proteins to separate NASH from NAFL-patients. No inflammatory serum imprint was seen when comparing NAFL and healthy controls, which is in line with the absence of liver inflammation in these two groups. In addition, the co-expression patterns of serum proteins were analyzed, and several dysregulated cytokine pathways were identified using pathway analysis. To conclude, using pre-existing datasets of liver transcripts from NAFLD patients we mapped the expression of the serum proteins to liver macrophages and periportal hepatocytes. We also identified clusters of NASH-patients with different transcriptional profiles, possibly indicating subgroups of NASH patients.

Interestingly, while we detect inflammation in the form of increased white blood cell count (WBCs) and hsCRP, our study suggests lower levels of several inflammatory proteins in the circulation in NASH patients. This might seem controversial since some of them, like IL-18 and IL-6 have been associated with the metabolic syndrome. Several studies have found increased levels of IL-18 in obesity and other metabolic diseases, such as insulin resistance[184,185]. This overlap with a common comorbidity makes the results on increased IL-18 in NAFLD more difficult to interpret. Studies on IL-18 in NASH are scarce and have not always taken the presence of obesity nor insulin resistance into account or distinguished between simple steatosis and NASH[186,187]. When NASH-patients have been separated from NAFL-patients, no difference in IL-18 have been detected[188]. The situation is similar regarding IL-6 where increased levels in NAFLD patients, as well as in obese patients with different conditions such as type 2 diabetes, have been reported. The literature on this topic however is not consistent. Even though there are studies that report increased levels of IL-6 in NASH-patients compared to NAFL[189,190], one study has reported a similar decrease of IL-6 as seen in our study.[191]. This might be due to a substantial difference in BMI in the cohorts measured, since IL-6 is produced by adipocytes and is often found elevated in obese individuals[192,193]. On a similar note, a previous study that reports an increase of IL-6 transcripts in NASH-livers that were not mirrored by an increased levels in adipose tissue or circulation[194]. In addition, a recent study showed increased levels of soluble IL-6 Rα in NASH. IL-6 can bind to sIL-6 R-a and send activating signaling through trans signaling[195]. Thus, it is possible that IL-6 is bound to its receptor and thus not detected in our assays. In addition, it is important to remember that much cytokine signaling take place in an autocrine

or paracrine manner locally in the inflamed tissue. While IL-6 is considered one of the important cytokines in obesity, the proinflammatory role of IL-6 is controversial, with studies suggesting a protective, role of IL-6 in adipose tissue homeostasis and alternative activation of macrophages[196]. Interestingly, an adipose tissue resident NK cell population also display IL-6R[147]. The differentially expressed gene transcripts of hepatic IL-6, indicating heterogeneity among NASH-patients, along with the association between IL-6 and obesity, provide a possible explanation to the contradictory results[197,198].

Due to the many comorbidities and possible confounders when studying obese individuals, we carefully assessed whether our results were affected by comorbidity. But neither obesity, age, presence of type 2 diabetes, hypertension or obesity did alter our results. However, these results are preliminary, and our cohorts are relatively small. Thus, these results would need to be validated in a larger cohort, including more patients with comorbidities such as type 2 diabetes. It would also be interesting to follow the inflammatory imprint over time, e.g., in relation to development of HCC, which would be possible e.g., during the large ongoing clinical trials in NASH.

By analyzing co-expression patterns of the measured proteins and comparing the different groups, we could identify several clusters of co-expressing proteins that differed among the groups. Further analysis using a pathway analysis revealed that the ten most dysregulated clusters in NAFLD were involved in IL-4, IL-10 and IL-13 signaling. IL-4 and IL-13 can, in a liver context, contribute to alternative activation of macrophages towards a profibrogenic phenotype, which can also produce anti-inflammatory cytokines[75]. However, in addition NASH-patients displayed deregulations in IL-18 and NFκB-signaling. Our results highlight inflammatory pathways in Th2-immunity in NAFLD. This is in line with previous studies with pro-fibrotic effects of Th2 in NAFLD, both murine and human results[64]. This might seem contradictory since adipose tissue inflammation often coexist in obese patients with NAFLD. In adipose tissue, Th2 immunity is connected to maintenance of anti-inflammatory homeostasis while it in NASH livers is connected to NAFLD exacerbation and fibrosis development[111]. This emphasizes that even though inflammation tend to coexist in the adipose tissue and liver in the context of NAFLD, the pathogenic effect can be very different. In addition, in the context of mixed inflammatory states with both Th1 and Th2 activity, localization of cells into different niches can affect the outcome. One such instance can be Th1 cells in liver parenchyma while Th2 cells stay in perivascular areas near the pro-fibrotic hepatic stellate cells[199]. How this affects NASH pathogenesis remain unknown but could contribute to the, sometimes, contradictory results from immunological studies of NAFLD.

Since our serum signature was distinct to NASH-patients, we wanted to see what it could teach us about the pathogenesis of NASH. Using pre-existing data sets of healthy livers we could identify expression of IL-18, ST1A1 and S10012 (EN-RAGE) in a subset of periportal hepatocytes and a subset of myeloid cells. Taken together, it would be interesting in future studies to further explore these cell subsets in NASH-development.

Finally, we used our six serum proteins and analyzed their corresponding gene transcripts in pre-existing data sets of transcriptomes from NASH-livers. This revealed eight different clusters depending on how these transcripts were expressed. While some clusters were similar, this clustering also revealed highly different clusters, e.g., cluster A, B, F and G. This heterogeneity among the patients is interesting and could be an explanation to the very heterogeneous prognosis and treatment responses that are seen in some drug trials[200,201]. Other studies have also implied the presence of subgroups in NASH. In 2017 two different subtypes of NAFLD was identified based on metabolomics. By comparing serum from mice and NAFLD patients three different groups were identified: M-subtype, non-M subtype as well as indeterminate subtype. Since hepatic steatosis, though histologically similar, can arise from a variety of reasons, it is possible that these patients may have different etiology of disease[59]. However, these mice data are preliminary, and the subtypes does not follow the NAFL/NASH dichotomy where patients with NASH and advanced fibrosis usually are the ones under consideration for treatment.

Interestingly, some clusters had high levels of *SULT1A1* (ST1A1), which was found in increased levels in NASH-patients. Sulfotransferase A1 is an enzyme found increased in liver and is involved in many different biological processes. We also identified a similar rise of ST1A1 to the one seen in the serum of NASH-patients in a model of NASH-organoids. While ST1A1 is linked to other inflammatory diseases[202], it has not previously been described in NAFLD. Thus, in study III, we identify novel proteins that might be involved in NASH pathogenesis.

Luo et al performed a proteomic scan that identified several biomarkers that associated with liver fibrosis in patients with NASH[81]. This is highly relevant since patients with high levels of fibrosis are the ones considered for treatment now, due to their prognosis. Several scoring systems exist for the measurement of fibrosis in NASH, such as FIB-4, NAFLD fibrosis score and the use of Fibroscan, but scoring systems or biomarkers that are related to the levels of inflammation are still lacking, which is addressed in **study III**. Our main goal in this paper was to study the systemic inflammatory imprint, not fibrosis, even though several studies have showed that fibrosis deposition is linked to worse prognostic outcomes[88,89]. It is possible that the collagen accumulation is a more stable measurement, and that inflammation is more rapidly fluctuating. However, there is a lot of knowledge of interactions between the immune system and hepatic stellate cells, the main producers of collagen in the livers. In NASH, several waves of acute hepatic inflammation are followed by regenerative, reparative phases that lead to fibrosis deposition[203]. This can lead to an alternative activation of macrophages towards an immune suppressive but pro-fibrogenic phenotype. These cells can produce high levels of IL-13 and TGF-β1 contributing to fibrosis development[75]. Thus, it is plausible that presence of inflammation would be linked to fibrosis development, and it is known that long term inflammation is detrimental in other chronic inflammatory diseases. But due to the difficulties in measuring often and repeatedly with biopsies this is difficult to study in NAFLD. However, with the large clinical trials that are ongoing worldwide, trying out drugs

to hamper NASH with advanced fibrosis, new opportunities to study the progression of the disease has arrived.

4.4 GENERAL DISCUSSION

One potential limitation of our studies of NASH is that the presence of inflammation is based on the rather blunt lymphocyte count included in the histological NAFLD activity score. We use this to study inflammation in the circulation, in both project I and III. While in line with diagnostic criteria, one could argue that liver biopsies risk not being representative of the overall liver histology since a very limited volume of liver tissue is measured. There are some imaging techniques that can, noninvasively, define a more global imprint of liver inflammation and it would have been interesting to compare our results using such a technique. However, this is a limitation that is inherent due to the diagnostic criteria of the disease.

Many studies on obesity-induced inflammation have been performed in mice. A healthy inflammatory response resolves after clearance of the harmful stimuli. However, obesity-induced inflammation is often chronic. In experimental models, metabolically activated adipose tissue macrophages, activated by free fatty acids (FFAs), have been shown to perform both detrimental and beneficial functions during diet-induced obesity, depending on the timing and duration of obesity[129]. This emphasizes the importance of timing when studying human obesity-induced inflammation. However, this is a considerable challenge when studying humans where patients often tend to be in different disease phases, e.g., years after onset of disease, which can be difficult to measure at this time. In addition to the time aspect, murine and human immunity differs greatly, why it is important to improve the knowledge of the human immune system, something that the studies included in this thesis has strived towards.

When thinking about immunity and studying the immune system in a disease context, it is easy to forget that an adult human has encountered many different microbes and immunological events during their lifetime. One of the microbes that is known to greatly affect NK cells is CMV, which is a very common infectious agent creating latent infection in humans. In addition, obesity itself is often complicated with several comorbidities, such as cardiovascular disease, atherosclerosis and type 2 diabetes. This is a major difference when comparing human studies to mice, since mice often have a completely identical genetic background, as well as a homogenous immunological history by have been bred in a sterile, microbiota free environment. Thus, that human studies show a more complex, and sometimes contradictory results, compared to the clear, reproducible results in mice, may not be surprising.

While the link between obesity-induced chronic inflammation and metabolic is widely accepted, whether this represents a causal connection is not fully known. In murine models, anti-inflammatory treatments or blockage of inflammatory mediators have been very successful to treat metabolic diseases[108]. However, when metabolic diseases have been

treated with anti-inflammatory treatments in patients the effects on insulin resistance have been modest. Whether this is due to factors such as study design (randomization, cohort size, dose/length of treatment, target) is not known and clinical trials are still ongoing[204]. Recent studies have also implied non-inflammatory roles of macrophages in NAFLD[205] and it has long been known that e.g., IL-18 can have impact on metabolism beyond immune mediated effects. Thus, while this thesis has focused on the immune-mediated effects of obesity, the field requires future studies in order to further elucidate this causality. In addition, this thesis has not focused on the differences displayed within the obese population, where some individuals display a more "metabolically healthy phenotype" with less presence of metabolic disease.

The studies in this thesis have mainly been performed using flow cytometry. However, during the years of this PhD, the methods to study immune cells have been revolutionized. Future studies, using integrated multi-omics analysis as well as single cell spatial transcriptomics have the potential to further increase our knowledge, answering questions that previously was not possible, especially in such a complex field as obesity-induced tissue inflammation.

5 CONCLUDING REMARKS

Innate immunity plays an important part in obesity-induced inflammation. In this thesis, different parts of the innate immune systems, (NK cells, macrophages and circulating inflammatory mediators) have been investigated to provide increased understanding of the pathological processes involved in the different disease manifestations linked to the presence of obesity. In summary, our key findings are:

- While NKG2D, was increased on NK cells in NASH compared to healthy controls, the circulating NK cell population remains at large unaltered in NAFLD (**Study I**).

- Adipose tissue contains a population of NK cells displaying the tissue residency markers CD69 and CD49a (**Study I**).

- Several novel surface markers associated with a proinflammatory (CD85a, CD48 and CD371), or anti-inflammatory phenotype (CD51 and integrin α9β1) of adipose tissue macrophages were detected (**Study II**)

- Depot-specific immunophenotypes in SAT and VAT were identified (**Study II**).

- Insulin resistance was positively correlated to the pro-inflammatory/anti-inflammatory macrophages ratios in both SAT and VAT (**Study II**).

- A NASH-specific inflammatory imprint with 13 serum proteins could, independent of comorbidities and fibrosis stage, differ between NAFL and NASH patients (**Study III**).

- Several pathways were dysregulated in NAFLD, including IL-18 and NFκβ-signaling (**Study III**)

- The differentially expressed inflammatory proteins in serum of NASH-patients can be used to further subgroup NASH-patients (**Study III**)

Taken together, the studies in this thesis have provided new knowledge on how obesity could affect innate immunity, but there are still many outstanding questions that remains to be addressed.

6 POINTS OF PERSPECTIVES

Obesity is a comorbidity that complicates many different diseases and affect patients that are found in all different areas of medicine, from cardiology and oncology to autoimmune or infectious diseases. Thus, it is important to increase our knowledge of how obesity affect our immune system, not only due to its high prevalence but also because of the effect it can have on our immune system, including the defense against viral infections or against malignancies. While much remain to be explored, the aim of this thesis has been to add some pieces of new knowledge to the complex puzzle of human immunity. The important evolutionary conserved interactions between metabolism and immunity are demonstrated by a strong link between inflammatory mediators and insulin resistance. Many inflammatory mediators e.g., toll-like receptor (TLR)-signaling or IL-6, can directly interfere with insulin signaling, likely to keep glucose in the blood stream as energy sources for immune cells during infection. Several connections exist between metabolic needs and inflammatory pathways, including NLPR3 inflammasome activation[206]. Some drugs, such as the anti-inflammatory corticosteroids, exist in the borderland between immunology and metabolism.

One example of where obesity can be detrimental to the immune response is viral infections. Murine models have demonstrated an increased mortality in obese mice from viral infections such as influenza, and that this is in part due to decreased NK cell cytotoxicity[207]. It is also known that obesity is linked to increased risk of developing certain types of cancer. Many studies hypothesis that this could, in part, be due to decreased immune functions. A decreased NK cell function, as reported in several studies of NK cells in obesity, could possibly have a detrimental effect on both the anti-viral as well as the anti-tumor defense. It could potentially also affect metabolic disease, as repeatedly demonstrated in mice. Many new cancer therapies against hematological malignancies are based on modified immune cells, including NK cells[209,210]. Perhaps further knowledge of the detrimental effect of obesity induced inflammation on the immune system in the future could be harnessed to improve the treatment of patients with e.g., malignancies or severe viral infections that also suffer from obesity. Thus, the aim of this thesis has been to contribute a part to the field of immunometabolism.

7 ACKNOWLEDGEMENTS

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