Characterization of adipose tissue and liver innate immune cells in obesity-related insulin resistance

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This study was conducted from January 2017 to September 2020 mainly at the Hormone Laboratory Research Group, Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital and Department of Clinical Science, University of Bergen, Bergen, Norway. Parts of the study was also conducted at Centre for Infectious Medicine, Department of Medicine, Huddinge, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden. The work was carried out under the supervision of Johan Fernø, Niklas Björkström, and Gunnar Mellgren.

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Kristina Strand

Abbreviations

APC	Adipocyte progenitor cells
AT	Adipose tissue
ATM	Adipose tissue macrophages
BAT	Brown adipose tissue
BMI	Body mass index
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CLS	Crown-like structures
CVD	Cardiovascular disease
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DEG	Differentially expressed gene
DPP4	Dipeptidyl peptidase 4
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FFA	Free fatty acids
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HFD	High-fat diet
HLA	Human leukocyte antigen
HOMA-IR	Homeostatic model assessment of insulin resistance
ICAM-1	Intracellular adhesion molecule -1
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cells
iNKT cell	Invariant NK T cell
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
IκK	Iκ B kinase β
JAK	Janus kinase
JNK	c-JUN N-terminal kinase
KIR	Killer cell immunoglobulin-like receptor
LAM	Lipid-associated macrophages
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide

MAIT cell	Mucosal-associated invariant T cell
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony stimulating factor
MIP	Macrophage inflammatory protein
MMe	Metabolically activated macrophages
mTORC	Mammalian target of rapamycin complex
NAFLD	Nonalcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCR	Natural cytotoxicity receptors
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NKG2D	Natural killer group 2, member D
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PI3	Phosphatidylinositol 3
PKB	Protein kinase B
PPAR	Proliferator-activated receptor
PRR	Pattern-recognition receptor
S1PR1	Sphingosine-1-phosphate receptor
SAT	Subcutaneous adipose tissue
scRNA-seq	Single cell RNA-sequencing
SFA	Saturated fatty acids
STAT	Signal transducer and activator of transcription
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TG	Triglycerides
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
TREM	Triggering receptor expressed on myeloid cells
UCP-1	Uncoupling protein-1
UPR	Unfolded protein response
VAT	Visceral adipose tissue
WAT	White adipose tissue
WC	Waist circumference
WHO	World Health Organization
WHR	Waist-to-hip ratio

Abstract

Obesity is a highly prevalent disease associated with a number of chronic and noncommunicable diseases including type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD). Obesity is associated with a chronic low-grade systemic inflammation, which is thought to cause insulin resistance as well as other co-morbidities. The systemic inflammation may originate in the adipose tissue (AT), and is characterized by an infiltration of immune cells into the tissue and paracrine and endocrine secretion of pro-inflammatory cytokines. Adipose tissue macrophages and NK cells has previously been shown to be important for obesity-associated AT inflammation, and NK cells have also been linked to liver inflammation in mice models of (NASH).

In paper I, we investigated circulating NK cells in patients with NAFLD and NASH and found that the proportions of $CD56^{dim}$ to $CD56^{bright}$ NK cells were similar to healthy controls. Further, NK cells from patients and healthy controls showed similar expression levels of activating and inhibitory receptors, with the exception of NK cells from patients with NASH, which showed increased expression of the activating receptor NKG2D. NK cells from NAFLD and NASH patients also had retained functionality compared to healthy controls, and the frequency of liver and AT NK cells were unaltered in the patients.

In paper II, we characterized ATMs in individuals with obesity, and found that the visceral adipose tissue (VAT) harboured relatively more pro-inflammatory M1-like macrophages than the subcutaneous visceral tissue (SAT). The pro-inflammatory ratio between M1- and M2-like ATMs correlated positively with the degree of insulin resistance and dyslipidemia. For other markers of inflammation, such as expression of inflammatory genes or the numbers of crown-like structures in the AT, we did not find any signficant associations with the metabolic disease parameters. Interestingly, improved insulin sensitivity after weight loss following bariatric surgery was not correlated to a simultaneous decrease in circulating inflammatory factors, suggesting that these two events were not linked.

Lastly, in paper III we characterized the surface proteome of ATMs and adipocyte progenitor cells (APCs) and found subpopulations of the macrophages and progenitors with distinct expression of surface proteins. We compared our surface protein data with an available scRNA-seq dataset, and found enrichment of both ATM and APC-specific markers in macrophage and progenitor clusters. We also used the scRNA-seq data to explore the local interactome of the macrophage and progenitor cells in adipose tissue, using a computational method guided by our surface protein expression data.

List of publications

Paper I

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

Paper II

Strand, K., Stiglund, N., Haugstøyl, M., Kamyab, S.Z., Laupsa-Borge, J., Langhelle, V., Hjellestad, I.D., Nielsen, H., Njølstad, P., Mellgren, G., Björkström N., Fernø, J. Distinct patterns of local compared to systemic inflammation in patients with obesity-induced insulin resistance. *Manuscript*

Paper III

Strand, K., Haugstøyl, M., Stiglund, N., Dyer L., Busch, C., Cornilliet M., Mellgren, G., Björkström, N.K., Fernø, J. Identification of subtype-specific surface proteins on adipose tissue macrophages and adipocyte progenitor cells. *Manuscript*

Related papers not included in the thesis

Fernø, J., **Strand, K**., Mellgren, G., Stiglund, N., Björkström, N.K. Natural Killer Cells as Sensors of Adipose Tissue Stress. *Trends Endocrinol Metab* 2020;31(1):3-12

Contents

1. Introduction

1.1 Obesity

1.1.1 Definition and prevalence

Obesity is considered a major global health problem with increased risk of developing co-morbidities such as insulin resistance and type 2 diabetes (T2D), cardiovascular disease (CVD), nonalcoholic fatty liver disease (NAFLD) and certain cancers. Obesity is also associated with increased mortality compared to normal weight (1), also independently of physical activity levels (2). According to the World Health Organization (WHO), the rate of worldwide obesity has almost tripled since 1975, while the prevalence of obesity in Norway had nearly doubled between 1980 and 2015 (3). The WHO stated that in 2016 more than 650 million adults worldwide were defined as being obese, with estimates of more than 1.12 billion obese individuals by 2030. Further, 1.9 billion adults were classified as being overweight in 2016 (4).

WHO defines obesity and overweight as excessive accumulation of fat that may have negative impact on health. Body mass index (BMI) is a common way to measure obesity and overweight and is calculated by dividing the weight of an individual in kilograms by the square of its height measure in meters (kg/m^2). For adults, overweight and obesity is classified as having a BMI of 25 and above and 30 and above respectively (5). Further obesity is classified into three categories depending on BMI (Table 1).

The risk of comorbidities is expected to increase with increasing BMI and even moderate weight gain in adults are shown to be associated with a higher risk of death (2), which is also true for people characterized as overweight (6,7). BMI is a useful measure of obesity on a population level; however, it does not take into account the distribution of body fat nor if a higher weight is due to increased muscle mass rather than increased fat mass. Thus, individuals with the same BMI may not have the same level of excess fat, the same degree of obesity or the same obesity-associated health issues (5).

Table 1: Obesity classification according to BMI[a]

Classification	**BMI (kg/m^2)**
Underweight	< 18.5
Normal range	18.50 – 24.99
Overweight	≥ 25.00
Preobese	25.00 – 29.99
Obese class I	30.00 – 34.99
Obese class II	35.00 – 39.99
Obese class III	≥ 40.00

[a]According to WHO (reference, fact sheet)

As increased fat accumulation in the abdominal region is associated with increased risk of obesity-related co-morbidities (8) the waist circumference (WC) or the waist-to-hip ratio (WHR) has been suggested as alternative measures to determine obesity and risk for comorbidities. The WHR is found to predict diabetes and CVD better than BMI (9,10), and WC is also considered one of the parameters defining the metabolic syndrome (11). WC, rather than BMI, was also found to predict obesity-associated co-morbidities. Consequently, similar health risk was seen for individuals with the same WC regardless of whether they were defined as lean, overweight or obese according to their BMI (12). Additionally, the waist-to-height ratio is also used to predict risk of obesity-related diseases and had been shown to be better predictors of CVD and T2D than BMI (13). Additionally, increased body fat percentage is seen in individuals with prediabetes or T2D that are considered lean according to their BMI. However, BMI is still widely used as inclusion criteria in for instance clinical studies or to be eligible for obesity treatment with bariatric surgery (14).

1.1.2 Comorbidities of obesity

Obesity is associated with a number of comorbidities, including CVD, NAFLD, T2D, and several types of cancer. The metabolic syndrome is a term used to describe a combination of metabolic disturbances associated with obesity. It was first described in the 1920's and has been defined in various ways later. However, the current definition describes it as cardiovascular risk factors that is associated with a high risk of developing T2D. The International Diabetes Federation had defined the metabolic syndrome as the presence of central obesity as well as the occurrence of two or more of the following: elevated levels of triglycerides (≥ 1.7 mmol/L), decreased levels of HDL-cholesterol (< 1.03 mmol/L in males and <1.29 mmol/L in females), elevated blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic) or increased fasting blood glucose levels (≥ 5.6 mml/L or previously diagnosed T2D) (15).

Cardiovascular disease

Obesity is known to increase the risk of CVD, and weight gain may increase the risk of death from CVD (2). Cytokines and other inflammatory factors secreted by adipose tissue during obesity can affect blood lipid levels and promote atherosclerosis. Visceral adiposity is associated with CVD (16), hypertension, (17) and higher levels of plasminogen activator inhibitor-1 (PAI-1), which is a pro-coagulant. Further fat storage in other places than the AT, such as in the heart, blood vessel,s and kidneys has been found to increase CVD risk (18).

Accumulation of abdominal fat is associated with increased CVD risk as both the WC and WHR are independently associated with an increased risk of coronary heart disease. Obesity is also associated with increased levels of leptin, which can induce platelet aggregation and arterial thrombosis (19). Increased leptin levels are also linked to impairments in vascular functions (20). Low levels of adiponectin, which is seen in obese individuals, has also been associated with impaired vasodilation (21). Thus, the circulating levels of the adipokines leptin and adiponectin might be a link between obesity and CVD.

Dyslipidemia, hypertension, and glucose dysmetabolism is thought to be obesity-induced factors that can increase the risk for cardiovascular disease. Further, adipokines, pro-inflammatory cytokines, and fibrinolytic factors secreted from adipocytes or adipose tissue macrophages might also increase oxidative stress and endothelial dysfunction, which in turn can lead to atherosclerosis (22).

Type 2 diabetes and insulin resistance

Obesity is a major risk factor for development of T2D and insulin resistance. Diabetes is a disease characterized by elevated levels of blood glucose. The two major forms of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is an autoimmune disease where the insulin-producing β cells in the pancreas is destroyed leading to insulin deficiency. T2D, on the other hand, is a multifactorial disease which is characterized by a combination of insulin resistance and pancreatic β cell failure. The insulin resistance leads to higher levels of insulin being secreted by the pancreatic β cells, which compensates for the lack of responsiveness to insulin in the tissues and maintain normal or almost normal glucose levels. Eventually, the β cells start to fail and can no longer secrete high enough amounts of insulin, leading to hyperglycemia and T2D (23).

The prevalence of diabetes is thought to be about 463 million people and is estimated to increase to about 578 million by 2030. T2D accounts for around 90 % of diabetes cases worldwide. At the same time, half of the world's 493 individuals with T2D are unaware of their condition (24). WHO recommends four ways to diagnose diabetes and those include: fasting plasma glucose values ≥7.0 mmol/L, plasma glucose ≥11.1 mol/L 2 hours after glucose load, HbA1c ≥48 mmol/mol (6.5%) or a random blood glucose ≥11.1 mmol/L in the presence of symptoms of diabetes (25). HbA1c is glycosylated hemoglobin and reflects the average blood glucose levels the past 8 to 12 weeks (26).

Prediabetes is characterized by impaired glucose tolerance and/or impaired fasting glucose and is a risk factor for development of T2D (26). WHO defines impaired glucose tolerance as fasting plasma glucose of < 7.0 mmol/l or 2-hour plasma glucose

≥7.8 and < 11.1 mmol/L and impaired fasting glucose as both a fasting glucose level between 6.1 and 6.9 mmol/L and a 2-hour plasma glucose of <7.8 mmol/L. Further, the American Diabetes Association also includes HbA1c between 39 and 47 (5.7-6.4%) in their criteria for prediabetes (26). Diabetes is a risk factor for CVD through mechanisms such as insulin resistance, inflammation, and endothelial dysfunctions. Further high blood glucose levels can have toxic effects om microvasculature.

Insulin resistance occurs when a normal dose of insulin cannot stimulate its regular responses thereby decreasing insulin signaling. This leads to a reduction in insulin sensitivity. In the adipose tissue, insulin resistance leads to increased lipolysis, stimulating re-esterification of lipids in other tissues, such as muscle and liver, further worsening the insulin resistance. Lipid accumulation in muscle cells can impair glucose uptake resulting in increased glucose to the liver, while in the liver, lipid accumulation reduces the capacity of insulin to activate glycogen synthesis and regulate gluconeogenesis (27).

Insulin resistance can be assessed using the hyperinsulinemic-euglycemic clamp technique, which is considered the gold standard method to measure insulin sensitivity. This technique involves insulin being administered intravenously at a constant rate. Subsequently, glucose is intravenously infused to maintain fasting levels of glucose in the blood. Thus, the amount of glucose infused is equivalent to the amount of glucose needed to compensate for the hyperinsulinemia. The steady-state glucose infusion rate then reflects the level of insulin sensitivity (28,29). The hyperinsulinemic-euglycemic clamp technique is a complicated and labor-intensive way to measure insulin resistance. Thus, the homeostatic model assessment of insulin resistance (HOMA-IR) is frequently used as a more feasible alternative to measure insulin resistance in larger cohorts. This method, first described in 1985 (30), uses fasting glucose and insulin levels (measured in mmol/L) to determine insulin resistance and β-cell function (%B), which can be measured using simple formulas: HOMA-IR = (FPI x FPG)/22.5 and HOMA%B (20 x FPI) / (FPG – 3.5), where FPI and FPG is fasting plasma insulin and fasting plasma glucose respectively (31). Later a computer model was developed to more accurately determine HOMA-IR and β-cell function using fasting plasma glucose

and fasting plasma insulin or C-peptide levels across a range of 1-2200 pmol/L for insulin and 1-25 mmol/L for glucose (32). Currently, no cut-off values for HOMA-IR has been set to define insulin resistance.

Nonalcoholic fatty liver disease and non-alcoholic steatohepatitis

NAFLD is the most common chronic liver disease with a prevalence of about 24 % in 2016 (33). NAFLD can progress to a more severe form of liver disease, non-alcoholic steatohepatitis (NASH), with the risk of developing liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (34). Development of NAFLD is driven by a combination of genetic and environmental factors and is linked to obesity and the metabolic syndrome. There is currently no effective medication against NAFLD (35), and it is thought that this disease will become the primary cause of liver transplantation in the coming years (36). Progression to NASH might involve reduced hepatic levels of neuregulin 4, a factor secreted by adipocytes. Further, type II inflammatory responses involving transforming growth factor β (TGFβ) is thought to promote fibrosis in NAFLD. Patients with NASH fibrosis is found to have more IgA+ plasma cells in their liver relative to patients without NASH fibrosis. These cells may inhibit CD8+ T cell activity leading to increased risk of HCC (35).

1.1.3 Treatment of obesity

Lifestyle interventions

The WHO recommend a weight loss of 10% in order to improve health and reduce co-morbidities (37). Traditional treatment of obesity is lifestyle interventions, including diet, exercise, and behavior therapy. However, while some studies have shown substantial weight loss for obese individuals undergoing weight-loss interventions, a meta-analysis showed that most studies reported a weight loss less than 5% with little or no beneficial effects on metabolic parameters (38).

Pharmacological treatment

Pharmacological treatment of obesity is often a supplement to lifestyle interventions leading to additional weight loss. Many drugs used in the treatment of obesity is

affecting hunger and satiety. In Norway there are currently three drugs approved for treating obesity: liraglutide, orlistat, and combination of naltrexone and bupropion (39). Liraglutide is a GLP-1 analogue primarily used in treatment of T2D. GLP-1 is a peptide hormone that is involved in appetite control and liraglutide is thought to decrease appetite and thereby reduce food intake. Orlistat is a lipase inhibitor and works by reducing the absorption of fats from the diet in the intestine. This drug is also found to reduce blood pressure, insulin resistance, and blood lipids. Naltrexone is an opioid receptor agonist also used in the treatment of alcohol and opioid dependence. Bupropion inhibits dopamine and norepinephrine transporters and is used in treatment of depression. These drugs are used in combination; however, their exact mechanism of action with respect to weight loss is unclear. It is thought that the combination increases the activity of the POMC neurons in the hypothalamus leading to both decreased food intake and increased energy expenditure (40).

Bariatric surgery

Bariatric surgery is considered the most effective treatment option for morbid obesity. The initial weight loss following bariatric surgery is usually quite large with the maximum weight loss seen during the first year after surgery, often followed by a subsequent increase in weight (41). The Swedish Obese Subjects (SOS) study involving 2010 bariatric surgery patients report a mean reduction in body weight at 23% after 2 years, and after 10, 15, and 20 years the mean reduction in body weight was 17%, 16% and 18% respectively. At the same time bariatric surgery was associated with an overall reduction in mortality and fewer cases of T2D, CVD, and cancer compared with an obese control group (42). Inclusion criteria for bariatric surgery in Norway is BMI>40 or BMI>35 with co-morbidites, such as T2D and hypertension (43).

Bariatric surgery reduces the size of the stomach and/or changes the anatomy of the gastrointestinal tract. The most commonly used procedures include sleeve gastrectomy and Roux-en-Y gastric bypass, with the latter associated with the best long-term outcomes for T2D remission (44) and a rapid improvement in postprandial glucose

homeostasis after surgery (45) Additionally, insulin sensitivity, measured by HOMA-IR, is found to decrease within days after surgery (46). The mechanisms involved in the weight loss effect of these procedures are both restrictive and/or malabsorptive. Roux-en-Y gastric bypass uses a combination of restriction and malabsorption. It includes disconnection of a large part of the stomach to create a small gastric pouch. Further, the small intestine is divided and the distal portion is connected to the gastric pouch. The lower portion of the stomach with the upper part of the intestine attached is then connected further down on the small intestine to allow pancreatic and biliary secretes to come in contact with the food passing through. Sleeve gastrectomy is a procedure where a large portion of the stomach is removed, leaving only a small tube of stomach left (47).

A remarkable feature of bariatric surgery is that most patients with T2D experience remission within days to weeks after the surgery, before any weight loss has occurred (48). Bariatric surgery patients are found to be more likely to undergo remission of hypertension, diabetes, and dyslipidemia compared to obese patients undergoing medical treatment, including lifestyle intervention. At the same time, surgery patients also had a lower risk for new-onset diabetes, dyslipidemia and hypertension (49). However, surgery is not without complications. Abdominal pain is a common complication following gastric bypass, the prevalence shown to be between 22 and 34% 3 to 5 years after surgery (50,51). Other side effects following bariatric surgery includes gallstones, kidney stones, hypoglycemia, diarrhea, dumping syndrome, fatigue, anemia, indigestion, and irritable bowel syndrome (51), at is it clear that careful considerations are needed when deciding on who should be recommended to undergo bariatric surgery.

1.2 Adipose tissue

1.2.1 Composition, functions and depots

Adipose tissue is the main organ for storage of fat and regulation of energy balance and whole-body homeostasis. In response to high calorie levels, the adipose tissue stores free fatty acids (FFAs) as triglycerides (TGs), which happens through esterification of

the fatty acids. The FFAs can then be released from the tissue back into the circulation when energy levels are low (52), but may also occur in a situation of surplus energy intake when adipose tissue storage capacity is exceeded (53). The fatty acids are stored in lipid droplets within adipocytes in the adipose tissue. In addition to mature adipocytes, adipose tissue consists of a number of other cells types including progenitor cells, preadipocytes, immune cells, endothelial cells, fibroblasts, and stem cells, which together make up the stromal vascular fraction (SVF) (54).

There are two main forms of adipocytes, white and brown, with a third and intermediate type of fat cell called beige adipocytes (54). Based on this, adipose tissue is broadly divided into two functionally distinct types, white (WAT) and brown (BAT) adipose tissue. Making up the majority of adipose tissue mass, WAT is the tissue that is mainly involved in storage and release of energy. BAT, on the other hand, utilizes stored energy to generate heat in a process called thermogenesis. This happens through the uncoupling protein-1 (UCP-1), which is a protein specific to BAT. Brown adipocytes contain a lot of mitochondria and UCP-1 localizes to the inner mitochondrial membrane. BAT is localized in smaller, more defined depots compared to WAT (55). Exposure of WAT to cold or adrenergic signaling may lead to the occurrence of UCP-1^{+} brown-like adipocytes within the WAT. These cells, derived from white adipocytes, are called beige or brite adipocytes, have similar morpholical features to brown adipocytes, however, they have a different gene expression signature (54).

In humans, white adipose tissue is localized in two main depots. The subcutaneous adipose tissue (SAT) is the fat tissue that is found under the skin and visceral adipose tissue (VAT) is localized in the abdominal region surrounding the inner organs (Figure 1). A high amount of VAT is associated with metabolic disease, whereas SAT is sometimes thought to have beneficial effects on metabolism (54).

In addition to its role as an organ for storage of excess energy, the adipose tissue is also an endocrine and immunological organ that secretes cytokines and hormones that regulate energy metabolism and maintain tissue homeostasis. The cytokines secreted by adipose tissue can be produced by the adipocytes themselves or from immune within

the tissue, such as macrophages and they can have both beneficial and harmful effects. In a state of obesity, adipose tissue can secret pro-inflammatory cytokines leading to a low-grade inflammatory environment in the tissue and that can affect insulin sensitivity systemically (56). Additionally, changes in adipose tissue mass in response to obesity can alter the endocrine and metabolic function of the tissue that further affects the physiology of the whole body (57).

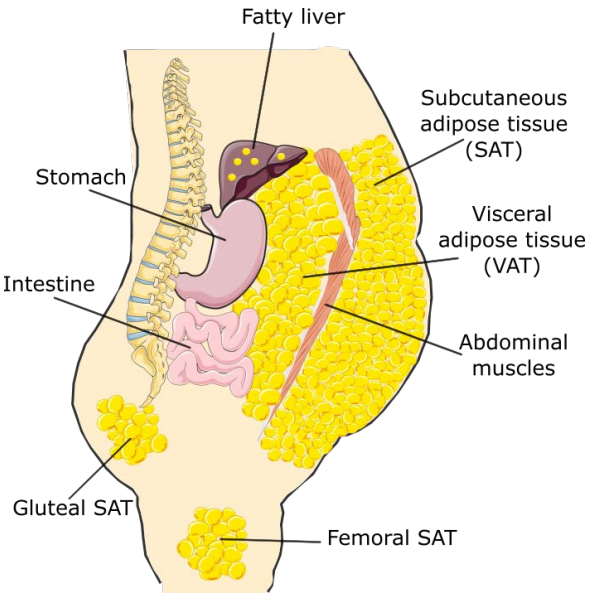

Figure 1. Main adipose tissue depots in humans

Human adipose tissue localized in different depots. Subcutaneous adipose tissue (SAT) depots are found under the skin and include the abdominal, gluteal, and femoral. The visceral adipose tissue (VAT) surrounds the inner organs such as the intestines and stomach. Figure adapted with permission from (58).

1.2.2 Adipocyte progenitor cells

Adipocytes develop from adipocyte progenitor cells (APCs) that arise from stem cells (59). APCs, sometimes also referred to as preadipocytes, are a part of the stromal vascular fraction (SVF) of the adipose tissue and, when cultured in the presence of a cocktail of hormones, are able to differentiate into mature adipocytes. This cocktail usually includes insulin, glucocorticoid, phosphodiesterase inhibitors, and often a proliferator-activated receptor γ (PPARγ) agonist such as rosiglitazone (54).

APCs from SAT show a greater ability to proliferate and differentiate to mature adipocytes compared to progenitors from VAT (60,61). At the same time APCs from VAT are more susceptible to apoptosis than APCs from SAT (62). The progenitors seem to have distinct gene expression patterns depending on the depot from which they arise (63,64). APCs from VAT appear to have a lower capacity to differentiate in culture, and addition of stimulatory factors such as bone morphogenic protein (BMP)-2 or 4 to the differentiation medium may improve the differentiation (63), indicating that the microenvironment is cruicial for proper adipocyte formation.

White and brown adipocytes are derived from different precursor cells. In fact, brown adipocytes originate from the same or very similar precursors as muscle cells, namely the $Pax7^{+}/Myf5^{5}$ stem cells. Development of brown adipocytes is further determined by the transcription factor PRDM16 (54,65). White adipocytes, on the other hand, are derived from $Pax7^{-}/Myf5^{-}$ stem cells. These precursors also give rise to beige adipocytes, which differentiate when stimulated by cold or other inducers such as β3 agonists (54).

Several studies suggest that APCs may be identified based on their surface expression of stem cell markers such as CD34 and Sca-1 (59). Others define human adipocyte progenitors as Lin^{-} $CD29^{+}$ $CD34^{+}$ $Sca\text{-}1^{+}$ $CD24^{+}$ cells. These cells may be sorted from the rest of the SVF based on their negativity for the markers CD31, CD45 and Ter119, which make up a linage-negative (Lin^{-}) population. About half of the SVF cells were found to be Lin^{-} $CD29^{+}$ $CD34^{+}$ $Sca\text{-}1^{+}$ and these cells did not express the markers CD105 or CD117, which separated them from bone marrow-derived mesenchymal stem cells and hematopoietic stem cells. In mice, the expression of CD24 did not enhance the ability of the progenitors to differentiate into mature adipocytes, however, gene expression analysis pointed towards the fact that the $CD24^{+}$ and $CD24^{-}$ preadipocytes were distinct populations (66).

Human studies have identifed three subtypes of APCs (67). Progenitors that did not express CD34 were found to be beige-like. Adipocytes derived from APCs expressing high levels of CD34 had high levels of lipid turnover, whereas low expression of CD34

gave rise to adipocytes with low lipid turnover. The composition of the different progenitor populations differed depending on their depot of origin as well as the diabetes status of the individuals they were derived from. The abdominal SAT and VAT depots had lower numbers of progenitors compared to the gluteofemoral adipose tissue. However, VAT had more $CD34^{hi}$ APCs compared to SAT. Individuals with T2D had a lower proportion of $CD34^{-}$ cells and in concordance a higher proportion of $CD34^{hi}$ APCs (67).

In a recent study, a stromal cell population expressing CD142 and that inhibited adipogenesis in a paracrine way was identified (68). In this study, they investigated Lin^{-} $CD29^{+}$ $CD34^{+}$ $Sca\text{-}1^{+}$ progenitor cells, which could be grouped in three clusters. One cluster of progenitors contained cells expressing stem cell markers, whereas another cluster of cells expressed genes involved in regulating early steps of adipocyte formation. The third group, making up around 10% of the APCs, contained the CD142-expressing cells that were unable to differentiate into mature adipocytes and removal of this group of cells from the other progenitors enhanced the ability of the other cells to differentiate (68). Another study, however, found that CD142-expressing APCs were able to differentiate into adipocytes (69). Cells expressing dipeptidyl peptidase 4 (DPP4), on the other hand, were highly proliferative cells but could not differentiate into mature adipocytes. Intracellular adhesion molecule -1 (ICAM-1) defined a committed progenitor population that expressed *Ppparg* and it was similar to the $CD142^{+}$ progenitor population. However, the $DDP4^{+}$ cells could differentiate into both $ICAM^{+}$ and $CD142^{+}$ progenitor cells that could further differentiate into adipocytes. The $DDP4^{+}$ cells were dependent on the transforming growth factor-β (TGFβ) signaling in order to remain as progenitor cells. The number of DDP4-expressing progenitor cells were reduced in response to obesity and insulin resistance, leading to a reduction in the differentiation potential of APCs, and this was especially seen in VAT (69).

1.2.3 Adipose tissue secretion of adipokines and hormones

Leptin

The peptide hormone leptin is expressed by adipocytes and is important for regulating body weight. During fasting, leptin levels are low to promote food intake and reduce energy expenditure while in a fed state, leptin levels increase to prevent overfeeding. Plasma levels of leptin are correlated with adipose tissue mass and adipocyte size and thus obesity is associated with elevated circulating levels of leptin (70). Leptin is also expressed at higher levels in SAT compared to VAT and women generally have a higher production of leptin due to estrogen stimulation (70,71). Leptin is encoded by the obese (*Ob*) gene and mice that lack leptin become extremely obese (72). Humans with deficiencies in in either leptin or the leptin receptor experience a fast weight gain after birth leading to severe obesity as well as insulin resistance (73). Further, mutations in the leptin gene leads to monogenic forms of childhood obesity (74).

Adiponectin

In contrast to leptin, adiponectin is an adipokine that is associated with insulin sensitivity and whose circulating levels are negatively correlated with adiposity, with lower circulating adiponectin levels seen in obesity. Similar to leptin, adiponectin levels are also highest in SAT compared to VAT and in women compared to men (75–77). Mice that lack adiponectin have high AT expression and plasma concentrations of tumor necrosis factor (TNF) and become insulin-resistant (78). The main function of adiponectin is to improve insulin sensitivity. Adiponectin also has anti-inflammatory properties promoting an anti-inflammatory macrophage phenotype, inhibiting differentiation of macrophages, and decreasing expression of TLR4. Adiponectin acts via receptors AdipoR1 and R2, increasing the activity of PPARα. This leads to increased fatty acid oxidation in the liver and skeletal muscle as well as increased glucose uptake by the liver and AT. At the same time, gluconeogenesis in the liver is

decreased. Activation of AdipoR1 and R2 by adiponectin also reduces levels of ceramide, a sphingolipid that is known to inhibit insulin signaling (79).

1.2.4 Adipose tissue in obesity

Adipose tissue dysfunction, hypertrophy and hyperplasia

Obesity is associated with impaired AT function that is thought to be the link between obesity and metabolic and cardiovascular disease. The dysfunction of the adipose tissue leads to hypertrophy, ectopic fat accumulation, hypoxia, inflammation, autophagy, as well as stress and impaired mitochondrial function within the adipose tissue (80). In a state of obesity, the expansion of the adipose tissue in response to excess nutrients occurs through an increase in the number of adipocytes (hyperplasia) as well as an increase in the size of the adipocytes (hypertrophy) (81). Adipocyte numbers are thought to be determined during childhood and stays constant throughout adulthood, both in lean and obese and also after weight loss (82). Thus, hypertrophy is thought to be the main mechanism for adipose tissue expansion in adults. In mice, the subcutaneous AT has limited capacity for hyperplasia and hypertrophy occurs only after 2 months on HFD. Visceral adipose tissue, on the other hand, displays features of hyperplasia as well as hypertrophy (83,84).

Lipotoxicity and hypoxia

Adipocytes in SAT may eventually reach their storage capacity, in which fat starts to accumulate, first in VAT and then in other tissues such as the liver, skeletal muscle, and pancreas. This ectopic fat accumulation leads to lipotoxicity as the lipids are toxic to these non-adipose tissues. Further, ectopic fat can disturb the normal insulin signaling promoting insulin resistance (85). As the adipose tissue expands, the supply of oxygen may become limited leading to hypoxia. The hypoxic state of the tissue may cause both oxidative and endoplasmic reticulum (ER) stress, which further drives the AT dysfunction. Additionally, hypoxia may promote the secretion of pro-inflammatory cytokines (80). The transcription factor HIF-1α is activated in response to hypoxia and leads to reduced oxygen consumption and a shift in the fuel consumption of the cells

from oxidative phosphorylation to glycolysis (86). HIF-1α is also found to induce fibrosis as well as inducing polarization of pro-inflammatory macrophages leading to increased inflammation in the adipose tissue (87,88).

Adipose tissue stress

Both hypertrophy and ectopic fat accumulation may induce adipose tissue stress. This stress may be in the form of oxidative stress, where enzymes involved in protection against such stress is found upregulated in adipose tissue of obese humans. Further, obesity is linked to ER stress, where the ER's protein-folding machinery is overloaded leading to activation of a signaling pathway called the unfolded protein response (UPR) (89). This UPR can be activated in obesity due to hypoxia or high influx of nutrients to the cell and the ER (80). ER stress is implicated in the development of insulin resistance as it may affect insulin signaling by activation of the JNK pathway as well as phosphorylation of the insulin receptor substrate-1 (IRS-1) (90).

ECM remodeling

Expansion of adipose tissue in response to nutrient surplus requires remodeling of the extracellular matrix (ECM). As the main component of the ECM is collagen, this remodeling involves breakdown and synesis of collagen. Hypoxia may drive angiogenesis and ECM remodeling to drive expansion of the adipose tissue and thus reduce hypoxia. The remodeling is necessary for the early adipose tissue expansion, however, long-term obesity, on the other hand, is pathological and may lead to fibrosis (91). Further, pro-inflammatory signaling is required for appropriate expansion and remodeling (92).

Autophagy

Autophagy, the process where intracellular components are targeted for lysosomal degradation, is upregulated in obesity, especially in the visceral adipose tissue. Studies in mice has shown that autophagy is involved in regulation of fat mass and knockout of autophagy genes leads to lean, insulin-sensitive mice that do not develop obesity.

Thus, it is hypothesized that autophagy could be a protective mechanism against adipose tissue dysfunction (80).

1.3 Insulin and insulin signaling

Insulin is a peptide hormone that is produced by β-cells in the pancreas and secreted in response to elevated levels of blood glucose. Insulin promotes uptake of glucose in skeletal muscle and synthesis of glycogen for storage in muscle cells. Insulin also stimulates the liver to store glucose as glycogen, it stimulates de novo lipogenesis, while at the same time inhibiting gluconeogenesis (27). In the AT, insulin stimulates the enzyme lipoprotein lipase to increase fatty acid uptake from circulating lipoproteins (93). Further, insulin leads to storage of triglycerides in the AT and suppression of lipolysis though inhibition of hormone-sensitive lipase (27). (Figure 2) Insulin also promotes differentiation of preadipocytes into adipocytes and induces lipogenesis in mature adipocytes (93).

Insulin signals through the insulin receptor on the surface of cells. This leads to recruitment and phosphorylation of tyrosine residues on the insulin receptor substrate promoting signaling through the phosphatidylinositol 3-kinase (PI3-kinase) and the protein kinase B (PKB)/Akt. PI3-kinase is an enzyme involved in phosphorylation of phosphoinositides and these phosphorylated inositols can activate the phosphoinositide-dependent kinase 1, which further activates the PKB/Akt through phosphorylation. Phosphoinositides also bind the plectrin homology domain of Akt and this causes recruitment of Akt/PKB to the plasma membrane. Activation of Akt/PKB induces translocation of the glucose transporter GLUT4 to the plasma membrane for uptake of glucose into cells (94).

In a fasted state, secretion of insulin decreases leading to an increase in gluconeogenesis in the liver and breakdown of glycogen to glucose that can be released into the circulation. In the AT, lipolysis increases leading to release of glycerol and FFAs. Glycerol may enter the circulation and be transported to the liver where it can be used in gluconeogenesis (Figure 2) (27).

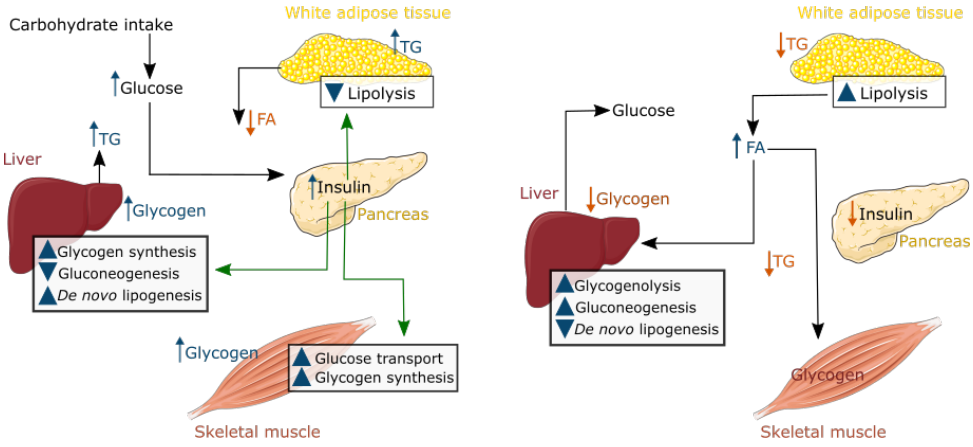

Figure 2. Overview of insulin action

Left: In a fed state, intake of carbohydrates leads to increasing concentrations of plasma glucose. This stimulates production of insulin by the β-cells in the pancreas. Insulin exert different effects on metabolic tissues. In the skeletal muscles, it promotes uptake of glucose and synthesis of glycogen for storage. In the liver, insulin stimulates de novo lipogenesis and storage of glucose as glycogen, as well as inhibition of gluconeogenesis. In the AT, insulin stimulates fatty acid uptake from circulating lipoproteins and storage of triglycerides (TGs) while suppressing lipolysis.

Right: In the fasted state, secretion of insulin is reduced, promoting gluconeogenesis in the liver and breakdown of glycogen to glucose for release into the circulation. In the AT, lipolysis is increased and this leads to release of glycerol and free fatty acids (FFAs). The glycerol can be used in gluconeogenesis by the liver. Figure adapted with permission from (27)

1.4 The immune system and inflammation

1.4.1 The immune system

The immune system is a complex group of cells and molecules that are specialized in defending the body against infections (95). The immune system is also involved in clearing of tumor cells and in maintaining tissue homeostasis (96). The immune system can respond to infectious agents through the innate or the adaptive immune system. The innate immune response is the first response against infections and occurs at the same strength each time. The adaptive or acquired response, on the other hand, improve and adapt with repeated encounter with the infectious substances. Phagocytic cells such as monocytes, macrophages, and neutrophils that produce different inflammatory

mediators as well as natural killer (NK) cells are all a part of the innate immunity. The adaptive immune response depends on specific T and B cells that proliferate in response to antigens (95,97).

1.4.2 Leukocytes

The cellular part of the immune system consists of leukocytes or white blood cells, which are derived from hematopoietic stem cell in the bone marrow that give rise to two types of progenitor cells namely the myeloid and the lymphoid progenitors. Leukocytes express the leukocyte common antigen also called CD45, and this surface receptor is therefore commonly used to identify leukocytes (98). Macrophages, dendritic cells, granulocytes, and mast cells all originate from the myeloid progenitor (97). Macrophages are derived from monocytes, which are found in the circulation and mature into macrophages upon infiltration into tissues. Macrophages are phagocytotic cells and an important part of the innate immunity. Dendritic cells, involved in antigen-presentation to lymphocytes, originate from immature dendritic cells that circulate in the blood and migrate to tissues. Mast cells also differentiate in tissue and activated mast cells influence vascular permeability. Granulocytes multiply in response to infections and travels to the sites of infection or inflammation. A type of granulocyte, the neutrophils, are also phagocytic and are one of the most important cell types in the innate immune system. Eosinophils are the main defense against infections with parasites (97).

Lymphocytes, consisting of T cells, B cells, and innate lymphoid cells (ILCs), are derived from the lymphoid progenitors. T and B cells have antigen receptors that are able to bind many different antigen-binding sites (97). ILCs lack antigen-specific receptors and are divided into three groups. NK cells are cytotoxic ILCs and belong to the group 1 ILCs (ILC1s), which produce the cytokine IFN-γ. NK cells depend on IL-15 for their development, whereas the other types of ILCs are dependent on IL-7, and hence they express the α chain of the IL-7 receptor. NK cell development is also dependent on the transcription factors T-bet and eomesodermin. ILC1s also require T-bet, whereas ILC2s rely on GATA3 and the ILC3s on retinoid-related orphan receptor γt (RORγt) for their development. The ILCs are also distinguished by the cytokines

they produce. As mentioned, NK cells and ILC1s produce IFN-γ, ILC2s produce IL-1 and IL-13, wheras ILC3s produce IL-17 and IL-22 (99,100).

The NK cells are the most studied of the ILCs. They are large, granular lymphocytes that can recognize and kill abnormal cells such as virus-infected cells and tumor cells. NK cells are characterized by their expression of CD56 and a lack of expression of CD3 (101). NK cells express several activating receptors that can recognize molecules associated with conditions such as viral infections or cellular stress. Activating receptors expressed on NK cells include natural killer group 2, member D (NKG2D), natural cytotoxicity receptors (NCRs) NKp46/NCR1 and NKp30/NCR3, and DNAX accessory molecule 1 (DNAM1 or CD226). Inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) and CD94-NKG2A. NK cells are targets of cytokines such as IFN-α and the interleukins (IL) 12, 15 and 18 promoting the NK cells to produce other cytokines and chemokines including IFNγ, TNF, and CCL4. NK cells are often divided based on their expression of the surface marker CD56. $CD56^{bright}$ NK cells are mainly cytokine-producing, whereas $CD56^{dim}$ NK cells are cytotoxic (102). The $CD56^{dim}$ NK cells express CD16, whereas expression of this receptor is absent on the $CD56^{bright}$ NK cells (101). NK cells may exert their cytotoxic functions through production of perforins and granzymes. Perforin is a membrane-disrupting protein while granzymes are proteases and together they can induce apoptosis of target cells (103).

1.4.3 Circulating and tissue resident-immune cells

As described above, many immune cell precursors circulate in the blood and differentiate as they enter different tissues. However, in recent years there is growing evidence for tissue-resident cells that exist in the tissue and that do not re-enter the circulation (102,104). Neutrophils and effector T cells are short-lived immune cells and they migrate to tissues in response to danger signals where they perform their functions and then die. Other immune cells such as ILCs, NK cells, DCs, macrophages, mast cells, and other types of lymphocytes may have to establish residency and adapt to the tissue environment (96).

Tissue-resident macrophages are mostly derived from yolk-sac macrophages or monocytes from the fetal liver and are established before birth. These tissue-resident macrophages are important for the normal tissue physiology and express tissue-specific residency-genes. They are highly adapted to the different tissues through unique transcriptional signatures, where genes encoding metabolic pathway proteins are especially important (96).

Tissue-resident lymphoid cells are associated with expression of adhesion molecules and a lack of chemokine receptors. The blood and lymphoid organs contain sphingosine-1-phosphate and CCL19/21 that promote re-entering of lymphocytes to the circulation through their receptors sphingosine-1-phosphate receptor (S1PR1) and the chemokine receptor CCR7. S1PR1 and CCR7 are downregulated on tissue-resident cells (105). The surface markers CD69, CD49a, and CD103 are involved in keeping lymphocytes in tissues and can thus be used to identify tissue-resident cells. CD69 was thought to be an activation marker for both NK and T cells but is now recognized for its role in maintaining these cells in tissues though inhibition of the S1PR1. Human circulating NK cells lack CD69, whereas many subsets of tissue-NK cells express this marker. Different types of tissues contain different amounts of tissue-resident NK cells. CD103 and CD49a retain tissue-resident cells in the tissue through their binding to E-cadherin and collagen, respectively. Their expression is controlled by transforming growth factor β (TGFβ) and they are found to be expressed on tissue-resident CD69+ T cells and on some subsets of tissue-resident NK cells (102).

1.4.4 The inflammatory response

Inflammation is a protective mechanism the immune system generates that can be triggered by both infectious and non-infectious agents. Infectious agents include bacteria, virus, and other microorganisms. Non-infectious agents might be either physical in the form of burns, trauma, frostbites etc., be in the form of chemical agents such as toxins, glucose, fatty acids, alcohol, or chemical irritants or biological in the form of damaged cells. The inflammatory response induces a signaling cascade to initiate a healing process (106). Inflammation is typically characterized by swelling, redness, heat, pain, and loss of tissue function. These symptoms are caused by an

increase in the permeability of the vascular endothelium leading to a leakage of components from the serum and recruitment of cytokine-producing immune cells leading to induction of the inflammation (107).

All inflammatory responses happen through the same mechanism involving four main processes. Firstly, the inflammatory response is triggered by infectious or non-infectious agents through pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) respectively. This leads to activation of pattern-recognition receptors (PRRs) that may be expressed on both immune and non-immune cells. Types of PRRs include the well-studied toll-like receptors (TLRs) as well as C-type lectin receptors, retinoic acid-inducible gene (RIG)-I-like receptors, and NOD-like receptors. Activation of PRRs triggers inflammatory signaling pathways that upregulate transcription of inflammatory mediators such as ILs and TNF. These mediators bind to their respective receptors activating important intracellular signaling pathways. Important pathways include the mitogen-activated protein kinase (MAPK), the nuclear factor kappa B (NF-κB), and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways. These pathways triggers production of inflammatory markers such as cytokines and chemokines leading to recruitment of inflammatory immune cells (106).

1.5 Adipose tissue immunology and inflammation

Adipose tissue homeostasis involves a number of immune cells such as macrophages, eosinophils, ILC2s, invariant NKT cells (iNKT) cells, NK cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and regulatory T cells (T_{reg} cells). These cells maintain an anti-inflammatory environment and prevent activation of pro-inflammatory macrophages. Several types of immune cells have also been implicated in AT in relation to obesity. The AT immune cells in lean and obese AT is summarized in Figure 3 (96).

1.5.1 Adipose tissue resident macrophages support adipose tissue homeostasis

Resident ATMs stimulate increased vascularization of the tissue thereby reducing hypoxia (96). Further, these ATMs remove apoptotic cells through efferocytosis. Lean AT contains mostly anti-inflammatory macrophages that have increased mitochondrial function and increased β-oxidation linked to improved lipid catabolism. The increased β-oxidation is necessary for clearing of the excess lipids that the macrophages obtain from the dead cells during efferocytosis (108). The macrophages are also involved in tissue repair, which is promoted by the apoptotic cells as well as the cytokines IL-4 and IL-13 that induce expression of tissue-repair genes (109). The IL-4-production in lean AT is mostly derived from eosinophils, which may produce up to 90% of the IL-4 (110). In mice, IL-4 and alternatively activated macrophages are also found to promote thermogenic gene expression, fatty acid mobilization, and energy-expenditure (111). Moreover, IL-4 and macrophage colony stimulating factor (M-CSF) is known to stimulate glucose metabolism during activation of M2 macrophages and glucose is found to be essential for alternative activation of macrophages. IL-4 and M-CSF signal through the mammalian target of rapamycin complex 2 (mTORC2) and interferon regulatory factor 4 (IRF4) to affect the glucose metabolism and this process is essential for activation of M2 macrophages (112).

Maturation of alternatively activated macrophages is dependent on PPARγ, which is also implicated in the macrophages' ability to maintain insulin sensitivity in mice (113). Resident ATMs may regulate tissue homeostasis through production of IL-10 and catecholamines. IL-10 is thought to block infiltration of macrophages and gene expression of genes encoding pro-inflammatory cytokines while the catecholamines may induce lipolysis in white adipocytes and expression of thermogenic genes in brown adipocytes (111,114). However, others have found that IL-10 does not reduce inflammation and insulin resistance and that it may suppress transcription of thermogenic genes, whereas IL-4 does not stimulate production of catecholamines by alternatively activated macrophages (115–117). Thus, the role of anti-inflammatory macrophages and the cytokines they produce, especially in humans, is not fully understood.

1.5.2 Adipose tissue T cell populations supports tissue homeostasis and have anti-inflammatory functions

T_{reg} cells are a group of $CD4^+$ T cells that express the transcription factor Foxp3. They have functions in autoimmune and autoinflammatory disorders, cancer, allergy, and acute and chronic infections. Further, T_{regs} are known to be involved in metabolic inflammation and tissue repair (118). A unique population of AT T_{regs} was identified in lean, but not obese mice. The percentage of T_{reg} was also higher in VAT compared to SAT. VAT T_{regs} expressed high levels of IL-10, which was found to affect adipocytes by inhibiting TNF-induced pro-inflammatory gene expression and reversing the effect of TNF to inhibit glucose uptake. Thus, IL-10 is thought to reduce insulin resistance (119). Further, T_{regs} found in VAT express elevated levels of genes encoding molecules important for lipid metabolism. Accumulation of T_{regs} in VAT as well as their phenotype and function are regulated by PPARγ (120).

Tissue-resident γδ T cells are also abundant in AT and are found to regulate the expansion of T_{regs}. These γδ T cells produce IL-17A that induce IL-33 production by AT stromal cells (121). γδ T cells are also found to be expanded in AT after a short-term ketogenic diet in mice. These γδ T cells were also tissue-resident, thought to be involved in adipose tissue remodeling and homeostasis and mice lacking these γδ T cells had impaired glucose homeostasis (122).

Tissue-resident iNKT cells in VAT is shown to produce IL-2 and IL-10, which controlled AT T_{reg} numbers and proliferation. They also promote alternatively activation of macrophages through their production of IL-4 (123). Further, AT iNKT cells are associated with decreased body fat, triglyceride and leptin levels as well as improved insulin sensitivity which is mediated by their production of anti-inflammatory cytokines such as IL-4 and IL-10. Mice lacking iNKT cells gain more weight, have larger adipocytes, more hepatic fat, elevated fasting blood glucose levels and higher levels of insulin resistance compared to mice with iNKT cells (124).

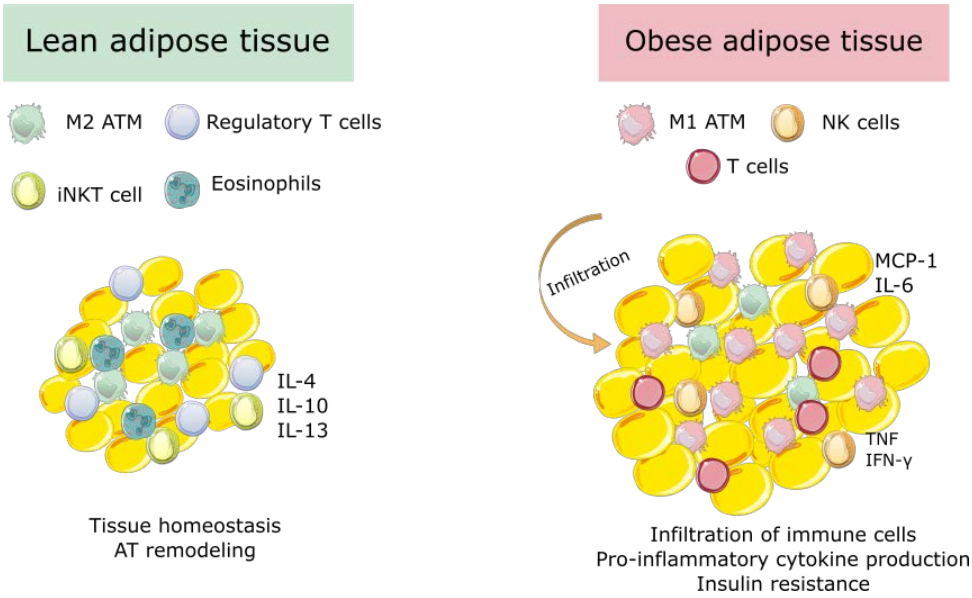

Figure 3. Lean and obese AT

In lean AT, Tregs, iNKT cells, and eosinophils promote anti-inflammatory signaling through production of IL-4, IL-10, and IL-13, stimulating the anti-inflammatory M2-like phenotype of the adipose tissue macrophages (ATMs). The immune cells maintain the tissue homeostasis and are involved in remodeling of the tissue. In an obese state, adipocytes are bigger and there is an infiltration of pro-inflammatory M1-like macrophages driven by the chemokine MCP-1. Further, M2-like macrophages polarize to M1-like macrophages. This leads to increased production of pro-inflammatory cytokines such as TNF and IL-6, which contribute to the development of insulin resistance. NK cells contribute to macrophage polarization trough production of TNF and IFN-γ. NK cells also has reduced capacity to kill M2-like macrophages, which can then polarize to M1-like macrophages. Figure adapted with permission from (109).

1.6 Obese adipose tissue immunology

The first reports suggesting that adipose tissue inflammation was linked to obesity and T2D came from studies revealing that expression of TNF is elevated in the AT of obese mice (125). Further, TNF can block insulin signaling, and mice that lack either TNF or its receptors have improved insulin sensitivity and experience increased insulin-stimulated glucose uptake (125,126). Many genes related to inflammation and macrophages are upregulated in adipose tissue of obese mice and in obesity there is an

accumulation of macrophages in the adipose tissue (127,128). Removing M1-like macrophages in mice is shown to reduce inflammation and improve insulin sensitivity in adipocytes (129). In addition to TNF, cytokines, and chemokines such as IL-6, IL-1β, monocyte chemoattractant protein -1 (MCP-1) also called C-C chemokine ligand 2 (CCL2) and macrophage inflammatory protein (MIP) are all secreted by ATMs. Transcriptionally, the production of these cytokines is controlled by two signaling pathways, namely the c-JUN N-terminal kinase (JNK)-activator protein 1 (AP-1) and Iκ B kinase β (IκK)-nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). These signaling pathways can be induced by ER and oxidative stress, saturated fatty acids (SFAs), and pro-inflammatory cytokines (130). Obesity associated activation of these two inflammatory signaling pathways can be achieved in several ways. Cytokines may activate their cell surface receptors or the signaling may be induced through activation of PPRs such as NODs or TLRs. The PPRs respond to PAMPs such as LPS or bacterial DNA or DAMPs, which includes SFAs. TLR4 especially has been implicated in promotion of SFA-induced macrophage inflammation (131). PAMPs and DAMPs associated with obesity are also associated with activation of the nucleotide-binding domain and leucine-rich-repeat-containing protein NLRP3 inflammasome (132). Reactive oxygen species, hypoxia, ER stress, and lipotoxicity are also shown to induce inflammatory signaling pathways (133).

1.6.1 Macrophages

During development of obesity, macrophages accumulate in the AT making it the most abundant type of immune cell in the obese AT. Adipose tissue macrophages (ATMs) make up around 10 – 15 % of the AT immune cells in lean mice and increase to 40 – 60 % in AT of obese mice (127,128). Most of the macrophage populations that emerge during obesity is thought to be derived from circulating monocytes and not from tissue-resident macrophages (134). The chemokine MCP-1/CCL2 is thought to be the main driver of macrophage infiltration into the AT during development of obesity (135–137). In mice, its expression is upregulated in obesity (135), overexpression leads to recruitment of macrophages and development of insulin resistance (137) while deletion of CCL2 or its receptor CCR2 prevents infiltration of macrophages and protects against insulin resistance (136).

Several studies also find that local proliferation of ATMs contribute to the increase in the number of ATMs in obesity, in both mice and humans. This proliferation might be driven by CCL2 (138), however, others find that this cytokine was not increased along with increased proliferation of macrophages (139). It has also been suggested that macrophages localized in crown-like structures (CLS) around necrotic adipocytes, proliferate and become more M2-like in their phenotype and this has been found in both mice and human AT (140). CD68 is considered the classical macrophage marker that is found to correlate with BMI and adipocyte size (127).

It is thought that most of the macrophages in obese AT localize in CLS. These CLS have been found in both mice and humans and some studies find that they mostly consist of M1-like or pro-inflammatory macrophages (141). The pro-inflammatory M1-like macrophages produce inflammatory cytokines such as IL-6, IL-1β, inducible nitric oxide synthase (iNOS), and TNF. Obesity reduces the expression of genes characteristic of M2-like macrophages and induces expression of genes encoding TNF and iNOS, which are markers of M1-like macrophages suggesting that obesity induces a phenotypic switch leading to polarization of macrophages from an anti-inflammatory M2 to a pro-inflammatory M1-like phenotype (142). M1-like macrophages are also found to be induced by lipopolysaccharide (LPS) and INFγ (143).

Human M1-like macrophages can be identified by their expression of the surface marker CD11c, which is not expressed on M2-like macrophages (141). M1-like macrophages also express CCR2, CD64, and CD206. Further expression of TLR4, CD40, and HLA-DR might also define M1-like macrophages. Human M2-like macrophages are defined as expressing CD206 and CD204 (144). Some also suggest they express CD163 (145), although this has been debated and CD163 has also been reported to be expressed on M1-like macrophages (144).

M1-like ATMs were also found to express the surface marker CD11c in mice. One study found that the CD11c+ ATMs did not change in numbers when the mice became insulin resistant after HFD-feeding. However, the macrophages exhibited a changed phenotype where markers of inflammatory pathways were no longer present and

instead markers of apoptosis and necrosis were increased. This suggest that M1-like ATMs may be highly plastic cells that are able to change their phenotype with a changing microenvironment (146).

Triggering receptor expressed on myeloid cells (TREM)-1 is found to be increased in AT, liver, and blood of individuals with obesity, both those with and those without diabetes. On the other hand, levels of TREM2 were decreased, both on a transcriptional level and also its protein expression in liver, SAT, and VAT (147). Some of these studies also find that TREM1 expression is higher in subjects with obesity and diabetes compared to subject with obesity, but without diabetes (147,148). Human ATMs may in some settings display a mix between a pro- and anti-inflammatory phenotype, where the ATMs express typical M2 surface markers, but at the same time are able to secrete high amounts of pro-inflammatory mediators such as TNF, IL-6, IL-1β, and MCP-1 (145).

1.6.2 Different types of ATMs detected in AT

It is thought that the M1/M2 classification is a simplification of the macrophage phenotypes. Accordingly, several types of ATMs have been identified and characterized in AT. Both monocytes and macrophages undergo remodeling during development of obesity. A recent publication showed that two macrophage populations in AT of mice showed a gradual expression of genes. One of these was the gene encoding the tetraspanin *Cd9*, a marker that has been described expressed on human ATMs (134,149). A population of CD9+ CD63+ macrophages seemed to be present during obesity and these macrophages were found in CLS around adipocytes (134).

A recent scRNA-seq of human adipose tissue cells revealed 5 different clusters of macrophages. One of the clusters had a higher expression of CD9 than the other subtypes of ATMs. This macrophage population was also enriched for genes connected to lipid metabolism. Further, CD9 expression was associated with BMI. Another subgroup of macrophages had a similar expression signature compared to this CD9-expressing population, however, this group also had increased expression of inflammatory genes. A cluster expressing M2-related genes was also identified,

whereas one of the clusters could not be further identified. One of the macrophage clusters turned out to be dendritic cells, while the last group was identified as $CD16^-$ monocytes (150). Hence, there is evidence that several subpopulations of ATMs exist in human AT.

There are still a lot of details about ATMs that are yet to be elucidated. More studies analyzing ATMs on a single-cell level will lead to increased insight into the different subtypes that might exist in the AT. Here some of the suggested macrophage subtypes that are shown be present in AT are presented (Figure 4).

Lipid-associated macrophages (LAMs)

A subset of ATMs express genes encoding molecules related to lipid metabolism and phagocytosis and thus they are referred to as lipid-associated macrophages (LAMs). This is a unique population of ATMs, found in both mice and humans, that express the marker Trem2, which is a marker that is not expressed on any other type of immune cell present in the AT (134). Trem2 is co-expressed with CD9 on the LAMs. LAMs in adipose tissue resembles disease-associated microglia and hepatic $Trem2^+$ immune cells in their gene expression signature, suggesting that Trem2 may define a specific type of macrophage across different tissues. LAMs can be identified by flow cytometry as $CD9^+$ $CD63^+$ macrophages and these cells contain intracellular lipids, further underlining their roles in lipid metabolism. In the absence of Trem2, mice did not accumulate LAMs in their AT and thus LAMs could not take up and store lipids in an obese state. Lack of Trem2 also leads to adipocyte hypertrophy in mice on HFD as well as increased fat accumulation, glucose intolerance, high serum insulin levels, and dyslipidemia. This suggests that Trem2 may be important for AT function and metabolic health (134). Furthermore, mice lacking Trem2 have lower circulating levels of MCP-1 and reduced infiltration of AT macrophages that were not able to form CLSs and clear dead adipocytes. Additionally, Trem2-deficient mice had severe hepatic steatosis following HFD compared to WT mice (151) TREM2-expressing LAMs have also been identified in human AT, expressing many of the same genes as mice LAMs. Further, LAMs were only found in AT of obese individuals and their proportion was

positively correlated with BMI. TREM2 was also only expressed on LAMs and not on any other AT immune cell type (134).

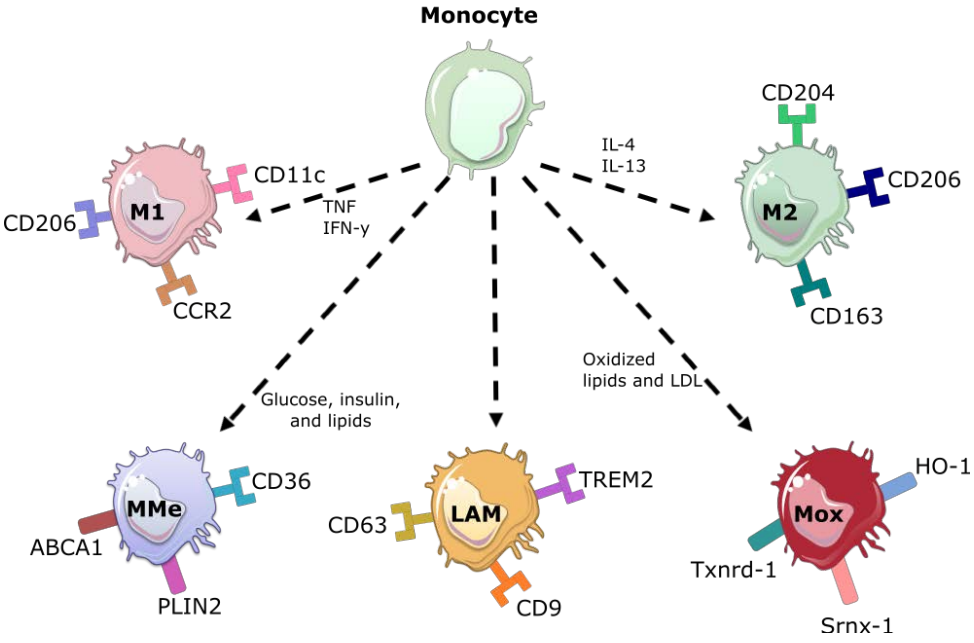

Figure 4. Subtypes of adipose tissue macrophages

Macrophages are derived from monocytes that, depending on the stimuli, can differentiate into different subtypes of macrophages. M1-like macrophages (M1) can develop after stimuli with interferon (IFN)-γ or tumor necrosis factor (TNF). These macrophages express the surface proteins CD11c, CD206, and CCR2. M2-like macrophages (M2) originate from stimulation with IL-4 and IL-13, and express surface proteins CD206, CD204, and CD163. The metabolically activated macrophages arise after stimuli with glucose, insulin or free fatty acids. Lipids can also stimulate the development of Mox macrophages. MMes typically express CD36, ABCA1, and PLIN2, while the Mox macrophages express heme oxygenase-1 (HO-1), sulforedoxin-1 (Srnx-1), and thioredoxin-1 reductase (Txnrd-1). M1-like macrophages, MMes and Mox macrophages all develop in response to obesity. This is also the case for the lipid-associated macrophages (LAMs), which are characterized by expression of CD9, CD63, and TREM2. Figure adapted with permission from (144).

Metabolically activated macrophages (MMe's)

Another type of macrophage, the metabolically activated macrophages (MMe) are found in AT of both mice and humans. These macrophages are activated by metabolic

stimuli such as high levels of insulin, glucose and palmitate. They do not express the classical M1 surface markers such as CD274, CD319, and CD40, but rather express distinct cell surface proteins compared to M1 macrophages. MMe macrophages express markers involved in lipid metabolism including ABCA1, CD36, and PLIN2. These markers are generally associated with M2 macrophages, however, M2 markers such as CD206 or CD163 are not expressed on the MMes. The surface markers induced in MMes are found to be regulated by PPARγ through its binding to the promoters for *PLIN2*, *CD36*, and *ABCA1* in both mice and humans. Treating macrophages with medium conditioned by adipose tissue induced expression of *ABCA1*, *CD36*, *IL1β*, and *TNF* (152).

MMe macrophages have both beneficial and detrimental functions. These macrophages are pro-inflammatory and able to secrete cytokines (detrimental function), but they are also involved in the clearance of dead adipocytes through lysosomal exocytosis (beneficial function), evident by their expression of cells surface proteins lysosomal-associated membrane protein (LAMP)1 and LAMP2. Their inflammatory cytokine production is regulated by TLR2, TLR4, NOX2, and MYD88 and macrophages lacking *Nox2*, *Tlr2*, and *Myd88* have impaired lysosomal exocytosis. The MMe phenotype can be detected in mice after eight weeks on an HFD, where the macrophages produce cytokines and expresse genes involved in lipid metabolism. However, hypoxia, apoptosis, CLS, or ectopic fat storage were not identified at this time. After 16 weeks on HFD, evidence of hypoxia and apoptotic adipocytes was detected in the AT. Further, lipid storage was seen in the liver and the MMes showed a more distinct MMe phenotype. This suggests that the clearance of dead adipocytes may only be needed after long-term HFD (153).

Hill and colleagues identified several unique ATM populations in mice, defined by surface markers CD9 and Ly6c (149). $CD11b^+$ $LY6c^+$ cells were assumed to be monocyte-derived macrophages, whereas $CD11b^+$ $LyC6^-$ macrophages also expressed macrophage markers F4/80 and CD64. Both of these cell types were increased following HFD. The $LyC6^-$ cells consisted of two different populations, one of these expressed high levels of *Cd9*. Consistent with findings by others (152,154), these $CD9^+$

cells also expressed genes associated with lipid metabolism, such as *Plin2* and *Lpl* and genes related to intracellular vesicle function such as *Lamp2* and *Cd63*. The $CD9^+$ ATMs were also found to accumulate in the AT following HFD, whereas CD9- ATMs did not. Further, the $CD9^+$ ATMs were localized in CLSs, contained intracellular lipids and secreted exosomes. Both the $CD9^+$ and the $LyC6^+$ ATMs were distinct from M1 or M2 macrophages in their transcriptome, however, the $CD9^+$ ATMs expressed proinflammatory genes as well as genes associated with lysosomal pathways. The $LyC6^+$ ATMs, on the other hand, expressed genes involved in vascular development and organization. $CD9^+$ ATMs were also found in human AT, and these cells also contained intracellular lipids and localized in CLSs. Moreover, BMI was positively correlated with the amount of $CD9^+$ ATMs in VAT (149).

Mox macrophages

A new macrophage phenotype was identified in atherosclerotic lesions that was different from M1 and M2 macrophages. These macrophages, called Mox macrophages can develop from M1 or M2 macrophages that undergo a phenotypic switching when treated with oxidized phospholipids. Mox macrophages have a different gene expression profile compared to M1 and M2 and their capacity for phagocytosis and migration is decreased. These macrophages are thought to develop in response to oxidative tissue damage. The Mox macrophages were associated with redox regulation and antioxidant activity and their phenotype was regulated by the transcription factor Nrf2 (155).

A cell population with a phenotype resembling the Mox macrophages was also identified in murine AT. Firstly, lean mice had an ATM population that was $CX3CR1^-$ /$F4/80^{lo}$ while AT from obese mice showed an accumulation of $CX3CR1^+$/$F4/80^{hi}$ cells. The resident $F4/80^{lo}$ population in lean mice was associated with the Mox markers Txnrd1 and HO1. These markers are redox homeostatic enzymes and their expression is driven by Nrf2. The $F4/80^{hi}$ population, on the other hand, expressed CD11c and CD206, in concordance with the inflammatory macrophages seen in human obese AT.

This population was also present in lean AT and increased during HFD while the Mox macrophage numbers were unchanged (156).

1.6.3 NK cells and ILCs

Obesity is associated with increased levels of AT NK cells, which is mostly seen in VAT and not in SAT (157–159). Ablation of NK cells decrease macrophage infiltration into VAT but not SAT and improve insulin resistance (160). Obesity may drive proliferation of and cytokine production by ILCs, which may further induce polarization of ATMs leading to development of insulin resistance. In mice, obesity is found to promote expression of stress ligands on adipocytes that can bind the activating NK cell receptor NCR1 (Figure 5). The human equivalent, NKp46 was also found to be expressed in human VAT, but not SAT (158). HDF leads to accumulation of IFN-γ- producing NK cells in the adipose tissue (158,161). IFN-γ is known to promote macrophage polarization during infections (162) and depletion of IFN-γ reduce insulin resistance in obese mice (163). The increase in NK cells during HFD also increased polarization of macrophages into an M1-like phenotype leading to insulin resistance. Depletion of NK cells, the NCR1 or IFN-γ, on the other hand, led to decreased numbers of both total and M1-like macrophages as well as improvements in AT inflammation and insulin sensitivity without affecting the body weight (157,158). Another study found that the AT NK cells in obese mice produced higher amounts of TNF than AT NK cells from lean mice. They also found that IL-15, produced by both sorted ATMs and epididymal fat, but not subcutaneous fat, drove proliferation and activation of AT NK cells (157). This suggests that NK cells, through their production of INF-γ or TNF, may play a role in macrophage polarization and development of obesity-induced insulin resistance, however human studies are needed.

A subset of group 1 innate lymphoid cells (ILC1s) is also identified in murine adipose tissue, which contribute to metabolic disease. These ILC1s are phenotypically and functionally different from AT NK cells, they are dependent on transcription factors T-bet and Nfil3 and they are tissue-resident. Diet-induced obesity led to an increase in ILC1 density, an increase that was small and transient in VAT and large and sustained in SAT, suggesting that SAT is the dominant depot for ILC1 accumulation during

obesity (161). Obesity also led to production of the cytokine IL-12, which induced proliferation and accumulation of ILC1s. This was dependent on signaling through the IL-12 receptor and STAT4. Additionally, the ILC1s were found to produce IFN-γ, leading to polarization of M1-like macrophages and development of insulin resistance (161).

Another type of ILC, called adipose type 1 innate lymphoid cells (AT1-ILCs) work by targeted cytotoxicity towards ATMs. These AT1-ILCs were found to kill the M2-like macrophages, which were assumed to be a way of removing M2-like ATMs that have fulfilled their homeostatic role and prevent them from develop into pro-inflammatory M1-like macrophages (164). The numbers of the AT1-ILCs were found to increase within the first days of putting mice on a HFD, however, as obesity was sustained, the levels of AT1-ILCs were found to decrease, both in humans and mice. In obesity, these AT1-ILCs also lost their ability to kill the M2-like macrophages and this led to an increase in ATMs and a shift towards more M1-like macrophages. ATMs were also found to express the NKG2D ligand Rae-1, and the expression was increased after HFD (164). NK cells and macrophages are known to interact with each other through NKG2D-Rae1 in infections or inflammation induced by LPS (165). It is unclear if ILC1s exsist in humans or if they rather correspond to $CD56^{bright}$ NK cells (166).

Several studies point towards altered NK cell phenotype and function in obesity (167–169). Obesity is associated with a shift from cytotoxic $CD56^{dim}$ to cytokine-producing $CD56^{bright}$ NK cells, which might explain the increased secretion of cytokines by AT NK cells associated with obesity. AT NK cells from individuals with obesity also showed decreased expression of NKp30 and NKp44 and unchanged levels of NKG2D (170). AT NK cells also possess an activated phenotype in human obesity, with increased frequency of $CD56^{bright}$ NK cells and a reduction in $CD56^{dim}$ NK cells within the total NK cell population in SAT and VAT compared to PBMC from individuals with obesity. Further, the frequencies of NK cells expressing CD158, NKG2D, NKp46 or CD27 as well as $CD16^{dim}$ NK cells are increased while $CD16^{bright}$ NK cell levels were decreased in VAT compared to PBMC(171). Development of T2D is associated with a lower frequency of inhibitory NK cells and a higher frequency of activated NK

cells seen by higher levels of NKG2D$^+$ NK cells and lower levels of NKG2A$^+$ and KIR2D3$^+$ NK cells in the T2D patients compared to controls (172). In mice, obesity is also associated with a subpopulation of NK cells expressing IL6Ra, which express the myeloid linage gene colony-stimulating factor 1 receptor (Csf1r). Depletion of NK cells expressing Csf1r prevents obesity and insulin resistance. Further, inactivation of IL6Ra or Stat3 in the NK cells limits the development of these Csf1r-expressing NK cells and this also protected against obesity and associated inflammation and insulin resistance (173).

Alterations in NK cell numbers are also seen in obesity. Childhood obesity is associated with reductions in peripheral NK cell frequency compared to normal weight children. Obese children had NK cells that were activated, metabolically stressed, and had reduced ability to respond to stimulus that lead to a loss of function (174). Several studies also show that the frequency of circulating NK cells is decreased in obesity (167,169,175). Studies in mice show that NK numbers are decreased or not changed in obesity (176,177), whereas one study found NK cell frequencies to be dependent on leptin as NK cell levels increased following HFD, however not in leptin-deficient (ob/ob) mice (178).

NK cells from individuals with obesity expressed elevated levels of activation markers CD69 and granzyme B and reduced levels of CD16. Further, reduced expression of the natural cytotoxicity receptor NKp46 and NKG2A/CD94 complex is seen on these NK cells. The NK cells also display a loss of function measured by their decreased ability for degranulation and production of MIP-1β and IFN-γ in line with increasing BMI (179). This is in contrast to findings by others suggesting an increase in the production of IFN- γ by NK cells in obese AT. Different obesity-related adipokines may affect NK cells. Leptin may influence proliferation and migration of NK cells, whereas adiponectin increases expression of the degranulation marker CD107a and the cytotoxicity of the NK cells. Both the leptin and adiponectin receptors are thought to be expressed on NK cells (180).

Altered NK cell function in relation to obesity is also suggested to be one of the links between obesity and cancer. Obesity stimulates metabolic reprogramming of NK cells, where PPAR-signaling cause accumulation of lipids in the NK cells, evident by obesity-driven upregulation of genes related to lipid metabolism, lipid-droplet formation and lipases as well as genes associated with lipid and glycerol uptake. Further, obesity also lead to downregulation of NK cell-mediated cytotoxicity. NK cells from obese individuals produce less IFN-γ and are able to kill tumor cells to the same extent as NK cells from lean individuals. Further, the NK cells experience a loss of perforin and granzyme B and this loss of function is thought to be due to lipid uptake by the NK cells in response to increasing concentrations of FFA in obesity. The mTORC1 pathway, which is essential for NK cell function, is also found to be impaired in obesity. Thus, obesity and the associated lipid-rich environment may impair the ability of the NK cells to perform their immunosurveillance function and their cytotoxicity against tumor cells seems to also be reduced in obesity (169).

1.7 Inflammation-mediated insulin resistance

M1-like macrophages may impair insulin signaling through secretion of cytokines such as TNF, IL-6, and IL-1β (181). These cytokines can induce insulin signaling through both transcriptional and posttranscriptional mechanisms. Some of these mechanisms involve the two serine kinases IKKβ and JNK (133,182) that can reduce insulin signaling by inhibitory phosphorylation of the insulin receptor (IR) and the insulin receptor substrate (IRS) (183,184). Further, activation of JNK activates AP-1 while IKKβ promotes translocation of NK-κB to the nucleus. These transcription factors stimulate expression of inflammatory genes such as those encoding TNF and IL-6, further promoting insulin resistance (185). Posttranscriptional mechanisms include those affecting mRNA or microRNA (miRNA). Stabilization of mRNA encoding inflammatory cytokines, which may happen through JNK signaling can also promote inflammation (186). TLR4-signaling may impair expression of the miRNA miR-223, promoting production of IL-6 and IL-1β (187).

TNF itself can increase lipolysis, leading to a release of FFAs that are able to bind and activate TLR4 and thus affect insulin signaling through IKKβ (133). TNF can also impair insulin signaling by decreasing the expression of GLUT4 or PPARγ (188,189). Obesity-induced inflammatory signaling also induce the synthesis of ceramides, and this group of bioactive sphingolipids may also assert negative effects on insulin signaling. Ceramides are found to block the recruitment of PKB/Akt to the plasma membrane and instead recruit and activate an atypical protein kinase C, the protein kinase C ζ. This leads to phosphorylation of the PH domain of Akt thereby decreasing the affinity of the PH domain for phosphoinositides and further inhibition of insulin-stimulated Akt-activation (190,191). Inflammatory mediators can also induce de novo lipogenesis in the liver leading to elevated levels of serum lipids (192). Obesity is also associated with elevated levels of fatty acid binding protein 4 (FABP4), a protein that can be produced by macrophages, however, is mainly produced by adipocytes. In macrophages FABP4 promotes induction of the IKK-NF-κB and the JNK signaling pathways, while in adipocytes this protein activates hormone-sensitive lipase to regulate lipolysis (193) (Figure 5).

There is still a lot to be known about immune cell composition in blood, liver and AT in human obesity and how different immune cell population associates with degree of NAFLD or insulin resistance. Further, markers identifying progenitor cells present in human AT and their functions and ability to differentiate to mature adipocytes is not clear.

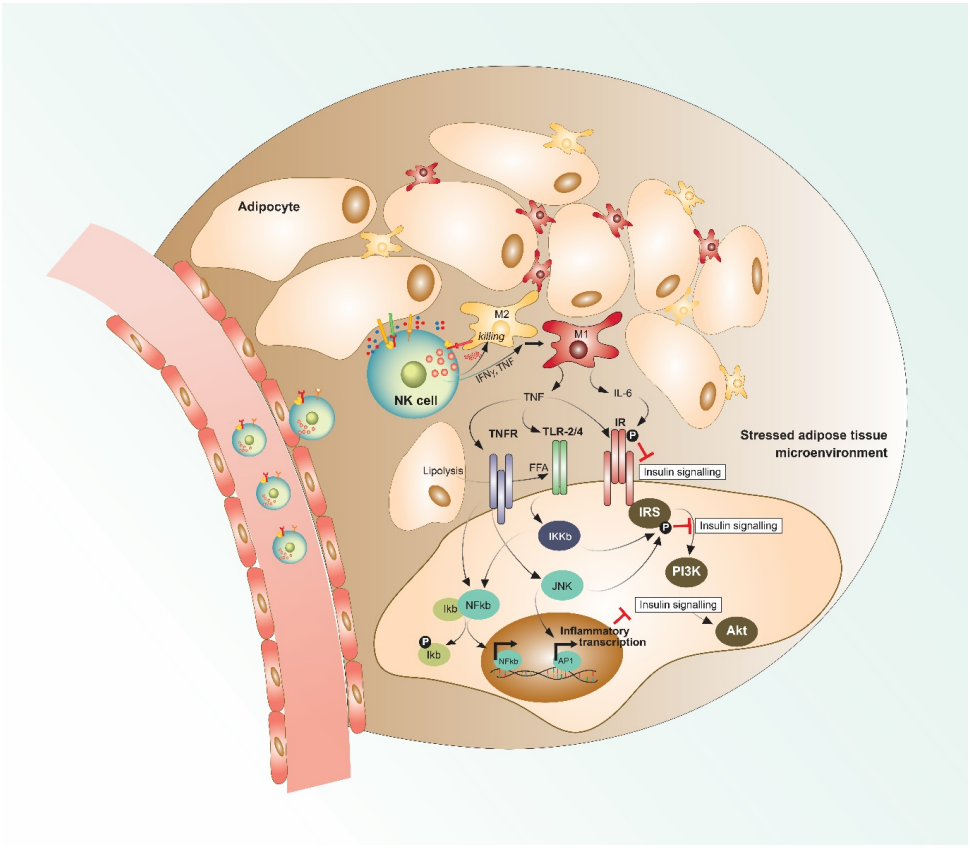

Figure 5. NK cells, ATMs and insulin resistance

In an obese state, NK cells secrete cytokines that drive polarization of M1-like macrophages. NK cells may also lose their ability to kill M2-macrophages. This leads to an increase in the numbers of M1-like ATMs. Pro-inflammatory cytokine production by ATMs drive development of insulin resistance though several mechanisms. IkBkinase-b (IKKb) and JUN N-terminal kinase (JNK) may phosphorylate insulin receptor (IR) and insulin receptor substrate (IRS) at inhibitory sites, which block insulin signaling. Further, activated IKKb leads to translocation of NF-kB to the nucleus and JNK activation can activate AP-1. The transcription factors NF-kB and AP-1 promote expression of inflammatory genes, such as TNF and IL-6, which can further induce insulin resistance. ATMs also produce TNF, which can promote lipolysis leading to release of FFAs that can bind TLR4 and affect insulin signaling. Phosphorylation of IRS can also inhibit signaling through PI3-kinase and the protein kinase B (PKB)/Akt pathway. In the end, this leads to reduced translocation of the glucose transporter GLUT4 to the plasma membrane, reducing uptake of glucose. Used with permission from (194).

2. Aims

The aims of this thesis were to characterize both circulating and tissue immune cells in individuals with obesity and to explore their associations with degree of NAFLD progression or insulin resistance. Further, the aim was to explore the heterogeneity of adipose tissue cells

Specific aims include:

1) Perform an in-depth phenotypic and functional analysis of circulating NK cells as well as characterize NK cells in liver and AT in patients with NAFLD and NASH.
2) Characterize ATMs phenotypically and measure circulating inflammatory factors in obese individuals with varying degrees of insulin resistance.
3) Explore the heterogeneity of adipose tissue myeloid cells and preadipocytes by performing a surface proteome screening and integrating this with analysis of single-cell RNA sequencing data of adipose tissue cells.

3. Comments on methods

3.1 Patient samples (Papers I-III)

The human studies were all approved by the local ethics committee and the subjects gave written informed consent. The studies in paper I were approved by the regional ethics committee in Stockholm (Dnr's 2010/678-31/3, 2006/971-31/1, 2006/229-31/3, and 2014/979-31/1). The first cohort in this paper consisted of 26 patients with liver biopsy-confirmed NAFLD. These patients were recruited from the out-patient clinic at the Upper GI Tract Department at Karolinska University Hospital, Stockholm. From these patients, peripheral blood was collected. As controls, this cohort also included peripheral blood from 15 healthy blood donors collected at the blood bank at Karolinska University Hospital, Stockholm. A second cohort consisted of 26 patients that underwent laparoscopic gastric bypass surgery at Danderyd and Ersta Hospitals in Stockholm. From these patients, peripheral blood as well as liver and adipose tissue biopsies were collected.

The study in paper II was approved by the Regional Committees for medical and health research ethics in Norway (REK, approval number 2015/2343). The first cohort in this study consisted of 57 morbidly obese individuals undergoing bariatric surgery at Voss Hospital. These patients donated fasting blood samples as well as subcutaneous and visceral adipose tissue biopsies. The samples from this cohort of patients were used for analysis by flow cytometry, gene expression analysis and immunohistochemistry. This cohort included 10 patients diagnosed with T2D. A second cohort in this study consisted of the 57 patients from the first cohort and an additional 10 T2D bariatric surgery patients as well as 25 lean volunteers (BMI > 25 kg/m^2). The blood samples from the lean controls were collected at Haukeland University Hospital. Blood samples from this cohort was used for analysis of inflammatory proteins and adipokines. The third cohort included 34 of the patients in cohort 2. These were bariatric surgery patients that underwent laparoscopic sleeve gastrectomy and that had follow-up blood samples taken 3 and 12 months after surgery. Blood samples from this cohort was also used to analyze inflammatory proteins and adipokines.

In paper III we used liposuction asiprates from patients undergoing plastic surgery. This study was approved by the Regional Committees for medical and health research ethics (REK, approval number 2010/502). The SVF was isolated and used for surface proteome screening. Additionally, buffy coats were used for isolation of PBMC. The buffy coat samples were collected from the blood bank at Haukeland University Hospital from anonymous donors and the samples were used in the surface proteome screening and as control samples for the flow cytometry experiment. The scRNA-seq data used in this study was retrieved from a previously published study (150). We only included the SAT-dervied cells that were obtained from 13 SAT samples collected from 14 individuals undergoing bariatric surgery.

3.2 Sample preparation

3.2.1 Isolation of peripheral blood mononuclear cells from blood samples

PBMCs were isolated from heparin blood collected from patients or controls or from buffy coats obtained from the blood bank. Isolation was performed using a density gradient centrifugation method in which PBS-diluted whole blood/buffy coat was carefully layered on top of either Lymphoprep (paper II and III) or Ficoll-Hypaque (paper I). After centrifugation at high speed with no brake and acceleration on the centrifuge, the leukocyte layer can be extracted. The isolated PBMCs were either used immediately for flow cytometry staining or cryopreserved in freezing media consisting of 90 % FBS and 10 % DMSO and stored in liquid nitrogen.

3.2.2 Isolation of the stromal vascular fraction from adipose tissue and immune cells from the liver

The SVF from the adipose tissue was isolated using two similar protocols depending on the starting material. Both involved enzymatic digestion of the tissue with either collagenase I (AT biopsies, paper II) or II (AT biopsies, paper I) or Liberase (liposuction material, paper III), which is a blend of different enzymes used to increase the quality of the tissue digestion. Liver biopsies (paper I) were digested using collagenase II. Adipose tissue biopsies collected during surgery as well as liver

biopsies were minced into smaller pieces before enzyme digestion in order to better dissociate the tissue. The smaller SAT and VAT samples obtained during bariatric surgery were left to separate in tubes, while the large amount of liposuction aspirates from plastic surgery patients were separated in a separating funnel. The SVF was then collected from underneath the floating adipocyte layer.

For paper II and III, the samples were used immediately after purification of the SVF as macrophages are fragile cells that are expected to have a rather low recovery rate when thawing the cells after cryopreservation (195).

3.3 Flow cytometry

Multi-color flow cytometry allows for analysis and characterization of cell populations at a single-cell level. Flow cytometry can also be used to assess DNA content of cells as well as cell numbers, size and granularity. Cells are usually stained with several fluorochrome-labelled antibodies and entered into the flow cytometer where they pass by a laser and a detector. As the cells pass through the laser, the scattered light and fluorescent signal produced is detected by photomultiplier tubes (PMT). The scattered light is in the form of forward scatter (FSC) that is proportional to the cell size, while side scatter (SSC) is a measure of the granularity of the cell (196). Most flow cytometers are made to detect fluorochromes in 15-18 channels in addition to FCS and SSC, however, newer flow cytometers allow analysis of up 50 parameters.

The emission spectrum of a fluorochrome is wide and, when several fluorochromes are used together, spectral overlap may be an issue. This leads to detection of signal from one fluorochrome in the detection filter for another fluorochrome. To correct for this signal overlap, compensation is performed. This involves staining with one antibody at the time to detect signal spillover into other detection channels, that will then be subtracted from these channels (196).. In our experiments, we used anti-mouse compensation beads, which are polystyrene microparticles that bind to any mouse antibodies, as well as beads that do not bind any antibodies, which were used as negative control. The beads are single-stained with each of the antibodies in the panel

and run on the flow cytometer. In the analysis, the single-stained samples are used to create a compensation matrix that is applied on all the samples run afterwards in order to compensate for signal spillover from the fluorochromes.

In this thesis, flow cytometry was used to assess NK cell phenotype and function in PBMCs from patients with NAFLD, assess phenotype of liver and AT NK cells from NAFLD patients, and phenotype of ATMs from obese patients. Additionally, we performed a flow cytometry-based surface proteome screening of monocytes and macrophage sin blood and AT as well as preadipocytes from AT.

The functional panel (described below) included intracellular staining, where after the initial staining of the surface proteins, a step involving both fixation and permeabilization, using a Fix/Perm buffer, was performed. Then, a second step of staining of the intracellular proteins was done. This allowed for staining of cytokines produced by the NK cells after the functional assay (described below). For the proteome screening, PBMCs and SVF were mixed together during the experiments. In order to be able to distinguish the PBMCs and SVF in the subsequent analysis, the cells were barcoded. This involved staining with CD45 antibodies, conjugated to different fluorochromes. The PBMCs were stained with CD45 BV650, whereas the SVF were stained with CD45 A700.

The flow cytometry data was analyzed using FlowJo, where the cell populations of interest are gated on based on their expression of or lack of expression of certain markers. High-dimensional data such as flow cytometry data is often visualized using an algorithm called t-Distributed Stochastic Neighbor Embedding (tSNE) or a similar algorithm called viSNE. This algorithm reduces the dimension of the data by taking each datapoints and giving it a location on a two or three-dimensional map (197). Thus, the data may be visualized by density plots or overlaying heat maps (198), where the phenotypically similar cell subsets tend to cluster together.

3.4 Functional NK cell assay

NK cell functions such as degranulation and cytokine production can be assessed using a functional assay. This involves overnight pre-stimulation of PBMCs with interleukins such as IL-12 and IL-18. The next day, K562 cells, which are target cells for NK cells are added at a ratio of 1:10 in relation to the amount of PBMCs. After one hour, protein secretion is inhibited by addition of Golgi plug (Brefelding A) and Golgi stop (Monesin). The cells are left for 5 more hours before staining for flow cytometric analysis. The ability of the NK cells to produce cytokines such as IFN-γ, TNF and MIP-1β, their ability to upregulate CD107a, CD69, CD44 and CD25, and to downregulate CD16 in response to the activation with ILs and target cells are then measured using flow cytometry.

3.5 Immunohistochemistry

Immunohistochemistry (IHC) is a method in which antibodies are used to detect specific types of cells in tissue sections. In contrast to flow cytometry, IHC allows for determination of tissue distribution and localization of certain antigens or cell types. However, using IHC to accurately quantify numbers of cells is not commonly done. Additionally, IHC is usually performed on only smaller parts of a tissue biopsy, and in thin sections, which may not be representative for the whole tissue as it depends on where the biopsy was taken. With flow cytometry, on the other hand, it is possible to analyze cells from a whole piece of tissue.

Formaldehyde or formalin is the gold standard fixative agent for fixing tissue for IHC. We fixed fresh AT biopsies in 4 % formalin for 24 hours before transferral to 70 % ethanol for storage until further processing. The tissue was then embedded in paraffin and sectioned using a microtome.

One issue with fixation in formalin is over-fixation, which may lead to unmasking of antigens. Further, fixation leads to cross-linking of the tissue and this may alter the structure of antigens making them hard to detect using antibodies. This obstacle is often overcome by introducing a step of antigen retrieval to the protocol, which is

often performed when tissue is fixed in formalin. The most common forms of antigen retrieval include protease-induced epitope retrieval, heat-induced epitope retrieval or retrieval using detergents (199). Our protocol used heat-induced epitope retrieval to retrieve the antigens and it involved heating the tissue slides at 98 ºC for 24 minutes followed by cooling to 58 ºC in a target retrieval solution. After antigen retrieval, the tissue sections were washed and incubated with H_2O_2 to block endogenous peroxidase activity. Then the sections were blocked to avoid unspecific binding of the antibody before overnight incubation with the antibody. We used CD68, which is a common macrophage marker (200). The next day the sections were washed and incubated with the secondary antibody, which was conjugated to horseradish peroxidase. After incubation, DAB substrate was added to the section. This substrate reacts with the horseradish peroxidase, creating a brown color. The sections were then counterstained with hematoxylin that stains cell nuclei blue and imaged using a microscope to determine $CD68^+$ macrophages in crown-like structures. Further, the images were processed using Image J with the plugin Adiposoft to determine adipocyte diameter (201).

3.6 Quantitative polymerase chain reaction (qPCR)

In order to analyze whole-tissue gene expression, RNA was extracted from frozen adipose tissue biopsies. The RNA was then reverse transcribed to cDNA using reverse transcriptase. Real-time qPCR was performed using SYBR Green and the LightCycler ® 480 system from Roche. SYBR Green is a fluorescent dye that binds to double stranded DNA (dsDNA). Upon binding to the dsDNA a fluorescent signal is detected that is proportional to the amount of dsDNA and this signal increases as the DNA is amplified in the PCR reaction.

In this study, the relative gene expression was calculated using the delta-delta Ct method. The Ct value of the target gene was first normalized to the Ct value of a reference gene, giving a ΔCt value for each sample. Then a calibrator was assigned and, in our case, this was the average ΔCt for all the SAT samples. The calibrator value was then subtracted from the ΔCt value, giving a ΔΔCt value for each sample. The

relationship between the Ct value and the amount of original mRNA in the sample is inversely and exponential. Thus, the normalized gene expression is calculated as $2^{-\Delta\Delta Ct}$. Lastly, the $2^{-\Delta\Delta Ct}$ values for all the samples were normalized to the average gene expression for SAT samples for each of the genes. This gave values for the SAT samples at 1 and values for the VAT samples were relative to the SAT samples making it easier to compare the expression between SAT and VAT for each gene.

3.7 Luminex assay

Luminex allows for simultaneous detection of multiple proteins in a sample. The Luminex system is based on magnetic beads coated with antibodies against the analytes of interest. Beads that target different analytes may be mixed together. The samples, in our case, serum samples were added to the bead mixture and incubated to allow for binding of the antibodies to their target. Detection antibodies, with biotin tags, are added and bind to the antigen. Then, phycoerythrin (PE)-conjugated streptavidin is added, which then binds to the detection antibodies. The sample is read on a detection instrument that has two lasers. The first laser is used to classify the bead and thus to determine which analyte is detected. The second laser can determine the strength of the PE-derived signal, which is proportional to the amount of the analyte in the sample.

4. Summary of results

Paper I

In this study, we first performed a global assessment of different circulating immune cells in patients with NAFLD and NASH and compared this to immune cell populations from healthy controls. Few differences were observed in the numbers of B cells, NK cells and total, as well as $CD4^+$, $CD8^+$, and γδ T cell populations between healthy controls and patients. However, there was a trend towards reduced MAIT cell frequencies in NASH patients compared to both healthy controls and NAFLD patients. Further, while monocyte and myeloid DCs levels were unchanged, we observed a decline in plasmacytoid DCs in NASH patients.

As NK cells have been implicated in liver inflammation, we assessed circulating and tissue-resident NK cell populations in patients with NAFLD and investigated any disease-specific changes within these NK cell populations. Phenotypic characterization of circulating NK cells revealed no changes in the proportion of $CD56^{bright}$ to $CD56^{dim}$ NK cells. Further, neither the disease severity of NAFLD or the presence of obesity influenced the surface receptor expression on the circulating NK cells, with the exception of NKG2D, which was expressed on higher levels on NK cells from patients with NASH. This increase was seen on both $CD56^{bright}$ and $CD56^{dim}$ NK cells and also when comparing obese and lean individuals. We also assessed the potential of the circulating NK cells to produce cytokines, upregulate CD107a, CD69, CD44 and CD25 and downregulate CD16 in response to stimulation with IL-15 and IL-18 and/or target cells. We found that NK cells from NAFLD and NASH patients had a similar capacity for degranulation, cytokine production and upregulation of activation markers as NK cells from healthy controls.

Finally, we characterized liver and AT NK cells from NAFLD and NASH patients. As expected, NK cells were enriched in the liver compared to blood and NK cells were also detected in the AT. The tissue NK cells had a higher ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells compared to blood, with up to half of all NK cells in the tissues being $CD56^{bright}$ NK cells. The frequency of AT and liver NK cells were unaltered in NAFLD

and NASH patients, and none of the investigated phenotypic markers showed a clear link to either NAFLD disease activity, level of liver fibrosis or the degree of insulin resistance (HOMA-IR) of the patients.

Paper II

In this study, we characterized adipose tissue macrophages (ATMs) in a cohort of morbidly obese subjects and found that while the total number of macrophages/monocytes were highest in SAT, VAT harbored more pro-inflammatory M1-like macrophages. The pro-inflammatory identity of the M1-like macrophages was supported by their expression of surface receptors CCR2, CD44, CD40, and HLA-DR. M2-like macrophages, on the other hand, displayed low levels of CCR2 and also somewhat higher levels of CD163, which supported their anti-inflammatory identity.

Further, we investigated the associations between the ATMs and insulin resistance and other metabolic parameters. The M1/M2 ratio in both SAT correlated positively with HOMA-IR. Further, elevated M1/M2 ratio was associated with dyslipidemia and this association seemed to mostly be driven by a correlation between M2-like macrophages and triglycerides and HDL cholesterol. We also assessed adipose tissue gene expression, measured adipocyte size and the presence of CLS in the AT. There were no clear depot-specific differences in the gene expression nor in the number of CLS between SAT and VAT and no associations were found between these markers of AT inflammation and clinical or biochemical markers of metabolic dysregulation.

Next, we measured a panel of adipokines and inflammatory factors in serum from lean and obese individuals. Most of the measured factors were elevated in obesity and distinct factors were associated with biochemical parameters and adipose tissue macrophages. Lastly, the same adipokines and inflammatory factors were measured in serum from obese individuals at the time of bariatric surgery as well as 3 and 12 months after surgery. All of the measured factors changed after bariatric surgery, however, the reduction in the inflammatory factors did not correlate significantly with reductions in HOMA-IR or other biochemical markers after bariatric surgery.

Paper III

There is a lack of consensus regarding which surface protein that best define M1- and M2-like adipose tissue macrophages. Further, there is no clear definition of surface proteins that describe adipocyte progenitor cells in humans. Thus, we performed a flow cytometry-based surface proteome screening of AT macrophages and progenitors.

The myeloid cells generally expressed higher levels of the analyzed proteins, however, we identified surface markers specific for both the progenitor and myeloid cells. The surface proteome was distinct in the $CD29^+$ compared to the $CD34^+$ progenitor subpopulation and in the M1-like compared to the M2-like ATMs as well as compared to the monocytes. The M1-like macrophages and monocytes were more similar in their surface protein expression compared to the M2-like macrophages.

Further, we used a previously published single-cell RNA sequencing (scRNA-seq) dataset to annotate clusters of the progenitor and myeloid cells. The $CD34^+$ progenitor-specific proteins mapped to one of the progenitor clusters, whereas the $CD29^+$ preadipocytes could not be annotated to any of the clusters. One of the two macrophage clusters seemed to be enriched for M1-like macrophages, however, this cluster also showed upregulation of genes associated with LAMs and MMe's. Lastly, we combined our protein expression data with the scRNA-seq data and explored the local interactome of the myeloid and progenitor cells.

5. Discussion

NK cell phenotype and function in NAFLD and NASH

In paper I, we found retained NK cell function in circulating NK cells, however, it would also be interesting to assess function of NK cells in liver and AT. Due to limited amounts of tissue available, only phenotypic characterization of tissue NK cells was performed. Other studies found that human AT NK cells have increased expression of activation markers compared to peripheral blood NK cells. AT NK cells expressed higher levels of several activation receptors compared to circulating NK cells, and amongst these were NKp46 (171). Our results also pointed towards higher expression of NKp46 on AT NK cells compared to circulating NK cells, however, this was due to the AT containing higher amounts to $CD56^{bright}$ NK cells. Our finding of higher levels of $CD56^{bright}$ NK cells in AT compared to blood is in line with findings by others (171). Several studies also report increased activation of NK cells as well as alterations in NK cell function in obesity (169,174,179). Obesity was associated with increased expression of surface receptors CD69 and production of granzyme B, indicating increased activation of NK cells. At the same time, the ability of the NK cells to degranulate and produce cytokines was reduced (179). In childhood obesity, NK cells were also activated compared to normal weight children. Additionally, the NK cells showed increased metabolism and a loss of functions such as proliferation and ability to lyse tumor cells (174). Another study also showed that obesity was associated with reduced function of NK cells due to metabolic paralysis of the cells (169).

It has been shown that NK cells can kill activated hepatic stellate cells (HSC) in the liver through NKG2D signaling in mice (202). A human study showed that NKG2D was the only marker investigated that was increased on AT NK cells from obese compared to lean individuals (171). Similarly, we found an upregulation of the activating receptor NKG2D on circulating NK cells in NASH patients compared to lean controls, on both $CD56^{bright}$ and $CD56^{dim}$ NK cells. In contrast to this, however, another study found that circulating NK cells from healthy controls had higher expression of NKG2D compared to NK cells from NAFLD patients. This increased

expression was observed on total NK cells, but also on $CD56^{bright}$ and $CD56^{dim}$ NK cells. This study also examined at NKG2D expression on hepatic NK cells, however, no difference in the expression of NKG2D was observed between patients with NAFLD and healthy controls (203). It is worth mentioning that this study only included liver samples from 3 healthy subjects. Our finding of increased expression of NKG2D on NK cells from NASH patients could indicate that also in humans, NKG2D might be involved in improving liver fibrosis, as is shown in mice (202).

Some studies have shown that the NKG2D ligands MHC class I chain-related protein A and B (MICA/B) are upregulated in the liver of mice with NASH (204). We assessed the expression of MICA and MICB on human hepatocytes, however, there was no difference in their expression between lean controls and patient samples. Further, we did not have the possibility to investigate the expression of NKG2D on NK cells from liver or AT. In the human uterus, NK cells are found to interact with macrophages via NKG2D on NK cells and MICA, expressed on macrophages (205). NK cells may also interact with macrophages in the AT, possibly through NKG2D or other receptors, however, this remains to be elucidated. Further, Kupffer cells in the liver has been shown to be involved in regulating hepatic NK cell function and phenotype and may decrease NK cell activation through their production of IL-10 (206). NK cells are also found to be able to regulate macrophage accumulation and phenotype in AT (157,158,160). Thus, it may be interesting to assess the expression of NKG2D and other activating receptors on NK cells from AT and liver and explore their interactions with other cell types, such as macrophages, in these tissues.

In paper I, we also performed immunophenotyping of the major immune cell subsets in PBMC from patients with NAFLD and NASH as well as healthy controls. This showed that only plasmacytoid DCs (pDCs) were significantly different between healthy controls and patients, with lower pDC levels in patients with NASH compared to healthy controls. In mice, liver DCs are found to be increased, which is thought to protect the liver from inflammation and fibrosis as DCs are involved in clearance of dead cells (207). Indeed, $CD103^{+}$ DCs specifically are found to improve liver steatosis and inflammation (208). Thus, it might be of interest to study DCs in human liver.

Another study investigating lymphocyte populations in PBMC and liver from healthy controls and NAFLD patients, found that NAFLD patients had increased frequencies of several $CD4^+$ and $CD8^+$ T cell subsets, including total $CD4^+$ T cells, compared to healthy controls. We did not find any differences in the frequencies of total, $CD4^+$ or $CD8^+$ T cells. On the other hand, we did find a trend towards reduced frequency of MAIT cells in NASH patients compared to controls. MAIT cells might be an interesting cell type to investigate further in terms of obesity and T2D, as obesity and T2D are both associated with decreased frequency of circulating MAIT cells (209–212), and bariatric surgery is associated with increased circulating MAIT cell levels (209). MAIT cells are also found to be more abundant in AT compared to blood (209,210). AT MAIT cells, but not blood MAIT cells were found to produce the cytokine IL-10. However, AT MAIT cells from obese individuals do not produce IL-10, but rather IL-17, a cytokine associated with insulin resistance (210). Reduced circulating MAIT cell levels in obesity is also associated wi th HbA1c and inflammatory markers. The decrease in MAIT cell abundance in obesity might be explained by elevated blood glucose levels as glucose is found to induce apoptosis of MAIT cells in vitro (211).

MAIT cells from NAFLD patients are found to express higher level of CXCR6 compared with MAIT cells from healthy controls. This suggests that the MAIT cells have a stronger ability to migrate to the liver. Circulating MAIT cells in both healthy controls and NAFLD patients are also found to express high levels of CCR5, indicating that they have a strong tendency to migrate to both the liver and AT.

Indeed, NAFLD was associated with increased frequency of MAIT cells in the liver and a higher NAFLD activity score was associated with higher levels of liver MAIT cells. The MAIT cells in the liver could be activated by Kupffer cells and the MAIT cells could also induce polarization of macrophages into a M2-like phenotype in vitro. Further, in mice, MAIT cell deficiency was associated with severity of hepatic steatosis and inflammation, suggesting that MAIT cells might protect against NAFLD inflammation (212). Thus, exploring the role of circulating and tissue MAIT cells in relation of obesity and NAFLD might be interesting.

ATMs and inflammatory circulating factors in relation to insulin resistance

In paper II, we analyzed ATMs in obese individuals and found that while the total number of macrophages and monocytes was higher in SAT, there were more pro-inflammatory M1-like macrophages in VAT. The literature on depot-specific differences in the numbers of ATMs in human obesity is somewhat contradictory, where some report higher M1-like macrophage levels in VAT (213,214). In contrast to this and to our findings, other studies find that M1-like macrophages are more abundant in SAT (141). Thus, the depot-specific differences in frequencies ATM are still not entirely clear.

While Wentworth and colleagues (141) showed that M1-like macrophages (CD11c+ CD206+) in the AT localizes to CLS, our data did not indicate an association between the numbers of M1-like macrophages detected with flow cytometry and the numbers of CLS detected as CD68+ macrophages surrounding adipocytes. CD68, while considered a general macrophage marker, might not be the optimal marker for detection of ATMs in general or specifically in CLS. A recent scRNA-seq analysis revealed that CD68 expression on macrophages varied considerably between individuals and that the expression was rather low. In fact, expression of CD68 was only seen on 19-52% of all ATMs (215). Others have used macrophage markers such as MAC-2/galectin-3(216) to detect CLS in adipose tissue. Another point to consider is using markers specific for detection of M1- or M2-like macrophages to identify these macrophage subsets specifically. Using subset-specific markers allows for identification of their localization in the AT and to investigate whether M1-like macrophages do indeed localize to CLS. One option is using CD11c, which is expressed on M1- but not M2-like macrophages. However, CD11c is also a marker of dendritic cells, and thus, a combination of markers might be more optimal for detection of different subtypes of macrophages and their tissue distribution. An interesting option would include immunofluorescent or imaging mass cytometry, which allows for staining with several antibodies at the same time.

In the analysis of ATMs, we did not examine the patients with T2D separately as only 10 of the 57 patients in the first cohort were diagnosed with T2D. It would be interesting to include more patients with T2D in order to analyze these individuals separately. Another issue is the fact that several diabetic drugs are anti-inflammatory (217,218) and could therefore influence the results. However, the diabetic group in the analysis of circulating factors, did not display levels of the factors that differed from the obese groups without T2D. On the other hand, when grouping the prediabetic individuals (HbA1c between 39 and 47) together with the T2D individuals, IL-18 was the only marker that were different between patients with obesity and patients with obesity and prediabetes or T2D. This is in line with findings by others reporting that IL-18 is elevated in serum of T2D patients (219) and associated with insulin resistance (220) and that higher IL-18 levels are associated with increased risk of developing diabetes (221) Thus, this circulating factor may be an interesting marker for T2D and/or prediabetes.

Another issue that potentially could affect the results in this study is the preoperative weight loss that the patients may undergo. It has been shown that a slight preoperative weight loss prior to bariatric surgery reduces the surgical complications (222). Thus, the patients are usually advised to lose weight during the months prior to surgery. The preoperative weight loss that the patients may experience could influence the inflammatory status at the time of surgery when both AT biopsies and blood samples were taken. It is shown that weight loss may influence inflammation in different ways.

Studies in mice show that elevated levels of total and CD11c+ ATMs persisted for up to 6 months following weight loss, even with a loss of fat mass (223). On the other hand, others find that while there in an initial increase in ATMs, the numbers of ATMs decreased with an extended weight loss period (224). Human data show that 7% weight loss after RYGB surgery or lifestyle intervention led to an increase in neutrophil numbers and elevated mRNA levels of IL-1β (225), whereas others studies demonstrate decreased AT expression of inflammatory genes following bariatric surgery (226). Another study reports no change in AT inflammation following surgery-induced weight loss after 1 month and even 12 months after surgery, when insulin sensitivity

was improved, no changes in pro-inflammatory AT gene expression was observed. This suggest that inflammation is a dynamic process that may be affected by length and/or pace of the weight loss (227). Another human study demonstrated that rapid weight loss after very low-calorie diet increased CLS density in SAT, without changing AT gene expression of inflammatory genes. At the same time, the weight loss was accompanied by a reduction in hsCRP and glucose levels (228), suggesting that weight loss have differential effect on AT and circulating markers of inflammation. This is in line with our findings were reductions in serum inflammatory factors do not correlate with reduction in insulin resistance (HOMA-IR).

One limitation of this study is the lack of lean controls in the part that assesses AT inflammation. As it is much easier to collect blood compared to AT biopsies from lean controls, we only included lean controls in the measurements of circulating inflammatory factors. However, after the initial work for this paper was completed, we started to receive blood samples and AT biopsies from lean patients undergoing cholecystectomy. Individuals with a high BMI are at increased risk of getting gallstones (cholelithiasis) or inflammation of the gallbladder (cholecystitis), which may lead to a cholecystectomy (229), thus many cholecystectomy patients are overweight or obese. Some studies report lower amounts of ATMs in lean cholecystectomy patients compared to indiviudals with obesity even when the lean control group had a median BMI of 26 kg/m^2. Other studies also include lean control groups with BMI < 30 kg/m^2 and show elevated infiltration of macrophages, higher AT gene expression of CC chemokines and increased serum concentrations of IL-6 and CRP in individuals with obesity compared to lean individuals (230). Thus, it seems as though a lean control group with a BMI below 30 kg/m^2 might be lean enough to detect differences compared to induviduals with obesity.

In the present study, we characterized ATMs in fresh adipose tissue biopsies directly after tissue fractionation as most of the macrophages do not survive after freezing and thawing. To get a cohort of patients with varying degrees of insulin resistance we had to analyze samples from a large number of patients. Thus, to characterize the degree of insulin resistance for these patients was not feasible. Therefore, the insulin resistance

status of the patients included in the study were defined by HOMA-IR, using fasting insulin and glucose levels, which were measured in fasting blood samples taken the day of surgery. HOMA-IR as a measure of insulin resistance has been criticized, however, it has been showed that HOMA-IR correlate well with other measures of insulin resistance such as the euglycemic clamp (31).

It has been suggested that insulin resistance of AT precedes insulin resistance in tissues such as liver and skeletal muscle, mainly because FFAs released from the AT may affect the liver and skeletal muscle and lead to development of insulin resistance. Insulin inhibits lipolysis and promotes storage of lipids and if this malfunction, it may lead to ectopic fat accumulation and lipotoxicity (231,232). Thus, it may be interesting to measure insulin resistance in the AT itself. Several methods have been described to quantify AT insulin resistance (232). Plasma levels of FFA or glycerol have been proposed as one simple method, however, there are larger inter-individual differences in the clearance of FFAs making these measures inaccurate. Another method is a multistep insulin clamp used in combination with an FFA tracer that determines a dose-response relationship between lipolysis rate and plasma insulin levels. This is a time-consuming method that may take up to 8 hours to perform. The hyperinsulinemic-euglycemic clamp technique can also be used and is less time-consuming than the multistep clamp method. However, this method can easily overestimate the IC_{50}, which is the concentration of insulin needed for 50% suppression of lipolysis. Another method is using the adipose tissue insulin resistance index (Adipo-IR), which is similar to HOMA-IR. Adipo-IR can be calculated based on fasting plasma FFA and insulin concentrations (Adipo-IR = FFA x FPI [nmol/L x pmol/L]). It is also found to be associated with hepatic fat content as well as ectopic fat accumulation. Patients with NASH are found to have increased Adipo-IR compared to total body adiposity-matched controls (231). The Adipo-IR index is also found to be reproducible and correlated with the multistep clamp technique, however, the Adipo-IR was found to be more sensitive to age and physical fitness of the individuals (233).

Surface proteome screening AT progenitors and macrophages

In paper 3, we performed a flow cytometry-based surface proteome screening of blood monocytes, ATMs and APCs, using the LEGENDScreen™ platform from Biolegend. This allowed us to screen for over 300 surface markers on the different cell types and subtypes. The LEGENDScreen™ kit can be coupled with a pre-staining of the cells that will be examined, allowing for analysis of surface markers on several different cell types or specific subtypes of cells, which is what was done in this study. One of the benefits of the LEGENDScreen™ is that is allows for extensive screening and thus there is a possibility for identification of new surface markers that have not previously been decribed. We identified expression of CD85a, CD48, and CD371 on the M1-like ATMs, proteins that to our knowledge has not previously been described to be expressed on ATMs. These surface markers might therefore define possible new subtypes of M1-like ATMs. Other surface markers might postulate specific functions to the cell types they are expressed on or serve as potential drug targets.

One of the challenges with this method is that it requires a large amount of starting material in order to have enough cells for analysis of all the markers. As ATMs are not abundant in AT, we had to test our backbone panel in order to ensure that we used enough cells to start with in order to end up with a sufficient number of macrophages in the end. Thus, analyzing rare cell populations using LEGENDScreen™ might be challenging and require prior testing to ensure sufficient starting material is used. In the study, we focused on the proteins that were differentially expressed between subpopulations, e.g. proteins that were highly expressed on $CD34^+$ progenitors and expressed at low levels on $CD29^+$ progenitors. However, as we analyzed the percentage of cells expressing each marker, a low to medium expression might not indicate low overall expression but rather that there are two distinct populations present, one population positive for a certain protein and one that is negative for the protein. Hence, we do not know if expression of a surface protein on a small population might define an important subpopulation. An example of this is $CD142^+$ progenitor cells that was found to only make up a small part of the total AT progenitor cells, however, it had potential to inhibit adipogenesis of the other progenitor populations in the AT (68).

LEGENDScreen™ is also limited to screening of surface proteins and detection of intracellular proteins such as transcription factors or signaling molecules is not possible. Another limitation to the method is that validation of the protein expression is needed as usually material from only one or very few donors is used in the screening. It may also be necessary to perform follow-up experiments to determine the function of certain markers expressed on a cell type or identify mechanisms behind expression or lack of expression of other markers. This may be achieved by performing functional or genetic analysis. In this study, it may be of interst to explore the implications of the expression of certain surface markers. Accordingly, it might be interesting to explore the differentiation potential of the identified progenitors and explore whether certain surface proteins define subpopulations that are more or less capable of differentiation to mature adipocytes. The protein CD142, for instance, has been shown to be expressed on progenitors that are not able to differentiate and that inhibit adipogenesis of other protenitors (68) however, it has also been shown to be expressed on progenitors that do differentiate (69). We found that this marker was expressed on the $CD29^+$ progenitors and thus, it might be of interst to explore the differentiation capacity of these progenitors. One of the studies also showed that $DPP4^+$ cells could become $CD142^+$ and then undergo adipogenesis, implicating that expression of other markers might regulate the function of another protein. Hence, investigating co-expression of proteins on the same cell type will also be important to determine the function of surface markers, which was not possible to do in the LEGENDscreen-experiment. Moreover, CD29+ progenitors are thought to be beige-like (67), which could be an interesting venue to explore further. It would then be possible to explore if this is true for all $CD29^+$ progenitors or if it is limited to certain supopulations of these APCs that are defined by other surface proteins.

It is also possible to use a combination of approaches to study the cell populations further, giving deeper insight into the different cell types in the AT. One study, for example, used a combinaton of LEGENDScreen™, microarray and liquid chromatography-tandem mass spectrometry to study T cells (234). Additionally, new methods are developed to combine surface proteome screening with scRNA-

sequencing allowing for simultaneous analysis of genes and proteins on a single-cell level (235).

In this study, we used a scRNA-seq dataset to validate our surface protein expression data obtained from the LEGENDScreen™. However, we were not able to validate expression of all the identified proteins on the specific clusters in the scRNAseq data, mostly due to low expression of a lot of the corresponding genes in the scRNAseq dataset. The LEGENDScreen™ platform is based on protein expression, where we analyze what is actually expressed as opposed to gene expression, which is subjected to epigenetics, post-transcriptional modifications etc. Thus, the RNA level is not always corresponding to the actual expression of the encoded protein and even if they show expression of genes coding for surface proteins, these proteins might not actually be expressed on the cell. However, the scRNA-seq can also detect many more genes than the protein screening was able to detect, including genes encoding intracellular proteins, which was not detect with the LEGENDscreen-method. Protein detection by flow cytometry is based on the availablilty of antibodies that target specific proteins, so identification of protein is limited by the antibodies included in the kit. The scRNA-seq, on the other hand, is limited to only the most highly expressed genes, which may explain the lack of overlap between our protein expression data and and gene expression data from the scRNA-seq.

An alternative to LEGENDScreen™ is mass cytometry (CyTOF), which allows for analysis of a large number of surface markers. While LEGENDScreen can analyze a higher number of total proteins, CyTOF can be used to detectct over 50 markers simultaneously at a single cell level. CyTOF is similar to flow cytometry, however, the antibodies used are conjugated to heavy metal ions rather than fluorochromes. The metal ions are detected in a time-of-flight mass spectrometer, which ameliorates the need for compensation (236).

This study is an example of integrating your own data with already exsisting data and new methods to complement and explore the data further. Further, it shows that there is potential little overlap between protein and gene expresson. Still, development of

sequencing methods with increased sequencing depth will allow for better resolution of scRNA-seq data. Other methods and combinations of methods may also be used to gain a better understanding of the heterogeneity of the AT.

6. Conclusions

In the present study, we characterized innate immune cells in liver and AT in individuals with obesity in relation to NAFLD or insulin resistance. We further performed a surface proteome screening of ATMs and APCs.

Patients with NAFLD or NASH did not display altered proportions of $CD56^{bright}$ to $CD56^{dim}$ NK cells in their circulation compared to healthy controls, nor was the functionality of the NK cells changed. However, NK cells from patients with NASH expressed higher levels of the activating receptor NKG2D. Liver and AT NK cells cells had a higher ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells compared to blood, however, none of the phenotypic markers investigated were associated with disease severity or the level of fibrosis or insulin resistance.

In obese patients with variying degree of insulin resistance as measured by HOMA-IR, we compared SAT and VAT and found that the SAT had a higher frequency of total myeloid cells, whereas the VAT had a higher frequency of pro-inflammatory M1-like ATMs. The M1/M2 ratio in SAT correlated positively with HOMA-IR as well as to dyslipidemia, an association that was driven mostly by a correlation between M2-like ATMs and TG and HDL cholesterol. However, no clear depot-specific differences were observed in AT gene expression or in the numbers of CLS, and these markers of AT inflammation were not related to markers of metabolic dysfunctions. We identified circulating inflammatory factors that were elevated in obesity and associated with biochemical parameters, however, despite redcutions in these factors after bariatric surgery, the reductions were not associated to parallel reductions in HOMA-IR.

A surface proteome screening of ATMs and APCs revealed distinct surface proteins on progenitor and macrophage subpopulations. Comparing the surface protein expression to a scRNA-seq dataset showed enrichement of some proteins associated to distinct clusters, however, overall the overlap between protein and gene expression was moderate. Lastly, we used our surface protein expression data to guide a computational tool that can use scRNA-seq data to explore the local interactome of the ATMs and APCs based on their cluster-specific gene expression.

7. Future perspectives

While we have studied both phenotype and function of circulating NK cells, we only had sufficient amounts of tissue available to characterize the phenotype of NK cells in liver and AT. Thus, it would be of interest to also investigate the function of tissue NK cells in terms of their ability to produce cytokines and kill target cells as well as study their activation state. Further, it would be interesting to more deeply characterize the surface receptors repertoire of AT and liver NK cells. Investigating the metabolic profile of the NK cells, both circulating and tissue NK cells, would also be interesting to pursue.

In regards to the macrophage study, it would be valuable to analyze ATMs from lean controls to compare with the results from the obese individuals. Here it would also be possible to include analysis of NK cells in AT from both lean and obese and possibly also study both ATMs and AT NK cells from the same individuals. Further, functional characterization of different ATM subsets might also be interesting. To achieve this, M1- and M2-like macrophages or even subtypes of these macrophages could be sorted from the rest of the SVF using FACS. The macrophages can then be stimulated with e.g. LPS or AT-specific stimuli such as FFAs and their secretion of cytokines into the medium can be analyzed. Additionally, we could investigate the metabolic profile of the ATMs. In this study, we also measured whole-tissue gene expression and it would be interesting to analyze gene expression in SVF and mature adipocytes or even in sorted ATMs to get a more specific overview of the gene expression in the macrophages and not just the whole tissue.

Further, we would like to include more patients with T2D to investigate their AT inflammation. It would also be of interest to include more men to be able to study gender-specific differences in AT inflammation. In our analysis of circulating factors, some of the factors showed opposite patterns in men and women, where e.g. the men had increased levels after surgery, while the women had decreased levels, whereas the whole group as a whole showed no change. In this study, we were not able to include

enough men to be able to group the studied individuals into men and women, but this could be done in future studies.

In regards to the progenitor cells, it will be valuable to validate the surface protein expression in several individuals and also in both SAT and VAT samples. Further, it might also be interesting to identify the progenitors that are most likely to differentiate into mature adipocytes *in vitro* and thus be able to use this as a cell model for future studies. As for the macrophages, we would also like to validate our findings from the screening and study these macrophages in SAT and VAT from both lean and obese individuals and relate them to the degree of metabolic disease in obese individuals. Additionally, we may further characterize the different progenitor and macrophage subtypes with regards to their functions and metabolism.

Source of data

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Paper I

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Retained NK Cell Phenotype and Functionality in Non-alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD), and the progressive stage non-alcoholic steatohepatitis (NASH), is the predominant cause of chronic liver disease globally. As part of the complex pathogenesis, natural killer (NK) cells have been implicated in the development of liver inflammation in experimental murine models of NASH. However, there is a lack of knowledge on how NK cells are affected in humans with this disease. Here, we explored the presence of disease-specific changes within circulating and tissue-resident NK cell populations, as well as within other major immune cell subsets, in patients with liver biopsy-confirmed NAFLD. Using 18-color-flow cytometry, substantial changes were observed in certain myeloid populations in patients as compared to controls. NK cell numbers, on the other hand, were not altered. Furthermore, only minor differences in expression of activating and inhibitory NK cell receptors were noted, with the exception of an increased expression of NKG2D on NK cells from patients with NASH. NK cell differentiation remained constant, and NK cells from these patients retain their ability to respond adequately upon stimulation. Instead, considerable alterations were observed between liver, adipose tissue, and peripheral blood NK cells, independently of disease status. Taken together, these results increase our understanding of the importance of the local microenvironment in shaping the NK cell compartment and stress the need for further studies exploring how NASH affects intrahepatic NK cells in humans.

Keywords: natural killer cells, liver immunology, adipose tissue immunology, NAFLD, obesity

INTRODUCTION

NK cells are an important part of innate immunity where they participate in the defense against viral infections and in tumor surveillance (1) Upon activation, NK cells perform cytotoxicity by the release of cytolytic granules. They can also contribute to a pro-inflammatory environment through the production of cytokines and chemokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (1). As part of the innate immune system, NK cells were believed to retain a static phenotype during their life span with little evidence of differentiation except for transition from

$CD56^{bright}$ to $CD56^{dim}$ NK cells (2). However, this view has been revised in the last decade and it is now clear that NK cells gradually undergo directed differentiation even after they have reached the $CD56^{dim}$ stage (2). In addition to their presence in the circulation, NK cells are found in numerous peripheral tissues and are especially enriched in the liver and uterus where they comprise up to 30 and 45%, respectively, of all lymphocytes (3, 4). However, compared to circulating NK cells, less is known regarding NK cells residing in tissues. In relation to the liver, studies have in recent years shown the importance of NK cells in the pathogenesis and clearance of chronic viral hepatitis infections in humans (5). However, the role of NK cells in many other liver diseases remains elusive.

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. A sub-group of NAFLD patients develop chronic inflammation in the liver, which over time can lead to liver fibrosis. This stage of the disease is known as non-alcoholic steatohepatitis (NASH) (6) and these patients are at risk of developing liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (7). The increased rates of obesity in many countries has contributed to the drastic global increase in NAFLD prevalence in recent years (8). Thus, NAFLD complications, such as NASH, liver cirrhosis, and HCC, are posing a significant challenge to health care systems worldwide (6). Several theories exist as to why liver inflammation develops in patients with NAFLD. In more detail, disease development is believed to be influenced by an interplay between genetic and environmental factors, ranging from disturbances in lipid storage and metabolism, changes in dietary patterns and microbiota, to perturbed immune activation (6). However, the exact mechanisms as to why some patients progress in their disease whilst others do not still remain elusive.

Interestingly, murine models have revealed the importance of innate immunity, and in particular NK cells, during NASH-development (9). Many NK cell ligands are up-regulated in the liver of mice with NASH and this is followed by influx of activated cytotoxic NK cells (10, 11). Furthermore, NK cells can, via Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) production, promote a pro-inflammatory state in the steatotic liver and by this mechanism contribute to progression toward steatohepatitis (10). In addition, NK cell activation in response to IL-15 promotes NASH-development in mice (12) and NK cells are also thought to play an important role in regulating fibrosis development in NASH (13, 14). In addition, obesity itself can also alter NK cell phenotype, metabolism, and function (15–18). Indeed, several recent studies in mice have suggested NK cells to be important for development of insulin resistance (19–21). However, there is a lack of knowledge on how NK cells are affected in humans with NASH, both with respect to circulating NK cells as well as to NK cells residing in metabolically active tissues such as liver and adipose tissue. To address this, we here performed an in-depth phenotypic and functional analysis of circulating NK cells in NAFLD patients as well as explored the NK cell compartment in liver and adipose tissue of these individuals.

Abbreviations: ALT, alanine transferase; AST, aspartate transferase; BMI, body mass index; HCC, hepatocellular carcinoma; HOMA-IR, homeostatic model assessment; HSC, hepatic stellate cell; IFN-γ, interferon-gamma; IL, interleukin; ILC1, innate lymphoid cell group 1; KIR, killer cell immunoglobulin-like receptor; MAIT, mucosa-associated T; mDC, myeloid dendritic cell; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NAS, NAFLD activity score; NK, natural killer; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; SDI, Simpson diversity index; SNE, stochastic neighbor embedding; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumor-necrosis factor.

MATERIALS AND METHODS

Clinical Cohorts

Several clinical cohorts were included in the current study. All studies were approved by the regional ethics committee in Stockholm (Dnr's: 2010/678-31/3, 2006/971-31/1, 2006/229-31/3, and 2014/979-31/1) and oral and written informed consent was obtained from all participants. First, peripheral blood samples were obtained from 26 patients with liver biopsy-confirmed NAFLD from the out-patient clinic at the Upper GI Tract Department, Karolinska University Hospital, Stockholm, Sweden. See **Table 1** for detailed patient characteristics. Secondly, as controls, peripheral blood from 15 healthy blood donors was collected from the blood bank at the Karolinska University Hospital, Stockholm, Sweden. Inclusion criteria in controls were normal body-mass index (BMI), normal liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], and no history of type 2 diabetes. Thirdly, in order to assess tissue resident NK cells, peripheral blood as well as liver and adipose tissue biopsies were collected from 26 patients undergoing laparoscopic gastric bypass surgery for morbid obesity at Danderyd and Ersta Hospitals in Stockholm. All patients had a BMI above 35 and had no previous history of liver disease. Included patients were not prescribed any low-calorie pre-surgery diet since this would influence degree of liver steatosis and possibly liver inflammation. A fraction of obtained liver biopsies was used for clinical scoring of liver histology according to the NAFLD activity score (NAS) and fibrosis stage. Based on the severity of the liver histology, patients were divided into three groups; patients with normal liver histology, patients with liver steatosis only (non-alcoholic fatty liver, NAFL), and patients with liver inflammation (NASH). See **Table 2** for more detailed characteristics on this patient cohort.

Isolation of PBMC From Blood Samples

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using density gradient centrifugation. Briefly, whole blood was diluted with phosphate buffered saline (PBS; Invitrogen, USA), carefully layered on top of Ficoll-Hypaque (GE Healthcare, UK), and centrifuged. The leukocyte layer was extracted, carefully washed, and cryopreserved in freezing medium [90% heat-inactivated fetal bovine serum (FBS; Sigma-Alderich, USA) and 10% dimethyl sulfoxide (DMSO; Life Technologies)] until flow cytometry experiments were performed.

TABLE 1 | NAFLD cohort characteristics.

	Healthy controls	NAFL[a]	NASH[b]
Subjects	15	11	15
Age, mean (years)	54	56	58
Male, n	10 (67%)	8 (73%)	9 (60%)
Female, n	5 (33%)	3 (27%)	6 (40%)
BMI[c] mean (kg/m^2)	24	35	31
ALT[d], mean (μkat/L)	NA	0.51	0.75
NAFLD Activity Score, median (range)	NA	3 (1–4)	5 (5–7)
Steatosis (0–3), median (range)	NA	1 (1–2)	2 (1–3)
Lobular inflammation (0–3), median (range)	NA	1 (1–3)	2 (1–3)
Hepatocyte ballooning (0–2), median (range)	NA	0 (0–1)	2 (1–2)
Fibrosis score (0–4)[e], median	NA	0 (0–1)	2 (0–3)

[a]*Non-alcoholic fatty liver.*
[b]*Non-alcoholic steatohepatitis.*
[c]*Body Mass Index.*
[d]*Alanine aminotransferase.*
[e]*Fibrosis score according to Kleiner et al. (22).*
NA, not applicable.

TABLE 2 | Bariatric surgery cohort characteristics.

	Non-NAFLD	NAFL[a]	NASH[b]
Subjects	8	10	5
Age (year), mean	43	45	43
Male	1 (12.5%)	3 (30%)	0 (0%)
Female	7 (87.5%)	7 (70%)	5 (100%)
BMI[c], mean (kg/m^2)	37	37	37
ALT[d] (μkat/L), mean	0.37	0.51	0.75
P-Insulin (IU[e]), mean	14.3	19.8	36.0
P-Glucose (mmol/L), mean	5.6	6.4	6.5
HOMA-IR[f], mean	3.6	5.7	10.5
NAFLD Activity Score, median (range)	0 (0)	3.5 (1–4)	5 (5)
Steatosis (0–3), median (range)	0 (0)	1 (1–3)	2 (1–2)
Lobular inflammation (0–3), median (range)	NA	1 (0–2)	2 (1–2)
Hepatocyte ballooning (0–2), median (range)	NA	1 (0–2)	1 (1–2)
Fibrosis score (0-4)[g], median (range)	1 (0–2)	1 (0–2)	2 (1–3)

[a]*Non-alcoholic fatty liver.*
[b]*Non-alcoholic steatohepatitis.*
[c]*Body Mass Index.*
[d]*Alanine aminotransferase.*
[e]*International Units.*
[f]*Homeostatic model assessment—insulin resistance.*
[g]*Fibrosis score according to Kleiner et al. (22).*

Isolation of Immune Cells From Liver and Adipose Tissue

The core liver biopsies were collected during surgery directly into complete cell medium [Hyclone RPMI (Invitrogen), 10% FBS, and 1 mM L-glutamine (Invitrogen)] and kept on ice until same-day processing. Tissue pieces were mechanically dissociated, followed by enzymatic digestion in collagenase II for 30 min at 37°C, and filtered through a 100 μm filter, before flow cytometry staining.

Antibody Staining Protocol

Cryopreserved PBMC were thawed in a 37°C water bath, immediately transferred to cell medium, washed, and resuspended in complete cell medium. Three million PBMC were stained in each test. Flow cytometry primary and secondary stainings were performed in flow buffer (PBS with 2 mM EDTA and 2% FBS) for 20 min in the dark at room temperature. After staining, cells were fixed for 15 min in the dark at room temperature using the Fix/Perm solution (eBioscience, USA). Finally, Fix/Perm solution was washed away, cells were resuspended in flow buffer, and kept at 4°C in the dark until they were acquired on the flow cytometer. For intracellular stainings, cells were permeabilized in Fix/Perm solution for 45 min and then stained for 30 min in permeabilization buffer (eBioscience, USA), diluted 1:10 with MQ water, before being washed and analyzed. All samples were run on an 18-color LSRFortessa (BD Biosciences, USA) equipped with 355, 405, 488, 561, and 639 nm lasers.

Functional Experiments

NK cell degranulation and cytokine production were assessed by co-culture experiments with target cells. PBMCs were pre-stimulated overnight with IL-12 (10 ng/ml, Peprotech) and IL-18 (100 ng/mL, Medical & Biological Laboratories) and then K562 cells were added at a 1:10 ratio. One hour after addition of target cells, Golgi plug (Brefeldin A, BD Biosciences) and Golgi stop (Monesin, BD Biosciences) were added and the assay was continued for an additional 5 h. NK cells were then stained for analysis using flow cytometry.

Microscopy

Liver biopsy specimens obtained from NAFL/NASH patients and patients undergoing gastric bypass surgery were stained with hematoxylin and eosin and graded in a blinded fashion by an experienced hepatologist according to the NAFLD activity score (NAS) (23). In addition, liver biopsies were stained with Sirius red to evaluate liver fibrosis and scored according to Kleiner on a 0–4 scale (23).

Flow Cytometry Analysis

To avoid bias from intra-experimental variability that could affect the flow cytometry analysis, samples from both healthy donors and patients were analyzed in each experiment. Acquired data was compensated using a compensation matrix generated based on antibody-stained control beads and analyzed using FlowJo Version 9.6.4 (Treestar, USA). Apart from conventional flow cytometry analysis, Barnes-Hut stochastic neighbor embedding (SNE) analysis, using an in-house built script (24) in R (The R Foundation for Statistical Computing), was performed in order to visualize potential differences not present in two-dimensional space. For SNE-analysis, 1,000 CD56^{dim} NK cells or 500 CD56^{bright} NK cells from each donor was included. The data was clustered based on median fluorescence intensity (MFI) of the following markers: CD16, CD25, CD44, CD49a, CD56, CD57, CD69, CD107a, HLA-DR, IFN-γ, KIRs, MIP-1β, NKG2A, NKG2C, and TNF.

Staining of Primary Hepatocyte

Primary human hepatocytes were isolated from three organ donors whose livers were not used for liver transplantation, acquired from the Karolinska University Hospital, using a protocol previously described (25). The cells were then stained fresh with antibodies against MICA, MICB, HLA-ABC, CD155, ULBP-1, ULBP-2, and ULBP-3 conjugated with PE and analyzed on a BD FACS Accuri.

Quantification of Soluble MICA and MICB by ELISA

MICA and MICB solid-phase sandwich ELISAs (enzyme-linked immunosorbent assay, Thermofisher) were performed according to the manufacturer instructions. Briefly, human sera were incubated 2, 5, and 2 h respectively diluted two times in provided buffers (capture phase). After washing, biotin-conjugated anti-MICA and MICB antibodies were incubated for 1 h and streptavidin-HRP was added after washing. Unbound streptavidin-HRP was removed by washing and substrate solution reactive with HRP was added. The reaction was stopped by acid after 30 min and absorbance measured at 450 nm.

Statistical Analysis

Data were analyzed using Prism Version 6.0 b (GraphPad Software Inc.; USA). The Mann–Whitney *U*-test or *t*-tests were used depending on if data sets were normally distributed, using D'Agostino-Pearson omnibus normality test. The threshold for statistical significance was set to $\alpha < 0.05$. For analysis of NK cell population diversity, Simpson Diversity Index (SDI) was calculated as previously described (26).

RESULTS

Global Assessment of Immune Cells in NAFL and NASH

To determine the impact of NAFL and NASH on the peripheral blood immune cell compartment, a broad profiling of major lymphoid and myeloid immune cells in patients and controls was initially performed (**Figure 1A**). Whereas, few differences were noted among CD4, CD8, and γδ T cells, a trend toward a decline in MAIT cells was observed in NASH patients as compared to NAFL patients and healthy controls (**Figure 1B**). A decrease of MAIT cells in NASH is in line with recent literature (27). Regarding NK cells, neither frequency (**Figure 1B**) nor absolute numbers (data not shown) were affected by the presence of obesity, NAFL, or NASH. With respect to the myeloid immune cell compartment few differences were observed for monocytes and myeloid DCs. However, a decline in the frequency of plasmacytoid DCs (pDCs) was noted in NASH patients (**Figure 1C**). Also, the absolute numbers of pDCs were decreased and the loss of pDCs correlated inversely with the degree of liver damage measured as serum alanine transferase levels (**Figure 1C**). Taken together, although alterations could be observed in certain innate immune cell subsets, the overall size of the peripheral blood NK cell compartment remained unaltered in NAFL and NASH.

Upregulation of NKG2D on NK Cells From NASH Patients

Since NK cells are far from a homogeneous population, a more in-depth immune-phenotyping of activating and inhibitory receptors on circulating NK cells was performed. The $CD56^{dim}$ to $CD56^{bright}$ NK cell relationship was unaffected in NAFL and NASH (**Figures 2A,B**). Next, we simultaneously assessed expression of 12 surface and intracellular markers on the NK cells (**Figure 2C**). As expected, $CD56^{dim}$ NK cells expressed higher levels of NKG2C, KIRs, and CD57, while $CD56^{bright}$ NK cells had a higher expression of NKG2A, CD161, CD44, and NKp46 (**Figures 2C,D**). Surprisingly, neither the degree of NAFLD disease severity (**Figure 2D**) nor presence of obesity (data not shown) had a detectable effect on the NK cell receptor repertoire on circulating NK cells, with the exception for expression of the activating receptor NKG2D. In more detail, both $CD56^{bright}$ and $CD56^{dim}$ NK cells from patients with NASH expressed significantly higher levels of NKG2D on their surface (**Figures 2E,F**). This was also observed when comparing normal weight with obese individuals (**Figure 2G**). However, since NK cells from NAFL patients had close to normal levels of NKG2D (**Figure 2F**), this would suggest that increased expression of NKG2D primarily associated with NASH. Furthermore, this increase was specific to NK cells since it was not observed on T cells from the same patients (data not shown). To dissect the role of NKG2D more in-depth in relation to the liver and NAFL we assessed presence of NKG2D-ligands. No difference in levels of soluble MICA and MICB was noted in patients as compared to controls (data not shown). Furthermore, primary human hepatocytes from healthy organ donors were negative for NKG2D-ligands whereas CD155 and HLA class I was expressed (**Supplementary Figure 1**).

Finally, we assessed NK cell differentiation, as determined by the expression of NKG2A, KIRs, and CD57, as well as NK cell diversity by calculating Simpson diversity index (SDI) (28, 29). However, both of these metrics for NK cell compartment composition remained unaltered in NAFL and NASH patients as compared to healthy controls (**Figures 2H,I**). In summary, the phenotype of the circulating NK cell population remains unaffected by NAFL and NASH with the exception of NKG2D being upregulated in patients with NASH.

Functional Capacity of Peripheral NK Cell Subsets in NAFL and NASH

Having determined the NK cell phenotype in peripheral blood, we next evaluated the functional capacity of NK cells in NAFL and NASH. To this end, NK cells were stimulated with cytokines (IL-12+IL-18) and/or K562 target cells and production of IFN-γ, TNF, MIP-1β, upregulation of CD107a, CD69, CD44, and CD25, as well as downregulating of CD16 as a consequence of activation was measured using flow cytometry (**Figure 3A**). As expected, stimulation with cytokines led to high levels of IFN-γ being produced as well as strong upregulation of CD25 and CD69 whereas K562 cell stimulation yielded a robust degranulation response and elevated levels of TNF and MIP-1β (**Figures 3A,B**). When assessing single functional responses, NK cells from

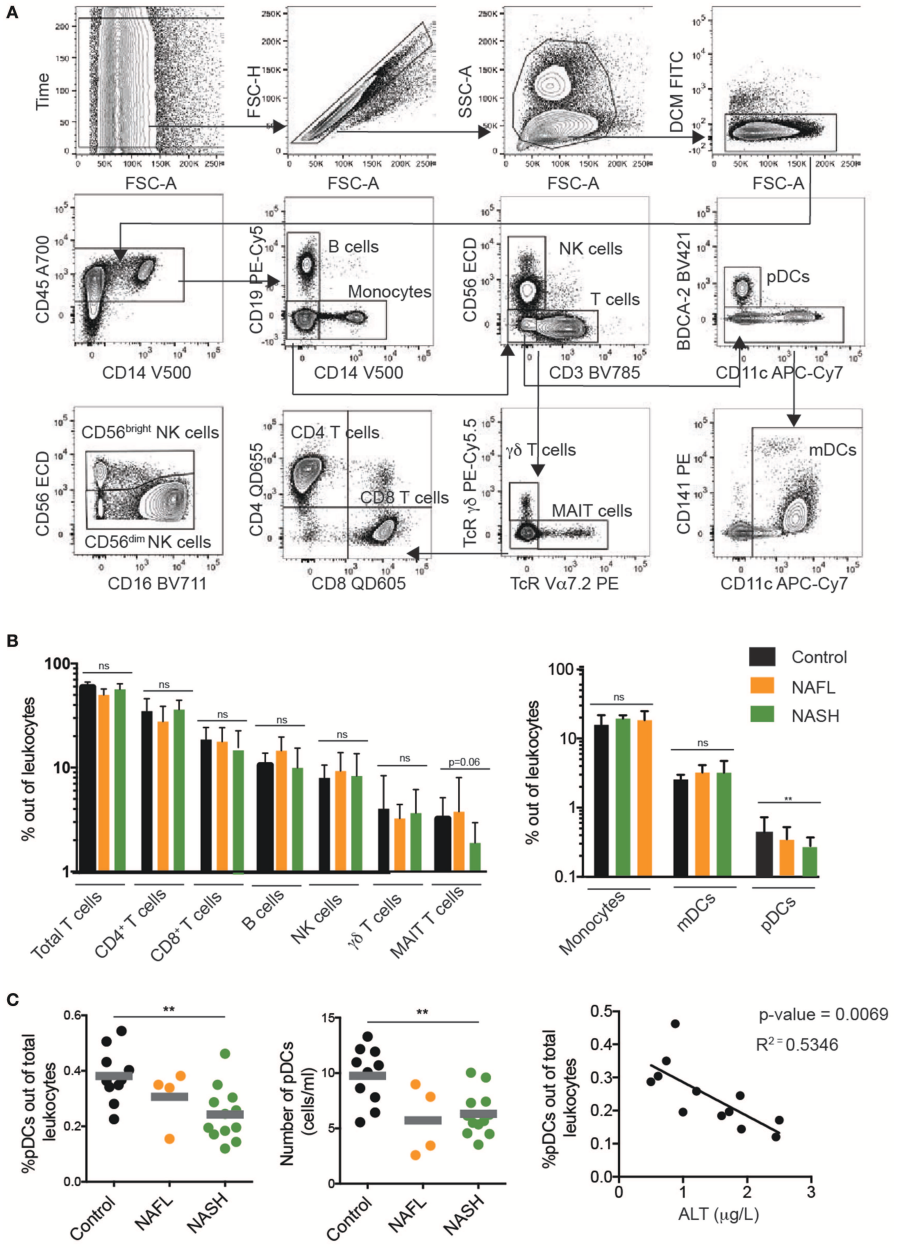

FIGURE 1 | Immunophenotyping of major peripheral blood immune cell subsets in NAFL and NASH. **(A)** Flow cytometry gating scheme used to identify the investigated immune cell subsets. Arrows indicate the sequence of gating. **(B)** Summary data for the frequency out of total leukocytes for the indicated immune cell populations in healthy controls ($n = 10$), NAFL ($n = 4$), and NASH ($n = 11$) patients. **(C)** Summary data of pDC frequency out of total leukocytes (left), absolute counts of pDCs (middle), and correlation between pDC frequency and ALT (right) in the indicated patient groups. Bars in **(B,C)** represent mean and error bars show SEM. $^{**}p < 0.01$.

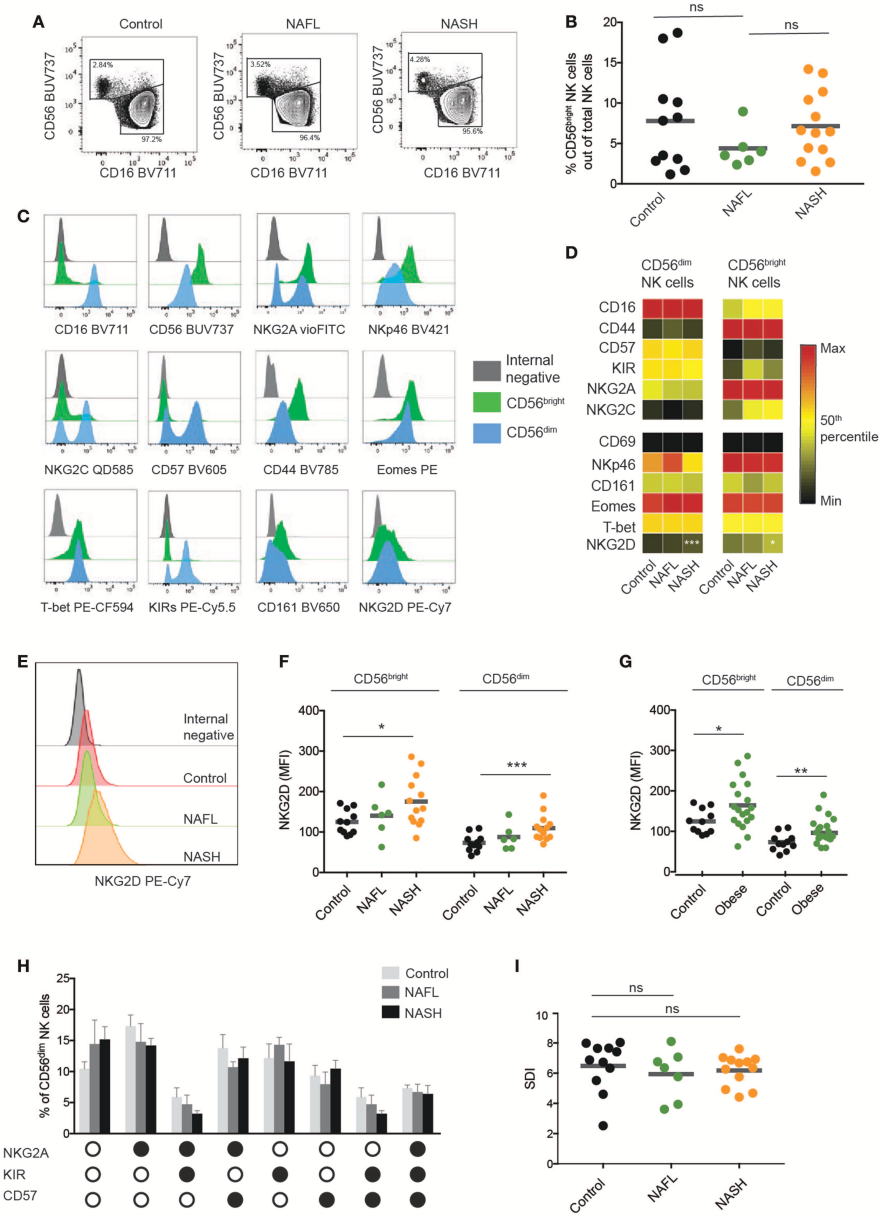

FIGURE 2 | Phenotypic characterization of circulating NK cells from NAFLD patients. **(A)** Representative flow cytometry plots of NK cells from healthy, NAFL, and NASH patients. **(B)** Frequency of CD56^{bright} NK cells out of total NK cells in peripheral blood of healthy controls (n = 13), NAFL patients (n = 9), and NASH patients (n = 16). **(C)** Representative histograms for the indicated markers on CD56^{bright} and CD56^{dim} NK cells as well as internal negative control. The plots represent stainings from one healthy donor. **(D)** Heat map depicting the mean frequency of NK cells expressing CD16, CD44, CD57, KIRs, NKG2A, and NKG2C as well as the mean MFI of CD69, NKp46, CD161, Eomes, T-bet, and NKG2D on CD56^{dim} and CD56^{bright} NK cells for the indicated groups. **(E)** Representative histogram of NKG2D

(Continued)

FIGURE 2 | expression on NK cells from healthy control, NAFL, and NASH patients respectively. **(F,G)** Scatter plots of NKG2D MFI on CD56^{dim} and CD56^{bright} NK cells from the indicated groups. In **(F)**, healthy controls ($n = 11$), NAFL ($n = 6$), and NASH ($n = 13$) patients, in **(G)** healthy controls ($n = 11$), obese individuals ($n = 18$). **(H)** Bar graph showing the frequency of CD56^{dim} NK cells that express different combination of NKG2A, KIRs, and CD57. Black circles indicate presence of the marker and white circles no expression. **(I)** Inverse Simpson Diversity index (SDI) analysis for healthy ($n = 11$), NAFL ($n = 7$), and NASH patients ($n = 12$). The Mann–Whitney *U*-test was used for comparison between groups. Bars in **(B,F,G,I)** show mean, error bars in H represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

patients with NASH had a similar capacity to degranulate, produce cytokines, and upregulate activation markers as their NAFL counterparts and the healthy controls (**Figure 3B**). Also, the ability of NK cells to perform multiple functions was retained in both NAFL and NASH patients as compared to healthy controls (**Figure 3C**).

The NK cell compartment consists of many different subpopulations and NK cells can produce a multitude of functions in a variegated fashion. Although single or multifunctional analysis of NK cell functional responses revealed no considerable alterations in function when comparing NASH with healthy controls it is plausible that functional difference might exist in multivariate space not allowing identification by conventional flow cytometry gating. To address this, we performed a SNE analysis of the NK cell functional responses and first generated SNE maps of responding compared to non-responding NK cells (**Figure 3D**). These SNE maps segregated considerably (**Figure 3D**, residual map) and by projecting the differences onto the single parameters that formed the basis of the SNE map the pattern of NK cell responses in multidimensional space could be revealed (**Figure 3D**, lower panels). In more detail this shows how certain NK cell subpopulations responded with many functions and others only with single function. Together, this validated the analysis approach and allowed us to compare patients with healthy controls (**Figure 3E** and data not shown). Although the residual plots revealed difference when comparing responding NK cells from healthy controls with NAFL or NASH patients (data not shown) or NAFL patients with NASH patients (**Figure 3E**), these differences could not be attributed to a specific phenotype when the residual plot was projected onto the single parameters (data not shown). This suggests that also in multi-dimensional space, the NK cell response was unaltered in NAFL and NASH.

Characterization of NK Cells in Adipose and Liver Tissue

To this end, characteristics of circulating NK cells have been analyzed. However, NAFL and NASH are diseases of the liver and also tightly coupled to obesity, adipose tissue dysregulation, and reduced insulin sensitivity. Thus, the analysis was focused on NK cells from liver and adipose tissue. In line with previous literature (9), NK cells were enriched in the liver compared to peripheral blood (**Figures 4A,B**) and a sizeable population of NK cells could also be detected in visceral adipose tissue. Furthermore, both types of investigated tissue had a skewing in the distribution of CD56^{bright} and CD56^{dim} NK cells as compared to circulation with CD56^{bright} NK cells representing up to half of all NK cells in liver and adipose tissue (**Figure 4B**). Also, whereas the differentiation status of adipose tissue NK cells, with respect to expression of NKG2A, KIRs, and CD57, mirrored that of circulating NK cells, differences were observed especially within liver CD56^{dim} NK cells in expression of these markers (**Figures 4C,D**).

SNE-analysis of the NK cell compartment identified that NK cells derived from liver and adipose tissue displayed a unique phenotype compared to peripheral blood NK cells (**Figure 4E**). The major distinction between NK cells derived from circulation and NK cells derived from tissues was a higher expression of the tissue residency marker CD69 (**Figure 4E**). As a consequence of liver and adipose tissue containing larger populations of CD56^{bright} NK cells, the SNE analysis also identified higher expression of NKp46, NKG2A, and CD56 but lower expression of CD16, CD57, and KIRs within the population enriched in the tissues compared to in circulation (**Figure 4D**). This is in line with the overall phenotypic differences observed when comparing CD56^{bright} with CD56^{dim} NK cells (**Figures 2C,D**). Finally, we confirmed expression of tissue residency markers on liver and adipose tissue by conventional flow cytometry gating (**Figures 4F,G**). As expected, the expression was primarily confined to the CD56^{bright} NK cells. Interestingly, while CD69 was highly expressed in both liver and adipose tissue, CD49a was only found on a small fraction of CD56^{bright} NK cells in the liver whereas higher expression was noted in adipose tissue (**Figures 4F–H**). These results highlight the importance of the organ-specific microenvironment in shaping the local NK cell population and emphasize the importance of studying the tissue-resident compartment when trying to understand the pathogenesis of diseases affecting peripheral organs.

The Phenotype of Tissue NK Cells Remains Unaltered in NAFL and NASH

Finally, we assessed the impact of NAFL and NASH disease stage and severity on the frequency and phenotype of liver and adipose tissue NK cells (**Figure 5**). Patients where data on tissue NK cells were available were stratified based on NAS score (non-NAFL which represented no steatosis present, NAFL, NASH), liver fibrosis (fibrosis score 0–1 vs. 2–3), and insulin sensitivity status (HOMA-IR <4.5 vs. >4.5). Although NK cells were more abundant in liver (**Figure 4B**) their frequency remained unaffected by clinical stage (**Figure 5C**). Also, the frequency of NK cells in adipose was unaltered by NAFLD disease activity, level of fibrosis, and insulin sensitivity (**Figure 5C**). Finally, the NK cell phenotype was assessed. Since CD16^{-} and CD16^{+} NK cells display distinct phenotypes (**Figures 2C,D**, **4E–G**), these were analyzed separately. For none of the investigated phenotypic markers, a clear link to the clinical parameters could be observed (**Figure 5D**).

Thus, whereas murine models suggest NK cells to play a distinct role in NAFL and NASH, both the human circulating and

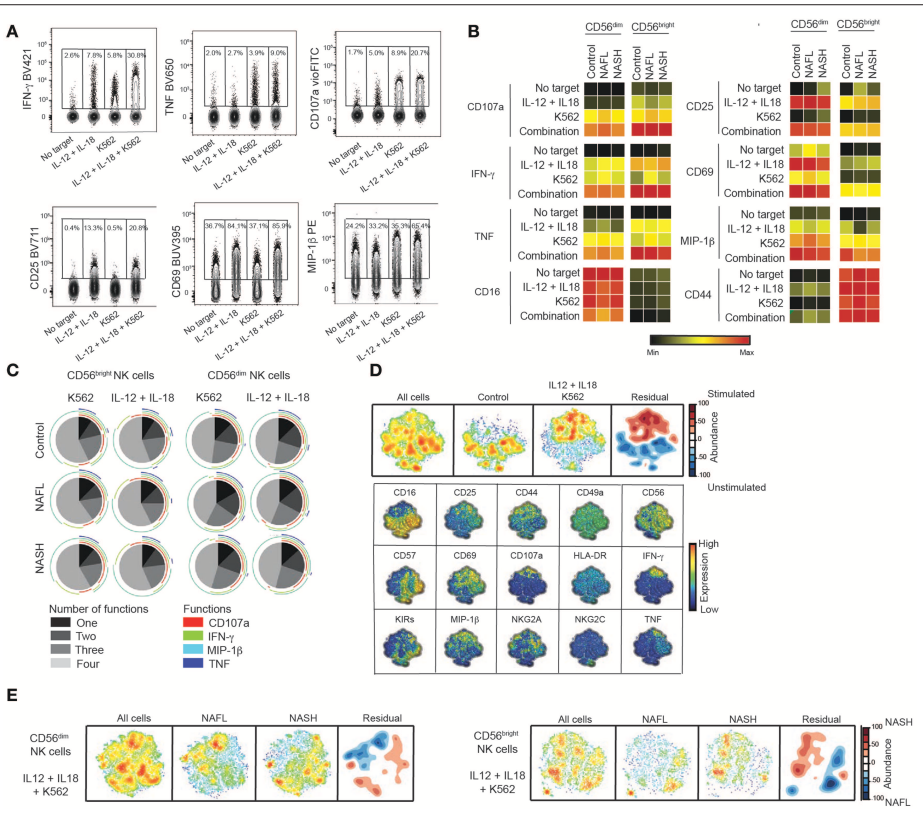

FIGURE 3 | Retained functional capacity of circulating NK cells in NAFLD. **(A)** Representative concatenated flow cytometry plots showing CD56^{dim} NK cell responses following the indicated stimulations. **(B)** Heat map summarizing the mean frequency of responding NK cells for the measured functions and indicated stimulations in healthy controls ($n = 15$), NAFL patients ($n = 10$), and NASH patients ($n = 16$). **(C)** Multifunctional analysis of the combination of degranulation (CD107a) and cytokine production (IFN-γ, MIP-1β, and TNF) responses for IL-12 + IL-18 stimulation and/or co-culture with K562 target cells. **(D)** SNE plots of total cells from healthy donors subsequently divided in stimulated and un-stimulated cells. The residual plot highlights the decreased (blue) or more highly expressed (red) areas in the stimulated cells. Underneath, individual plots of the expression of 15 single markers are shown. **(E)** SNE plots depicting the difference between CD56^{dim} (left) and CD56^{bright} (right) NK cells from NAFL and NASH patients after IL-12 + IL-18 + K562 stimulation where the density plots highlight the specific changes in NASH patients.

peripheral tissue NK cell compartments remain largely intact in these diseases.

DISCUSSION

NAFLD is the most common chronic liver disease worldwide and a subgroup of NAFLD patients develop chronic liver inflammation (NASH) with ensuing fibrosis and increased risk for HCC. Since little is known regarding NK cells in this disease and because NK cells are highly enriched in human liver, we here performed an extensive mapping of the NK cell compartment in NAFL/NASH using high-dimensional flow cytometry technology. We assessed the imprint of liver inflammation in NASH on circulating NK cells and show specific upregulation of the activating receptor NKG2D. In addition, by employing bariatric surgery as a human model, we also comprehensively mapped both liver and adipose tissue NK cells revealing substantial differences in NK cell population composition in-between tissues. However, no significant differences in the tissue-resident NK cell populations in patients with and without NAFL/NASH were detected.

NK cells have previously been investigated in other chronic liver diseases, especially in chronic viral hepatitis. These studies revealed that NK cells have decreased function in chronic hepatitis C (5, 30, 31). In obese individuals, decreased NK cell functionality combined with increased activation was reported (15, 16). A recent study showed that this reduced functionality was a result of metabolic paralysis of NK cells (18). However, our study detected no functional defects or changes in activation, despite both the in-depth and broad evaluation performed.

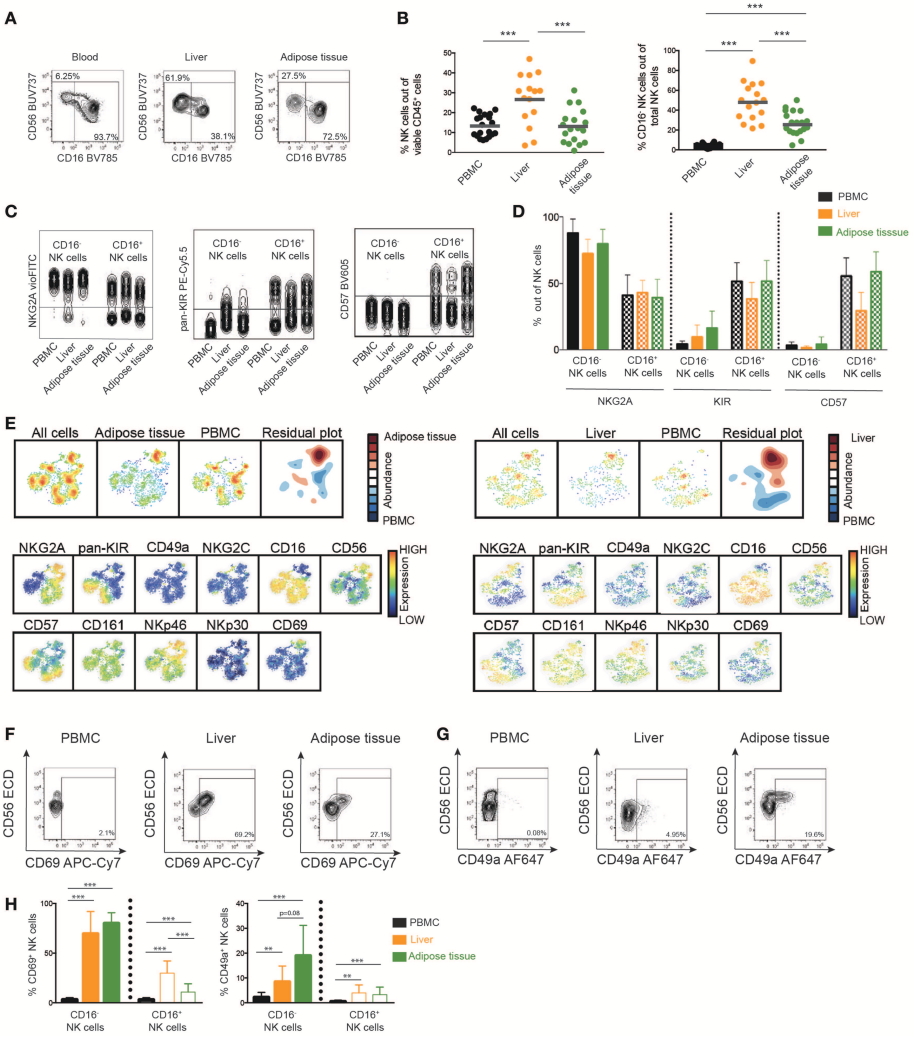

FIGURE 4 | Phenotype of NK cells in liver and adipose tissue. **(A)** Representative flow cytometry plots of NK cells derived from blood, liver, and adipose tissue. **(B)** Scatter plot of the frequency of total NK cells out of viable leukocytes and CD16$^-$ NK cells out of total NK cells in peripheral blood, liver, and adipose tissue. PBMC ($n = 22$), adipose tissue ($n = 19$), and liver ($n = 15$). **(C)** Representative concatenated flow cytometry plots showing expression of differentiation markers NKG2A, KIRs, and CD57 on CD16$^-$ or CD16$^+$ NK cells in blood, liver, and adipose tissue. **(D)** Bar graphs showing frequency of cells expressing the indicated differentiation markers in CD16$^-$ or CD16$^+$ NK cells from blood, liver, and adipose tissue. PBMC ($n = 22$), adipose tissue ($n = 19$), and liver ($n = 15$). **(E)** SNE plot of total NK cells from adipose tissue (left) or liver (right) compared to peripheral blood. The residual plot highlights the decreased (blue) or more highly expressed (red) areas within tissue NK cells. **(F,G)** Representative flow cytometry plots showing CD69 and CD49a expression on total NK cells from the indicated tissues. **(H)** Expression of CD69 and CD49a on CD16$^-$ and CD16$^+$ NK cells in peripheral blood, liver, and adipose tissue. In H, CD69 on PBMC ($n = 22$), adipose tissue ($n = 18$), and liver ($n = 15$), CD49a on PBMC ($n = 9$), adipose tissue ($n = 9$), and liver ($n = 7$). Bars in **(B,D,H)** represent mean and error bars in **(D,H)** show SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

These discrepancies might in part be based on methodological dissimilarities between the studies, with different target cells used but also on differing patient inclusion criteria. Indeed, our patients had, in general, mild fibrosis, with no patients suffering from cirrhosis, suggesting a more active inflammatory disease. This compared to other studies where many patients had cirrhosis (14) or higher BMI (15). Michelet et al identified functional defects as well as the loss of CD56^{bright} NK cells in

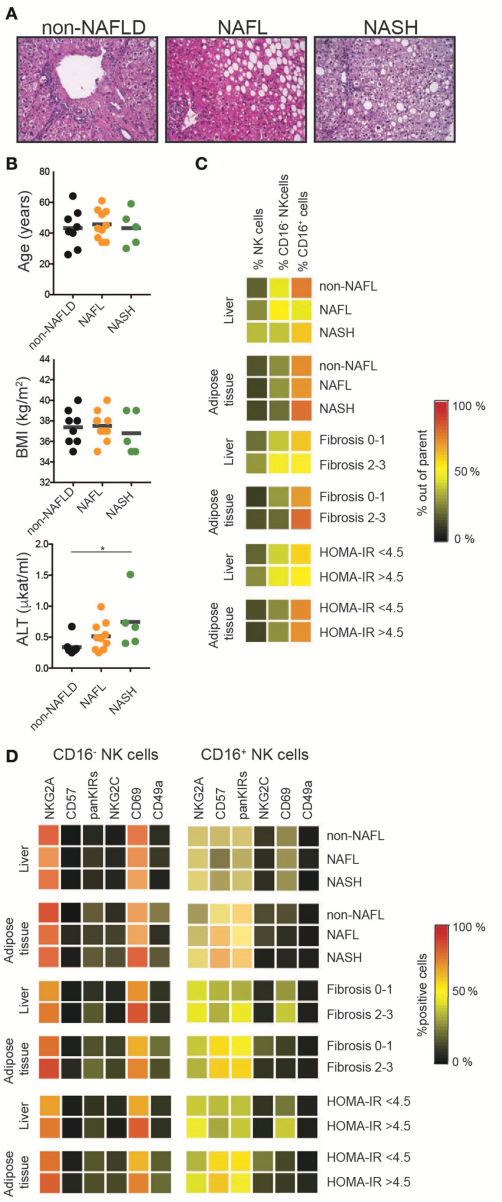

FIGURE 5 | Tissue NK cells in relation to patient disease characteristics. **(A)** Representative hematoxylin and eosin stainings on normal, NAFL, and NASH liver sections. **(B)** Distribution of the patients in relation to age, BMI, and levels of alanine transferase (ALT) as a proxy for liver damage. **(C)** Heat map showing mean frequency of total, $CD16^+$, and $CD16^-$ NK cells for the indicated groups. **(D)** Heat map showing mean frequencies of NKG2A, CD57, KIRs, NKG2C, CD69, and CD49a expression on $CD16^-$ and $CD16^+$ NK cells for the indicated groups. *$p < 0.05$.

obese patients (18). This is in line with a previous report (15), and common of these two studies was that included patients had BMI's of up to 50. Our cohort had a considerably lower average BMI of around 35. Together, this suggests that the functionality of NK cells and NK cell subset composition is retained in less severe obesity whereas alterations become evident in patients with morbid obesity. Atherosclerosis, hypertension, and adipose tissue inflammation are common comorbidities in NAFLD patients and thus potential confounding factors that should be controlled for. Our strategy to address this challenging issue was in one part to stratify patients into NAFL or NASH by the use of liver biopsies, considered the gold standard. In addition, we made a subgroup analysis based on the level of fibrosis and on insulin sensitivity. However, in none of these comparisons, neither in peripheral blood nor in liver or adipose tissue, any profound disease-related alterations in the NK cell populations could be observed. Of note, we focused our analysis on the major two subpopulations of NK cells: $CD56^{bright}$ and $CD56^{dim}$ NK cells. Apart from them, there are additional un-conventional NK cell subset, such as $CD56^-CD16^+$ and $CD56^{dim}CD16^-$ NK cells (32), that should be investigated in future studies.

A recent report demonstrated how chronic hepatitis C irreversibly causes decreased receptor repertoire diversity in circulating NK cells (26) Related changes have also been observed in chronic hepatitis D (33). Based on this, we wanted to investigate whether the chronic "non-infectious" inflammation in NAFLD could cause a similarly reduced diversity. However, NK cell repertoire diversity, determined by SDI, remained intact in NAFLD. Both NAFLD and chronic viral hepatitis are slowly developing liver diseases that take years to produce symptoms. However, many more changes in serum cytokines can be detected in hepatitis C patients as compared to NASH patients (34) and it is plausible that the general degree of inflammation in NASH is more low-grade as compared to chronic viral hepatitis. Thus, it might be that a larger inflammatory insult is needed in order to affect NK cell repertoire diversity.

In experimental models, NK cells have been shown to protect against liver fibrosis development, e.g., via NKp46-mediated macrophage activation (13) or by killing of hepatic stellate cells (HSCs) (35). Similar evidence exists in the human setting but primarily in relation to chronic viral hepatitis and fibrosis development (36). NKG2D is another NK cell surface receptor that may be protective against fibrosis development in mice by targeting activated HSCs (37). Furthermore, the NKG2D-ligands MICA and MICB are both upregulated in NASH-livers in mice (11). For NAFLD patients, level of fibrosis is an important predictor of long-term survival (38, 39). Interestingly, we could show that NK cells express higher levels of NKG2D in patients with NASH. This upregulated NKG2D expression could be a response to the increased hepatocyte stress, inflammation, and apoptosis that can be seen in NASH-livers (23). To determine if NKG2D influenced the degree of liver fibrosis in NAFLD patients, we compared levels of fibrosis to NKG2D expression on circulating NK cells but could not detect any association. This could be due to the fact that fibrosis is a dynamic process that occurs during many years in NAFLD. In the current study, we did not have the possibility to investigate NKG2D expression on

intrahepatic NK cells. Future studies should assess this and also specifically evaluate the capacity of NK cells to target HSCs (or hepatocytes) via NKG2D in NAFLD.

Apart from having a protective role in fibrosis development, adipose tissue NK cells, or ILC1-like cells, have also been shown to augment insulin resistance in experimental murine models (19, 20, 40). In more detail, it has been proposed that NK cells sense stressed cells in adipose tissue, respond with IFN-γ production, in turn causing macrophage polarization toward a pro-inflammatory phenotype, which subsequently leads to insulin resistance (19, 20, 40). However, little is known about human adipose tissue NK cells in general and also how they relate to obesity, insulin resistance, and liver disease. In this regard, we assessed visceral adipose tissue NK cell frequency and phenotype. Adipose tissue contained a similarly large population of NK cells as found in peripheral blood. However, adipose tissue was clearly enriched for $CD56^{bright}CD16^{-}$ NK cells expressing tissue residency markers. This profile with enrichment of $CD56^{bright}$ NK cells was similar to the phenotype of NK cells found in matched liver samples and also to NK cells in many other peripheral tissues in general (41). Interestingly, the specific subset of adipose tissue NK cells (or ILC1-like cells) that contribute to insulin resistance in mice express CD49a on their surface (21, 40). We here report that also human adipose tissue contains NK cells expressing CD49a. $CD49a^{+}$ NK cells had a $CD56^{bright}CD16^{-}$ phenotype, which is different from liver $CD49a^{+}$ NK cells (4), and were more prevalent in adipose tissue as compared to liver and peripheral blood. However, no link between the presence and levels of adipose tissue $CD49a^{+}$ NK cells and the presence of insulin resistance was noted in the investigated patients. Within the scope of this study, only CD49a and CD69 was studied as tissue residency markers. There are a number of additional surface markers as well as transcription factors that are linked to tissue residency, which should be investigated in future studies.

Our study design, with liver and adipose tissue biopsies acquired during laparoscopic surgery, enabled us to uniquely study NK cells from different peripheral tissues within the same individual. This analysis revealed interesting features, emphasizing the different phenotypes of tissue-resident cells from distinct tissues. We could show that NK cell differentiation status differed not only between liver and peripheral blood, in line with previous reports (41), but also between liver and adipose tissue-derived NK cells. The pattern of tissue residency marker expression was also distinct between liver and adipose tissue NK cells within the same individual. These data emphasize the importance of the specific local microenvironment in influencing the shape of the NK cell population. This also shows the importance of, although cumbersome, studying tissue NK cells in different human disorders.

Limitations of our study have also to be considered. First, the target cell line used in the functional experiments consist of the leukemia cell line K562. While being the gold standard for assessment of NK cells function, due to its lack of MHC class I, it is not representative for the NAFLD setting. However, it does express NKG2D-ligands (42), which primary hepatocytes derived from organ donors did not (**Supplementary Figure 1**). Ideally, future studies should assess NK cell responsiveness against primary target cells derived from livers of NAFLD patients, such as hepatic stellate cells or hepatocytes. Second, in addition to the target cells derived from NASH-livers, future work should focus on the function of tissue-derived NK cells since this study, due to practical reasons, only assessed the function of circulating NK cells. Third, within the scope of this study, NKG2D expression was only studied on NK cells derived from peripheral blood. This might not be representative of NKG2D expression on NK cells derived from liver and adipose tissue. Fourth, this study does not address the NKG2D-ligand expression in NASH livers which, in combination with the NKG2D expression on hepatic NK cells, could provide interesting insights into the disease mechanisms of NASH. Thus, the exact role of NKG2D in NAFLD still remains to be elucidated.

In summary, we here performed a comprehensive assessment of peripheral blood and tissue NK cells in relation to NAFLD, the most common chronic liver disease worldwide. Surprisingly, despite a substantial literature from experimental model systems suggesting a role for NK cells in NASH, we found a largely intact NK cell compartment in the human setting. Instead, our study reveals significant differences in composition of the NK cell compartment between human peripheral tissues and, thus, illustrates the importance of understanding the local microenvironment in shaping the NK cell repertoire.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Regional Ethics Committee of Stockholm, Stockholm, Sweden. All subjects gave oral and written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regional Ethical Review Board in Stockholm, Sweden.

AUTHOR CONTRIBUTIONS

NS designed the study, performed experiments, acquired and analyzed data, and drafted the manuscript. NB designed the study, performed data analysis, drafted the manuscript, and supervised the work. KS, MC, and CZ performed experiments and data analysis. JF and GM contributed to the data analysis, the discussion, and supervision. HH recruited patients and was instrumental in the histology assessments. PS and HH contributed with in-depth knowledge of NAFLD. AT, EN, SK, and HN recruited and sampled patients. All authors provided valuable contributions and insights into the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01255/full#supplementary-material

Supplementary Figure 1 | Expression of NKG2D ligands on primary human hepatocytes. Representative histogram of flow cytometry stainings of NKG2D-ligands as well as HLA-ABC and CD155, ligand of DNAM-1, on primary hepatocytes. One representative staining out of three.

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