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THE ROLE OF LIVER MYELOID CELLS IN OBESITY AND METABOLIC DISEASE

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The Role of Liver Myeloid Cells in Obesity and Metabolic Disease
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By

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Popular science summary of the thesis

Obesity is a high risk factor for fatty liver disease, a condition caused by the accumulation of lipids in the liver due to the inability of the adipose tissue to store excess lipids. Despite initially being considered a benign condition, the accumulation of lipids in the liver can lead to injury and dysfunction, as observed in people that consume large amounts of alcohol for long periods of time. This liver injury, called non-alcoholic steatohepatitis (NASH), often occurs in obese individuals even if they do not drink excessive amount of alcohol. According to the public health agency of Sweden, 50% of the adult Swedish population are overweight or obese. Thereby, the prevalence of NASH is not only increasing worldwide but also in Sweden. NASH can develop into scarring in the liver, so called cirrhosis, and cancer. Today there are no approved medications to treat fatty liver disease or NASH, and the diagnostic methods are insufficient, despite NASH being predicted as the most common cause of liver transplantation within the next 20 years.

Macrophages are innate immune cells with the ability to sense and respond to injuries in the body by producing inflammatory mediators that help eradicate the injury and restore the normal function of the tissue. Macrophages have previously been shown to play an important role for the regulation of liver function in patients with obesity. The aim of this research was therefore to understand the role of macrophages in fatty liver disease before it develops into NASH. In the first study, we identified a distinct population of liver macrophages that expands and acquire pathogenic functions in obesity and were found to contribute to metabolic stress in the liver. In the second study we identified a population of liver macrophages with a low turn-over and the ability to detoxify the liver from harmful radicals produced by liver cells during fatty liver disease. In summary, these studies suggest that macrophages are a versatile cell population that can acquire both beneficial and detrimental roles in the development of liver injury associated with obesity. Targeting specific macrophage populations in the liver could therefore represent a promising therapeutic strategy for liver and metabolic disease.

Abstract

Macrophages are tissue resident cells with diverse phenotypes and functions depending on their origin, localization and physiological context. In the liver, their main functions are to detoxify the blood from pathogens, debris and metabolic waste arriving to the liver through the systemic and portal circulation. However, liver macrophages have been shown to change in their composition, phenotype and function during obesity-associated metabolic disease such as non-alcoholic fatty liver disease (NAFLD). NAFLD is a heterogeneous disease and represents a variety of liver conditions ranging from liver steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and cancer. NAFLD has become the major cause of chronic liver disease, since it is associated with an increasing prevalence of obesity and type 2 diabetes. Macrophages contribute to the development of these metabolic disorders in obesity. However, while multiple populations of macrophages have been described in the liver, little is known about their functional role in response to metabolic disease associated with obesity. Herein, we characterized liver macrophages during the development of metabolic disease in mice and humans with obesity. In the first study we used single cell transcriptomics and metabolomics and identified two distinct populations of embryonic-derived tissue resident Kupffer cells (KC1 and KC2) at steady state. While KC1 ($CD206^{low}ESAM^-$) represented the largest population of Kupffer cells, KC2 ($CD206^{high}ESAM^+$) cells were characterized by their expression of genes associated with lipid metabolism. Functional characterization of KC2 by either depletion of KC2 cells in the liver or silencing of the fatty acid transporter CD36 in a murine model of obesity and liver steatosis demonstrated that these cells contribute to liver oxidative stress associated with obesity. In the second study we characterized liver samples from lean and obese humans by single cell RNA sequencing and flow cytometry and identified a distinct population of tissue resident myeloid cells, denoted LM2, that expressed high levels of genes regulating oxidative and metabolic stress in obesity, in particular the antioxidant Peroxiredoxin-2. Moreover, functional validation in human primary 2D and 3D *in vitro* cultures showed that the LM2 cells can reduce oxidative stress induced by lipid accumulation. In summary, these studies have improved our understanding of the composition, diversity and function of liver macrophages in homeostasis and metabolic disease associated with obesity.

List of scientific papers

I. Camille Bleriot, **Emelie Barreby**, Garett Dunsmore, Raphaelle Ballaire, Svetoslav Chakarov, Xenia Ficht, Giorgia De Simone, Francesco Andreata, Valeria Fumagalli, Wei Guo, Guochen Wan, Gregoire Gessain, Ahad Khalilnezhad, Xiao Meng Zhang, Nicholas Ang, Ping Chen, Cecilia Morgantini, Valerio Azzimato, Wan Ting Kong, Zhaoyuan Liu, Rhea Pai, Josephine Lum, Foo Shihui, Ivy Low, Connie Xu, Benoit Malleret, Muhammad Faris Mohd Kairi, Akhila Balachander, Olivier Cexus, Anis Larbi, Bernett Lee, Evan W. Newell, Lai Guan Ng, Wint Wint Phoo, Radoslaw M. Sobota, Ankur Sharma, Shanshan W. Howland, Jinmiao Chen, Marc Bajenoff, Laurent Yvan-Charvet, Nicolas Venteclef, Matteo Iannacone, Myriam Aouadi, and Florent Ginhoux. *A subset of Kupffer cells regulate metabolism through the expression of CD36*. Immunity, 2021 Sep 14;54(9):2101-2116.e6.

II. **Emelie Barreby**, Benedikt Strunz, Sebastian Nock, Léa Naudet, Joanne X Shen, Helene Johansson, Isabella Sönnerborg, Junjie Ma, Egon Urgard, Laura J Pallett, Yizhou Hu, Achilleas Fardellas, Valerio Azzimato, Ana Vankova, Laura Levi, Cecilia Morgantini, Mala K Maini, Per Stål, Stephan P Rosshart, Jonathan M Coquet, Greg Nowak, Erik Näslund, Volker M Lauschke, Ewa Ellis, Niklas K Björkström, Ping Chen and Myriam Aouadi. *Human resident liver myeloid cells protect against metabolic stress in obesity*. Nature Metabolism, 2023 Jul;5(7):1188-1203.

Contents

List of abbreviations

TLRs	Toll-like receptors
NASH	Non-alcoholic steatohepatitis
NAFLD	Non-alcoholic fatty liver disease
MAFLD	Metabolic dysfunction-associated fatty liver disease
VLDLs	Very low-density lipoproteins
TNF	Tumour necrosis factor
IL-1β	Interleukin-1 beta
CSF1	Macrophage colony-stimulating factor
IL-34	Interleukin 34
DLL4	Delta like canonical Notch ligand 4
TGFβ	Transforming growth factor beta
BMP	Bone morphogenic proteins
CEBPs	CCAAT-enhancer binding protein
AP1	Activator protein 1
LXRα	Liver X receptor α
ID3	Inhibitor of DNA binding 3
UMI	Unique molecular identifier
FACS	Fluorescence activated cell sorting
DTR	Diphtheria toxin receptor
GeRPs	Glucan-encapsulated siRNA particles
siRNA	Small interfering RNA
KC	Kupffer cell
LDL	Low-density lipoprotein
ROS	Reactive oxygen species
LM cells	Liver myeloid cells
PRDX2	Peroxiredoxin-2

Introduction

Macrophages are innate immune cells known for their ability to engulf large particles by phagocytosis. This key function allows them to "clean" their surrounding environment of cellular material and other waste products and are thus important for the regulation of tissue homeostasis and tissue repair. Macrophages are therefore present in essentially all organs of the body, throughout life (1). As resident cells in the tissue, they are highly plastic and react to signals from their local and systemic environment to adapt their function, morphology and phenotype (2). In this way, tissue resident macrophages gain specialized functions within the tissue in which they reside and are therefore essential for the homeostasis of the organ (3). In addition to these tissue-specific functions, macrophages share core immunoregulatory functions important for the defense against infection (2). Macrophages often serve as the first line of response against invading pathogens in a tissue and upon activation (e.g., by LPS or in response to interferons) they respond by producing a range of factors including cytokines, chemokines and growth factors (2). At the same time, macrophages also have the ability to acquire a tolerogenic phenotype by down-regulating receptors that recognizes pathogens (e.g., toll-like receptors; TLRs) (4). In this way macrophages are able to phagocytose large numbers of particles (e.g., apoptotic cells) without becoming activated and inducing a pro-inflammatory response in the healthy state. Furthermore, macrophages can both induce and prevent tissue inflammation and have therefore been shown to play an important role for the development and progression of multiple diseases, including metabolic disease and obesity-associated non-alcoholic fatty liver disease (NAFLD).

1 Literature review

1.1 Obesity

The worldwide prevalence of obesity has increased at an exponential rate during the past decades. Obesity does not only impact the overall health of an individual, but often leads to the development of metabolic diseases including type 2 diabetes and fatty liver disease, cardiovascular disease and cancer (5-7). In addition to these co-morbidities, individuals with obesity are also highly affected by the social stigma of obesity. This includes discriminative behavior and prejudice against individuals with overweight or obesity. This is particularly detrimental in healthcare settings where weight-based bias has been associated to increased weight gain and worsened health in patients with obesity (8, 9). A common misconception is that body weight is under voluntary control and that obesity is easily reversible by eating less and exercising more. However, research suggests that the main driving factors for the development of obesity are highly complex and include genetic predisposition, societal factors and our environment, rather than being the result of voluntary decisions taken on an individual level (10).

Simplified, obesity is caused by the long-term imbalance between energy uptake and energy expenditure. After eating, excess calories that are not instantly needed by the body are stored in the form of fat (so called triglycerides) in the adipose tissue, that can later be used as energy in times of food scarcity. Historically, storage of energy in the form of fat has been necessary for our survival (10). However, this evolutionary trait is no longer protective as many western countries today face a constant surplus and availability of calorie-rich food in combination with a more sedentary lifestyle. This has therefore led to a dramatic increase in the prevalence of obesity during the past decades (11, 12). In Sweden for example, more than 50% of the adult population (16-84 years) was reported to suffer from overweight or obesity in 2022, and the prevalence is further expected to increase during the coming years (13).

Moreover, during a sustained positive energy balance, the excess calories are primarily taken up and accumulate in adipocytes in the adipose tissue. To cope with this, adipocytes undergo both hypertrophy (increase in size) and hyperplasia (increase in number) leading to adipose tissue expansion of primarily the subcutaneous adipose tissue but also the intraabdominal visceral adipose tissue in humans (14, 15). There is growing evidence suggesting that this capacity of the adipose tissue to expand is impaired in individuals with obesity (16). Indeed, when the subcutaneous adipose tissue has reached its expansion limit, lipids start to accumulate in visceral adipose tissue as well as in other non-adipose tissue organs including the liver. In humans, the onset of obesity is typically gradual, and therefore take years to develop. It is also currently believed

that once obesity has developed, due to the dysfunctional regulation of energy balance, the body will try to sustain a higher body weight than previously (10).

1.2 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) results from the excessive accumulation of lipids, mainly in the form of triglycerides, in the liver. NAFLD is therefore highly associated with obesity (~50% of patients with NAFLD have obesity) and metabolic conditions associated with obesity (~20% of patients have type 2 diabetes and 70% of patients have dyslipidaemia) (17). With the increasing prevalence of obesity, NAFLD has become the most common liver disease in westernized countries (18). Although, a minority of lean individuals (~10%-30% in Western and Eastern countries) also develop NAFLD (19). Due to the many metabolic features associated with NAFLD, it has recently been proposed to use the term 'metabolic dysfunction-associated fatty liver disease (MAFLD)', as it would better describe patients with NAFLD (20).

NAFLD represents a spectrum of liver conditions and can further advance into the more aggressive form non-alcoholic steatohepatitis (NASH), cirrhosis, liver failure and hepatocellular carcinoma (21-23). NASH is further characterised by steatosis, hepatocyte damage, inflammation, and varying degrees of fibrosis (24). Patients with NALFD and NASH can present a variety of combinations of these clinical manifestations making it a highly heterogenous disease.

Hepatic steatosis, the accumulation of triglycerides in hepatocytes, is considered the first stage of NAFLD. A healthy liver typically contains 1-5% of hepatic triglycerides, which can increase up to 20-30% in NAFLD. These lipids can arise from circulating free fatty acids that are released from the adipose tissue during obesity and metabolic disease, but also from liver *de novo* lipogenesis that refers to the synthesis of fatty acids from excess carbohydrates (glucose and amino acids) (25). Hepatocytes take up the fatty acids, which are either esterified into triglycerides and stored as lipid droplets, used as energy by mitochondrial β-oxidation or packed into very low-density lipoproteins (VLDLs) and released into the systemic circulation (26). During obesity, the excessive uptake and metabolism of fatty acids by hepatocytes can lead to the generation of toxic by-products including diacylglycerol and ceramides causing the development of lipotoxicity. Lipotoxicity leads to the gradual accumulation of hepatocyte injury by several mechanisms including oxidative stress, insulin resistance, inflammation, endoplasmic reticulum stress and cell death (27, 28). Hepatocyte injury is also associated with an enhanced wound response and the stimulation of fibrogenesis (24, 29). This occurs from the activation of hepatic stellate cells into myofibroblasts that start to produce extracellular matrix proteins faster than they are degraded, leading to fibrosis. Over time, the gradual progression of fibrosis (from periportal to bridging fibrosis between one portal triad and another) eventually makes the liver stiff and impairs normal function, which drives the progression of NASH into cirrhosis.

NAFLD pathogenesis is highly associated with insulin resistance (30). In the adipose tissue, insulin resistance prevents the suppression of lipolysis (the breakdown of stored triglycerides into free fatty acids and glycerol) leading to increased synthesis and release of free fatty acids into the circulation. In this way, insulin resistance is one of the major contributing factors leading to the accumulation of free fatty acids in the liver (31, 32). Insulin resistance in the liver has also been shown to contribute to the accumulation of triglycerides by enhancing *de novo* lipogenesis (30). This could therefore lead to an increased toxicity of these additional stresses to the liver during NAFLD.

The diagnosis of NAFLD and the identification of patients with NASH is currently done by imaging or assessment of liver biopsies (**Figure 1**). Individuals with at least 5% of hepatic steatosis, that consume little or no alcohol and lacks a secondary cause of steatosis are diagnosed with NAFLD (33). However, the disease scoring and staging system used to diagnose patients with NASH are highly subject to sampling error. Moreover, most individuals with NAFLD are asymptomatic and individuals can therefore have NAFLD for years and might even develop cirrhosis until diagnosed (24).
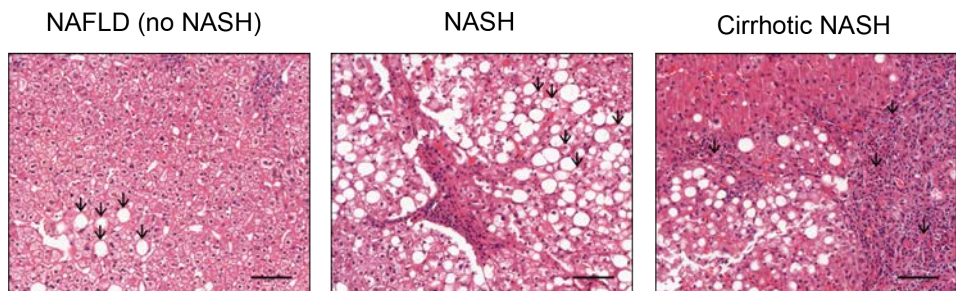
Figure 1. *Liver histology of human liver biopsies from patients with obesity and NAFLD diagnosis (scale bar, 100μm). Black arrows indicate hepatocytes with steatosis or lipid droplets (left), characteristic hepatocyte injury (so called hepatocyte ballooning or swelling; middle) and bands of fibrosis (right).*

The first line of treatment for NAFLD and NASH is lifestyle modifications, such as dietary changes and physical activity, and so far no approved pharmacological therapies exist (34). Due to the complex pathophysiology with the involvement of multiple organs and molecular pathways such as dietary factors, insulin resistance, adipose tissue dysfunction and altered gut microbiota, a vast number of studies are currently ongoing to evaluate different therapeutics targeting these various aspects of NAFLD pathogenesis (35, 36). However, the exact key drivers of the development of NAFLD and NASH are still not fully understood and more knowledge about the pathogenesis of these disorders are further needed to provide better diagnostics and treatment.

1.3 The liver

The liver has many functions and is not only a vital metabolic and detoxifying organ, but also harbors many important immunoregulatory functions for both host defense and immunological tolerance (37, 38). Hepatocytes represent the main cell population in the liver and make up the liver parenchyma together with cholangiocytes. The liver also consists of non-parenchymal cells including liver sinusoidal endothelial cells that lines the sinusoidal capillaries, hepatic stellate cells that locate at the space of Disse, the layer in-between the sinusoids and hepatocytes, and a large number of innate and adaptive immune cells (39). The liver is constantly exposed to the external environment and processes approximately 30% of all the blood in the body per minute, coming from both from the gastrointestinal tract (through the portal vein) and the hepatic artery (38). Therefore, it is exposed to a large number of antigens, microbial products, proteins and patrolling immune cells.

1.4 Liver macrophages at steady state

Macrophages represent the largest population of immune cells in the liver and are important gatekeepers for initiating or suppressing an immune response, as well as for maintenance of tissue integrity (40). Liver macrophages mainly reside within the liver sinusoids where they are in contact with the blood compartment, but also localize to intravascular and subcapsular (the space underneath the liver capsule) areas of the liver (41-43). By constantly sensing their environment through phagocytosis, liver macrophages are highly specialized in recognizing and clearing the blood from pathogens (e.g., by complement and pattern recognition receptors) and other toxic materials before it reaches the systemic circulation (44-48). In the absence of inflammation, liver macrophages preferentially induce immunological tolerance towards circulating and hepatocyte-derived antigens (39, 49, 50). In addition, liver macrophages are also important for the regulation of liver metabolism and are involved in both iron and cholesterol homeostasis (51-55). Due to these many specialized functions, liver macrophages have therefore been shown to play a central role during the development of multiple liver diseases (40).

1.4.1 Liver macrophage ontogeny

Liver macrophages consist of two major subsets, namely tissue resident macrophages (so called Kupffer cells) that are characterized by their tissue-specific functions, and recruited macrophages (so called monocyte-derived macrophages) (56, 57). Macrophages can further be distinguished by their developmental origin (ontogeny) and can either be embryonic or monocyte-derived (**Figure 2**). Fate-mapping studies in mice have demonstrated that the majority (~90%) of liver macrophages at steady state are Kupffer cells with an embryonic origin (58, 59). These are seeded from embryonic precursors from the yolk sac or fetal liver before birth and are maintained in adults by self-renewal through local proliferation (40, 60-62). Monocyte-derived

macrophages are derived from the bone marrow and arise from monocytes recruited from the systemic circulation. These are typically recruited to the liver in response to inflammation or after Kupffer cell depletion (57, 63). However, macrophage ontogeny is complex and recruited monocytes also have the ability to acquire a phenotype similar to that of embryonic-derived Kupffer cells with the ability to self-renew, referred to as monocyte-derived Kupffer cells (57, 64, 65).

Moreover, the differentiation of infiltrating macrophages into either monocyte-derived macrophages or monocyte-derived Kupffer cells is highly regulated by the local tissue environment. In the liver sinusoids, macrophages are in close contact with the sinusoidal endothelium and have been shown to partly extend into the space of Disse, where they make contact with hepatic stellate cells and hepatocytes (41). These non-parenchymal cells (referred to as the tissue niche) not only upregulate molecules for the recruitment and adhesion of infiltrating monocytes (e.g., CCL2 and VCAM1), but also secrete growth factors (e.g., CSF1 and IL-34) important for macrophage survival. In addition, they collectively generate signals (e.g., sterols, DLL4 and TGFβ) that induces the differentiation of infiltrating monocytes to macrophages (41, 66). These differentiation-signals have been shown to activate transcription factors in monocytes that are specific for inducing the macrophage identity (e.g., PU.1, CEBPs and AP1) as well as the tissue-resident Kupffer cell identity (e.g., LXRα, SPIC, ID3 and SMAD4) (41, 64, 66-68). In this way, signals from the liver niche influence the expression of transcriptional programs in macrophages that shapes their tissue resident phenotype in the liver, but can also induce other functional phenotypes (e.g., pro-inflammatory, anti-inflammatory or metabolism-related) depending on the physiological context (69).

In humans, little is known about the origin of liver macrophages due to the lack of tools to study their turnover, although a few studies have examined the turnover of liver macrophages in patients undergoing liver transplantation. One such study used immunohistochemistry to track the renewal of CD68^{+} liver macrophages in male patients that had been transplanted with donor livers from females (70). This identified a small proportion of liver macrophages that remained of donor-origin (XX[+]) for years after the transplantation, suggesting that this population of macrophages can self-renew independently of the recruitment of circulating monocytes. Although most liver macrophages (~80%) had been replaced by infiltrating (Y[+]) monocytes one year after the transplantation, further indicating that human monocytes also have the ability to differentiate into tissue-resident macrophages. This was further validated by a second study that differentiated resident and recruited macrophages by their different HLA-type and identified a small population of long-lived liver macrophages that remained of donor-origin years after the liver transplantation (71). Moreover, these observations are further corroborated by single cell RNA sequencing studies of human fetal tissues that have identified populations of tissue-resident macrophages at very early stages of development (72-76). In summary, these findings suggest that human liver macrophages likely consist of

both tissue-resident and recruited macrophages at steady state, that might be derived from both embryonic and bone marrow derived precursors.
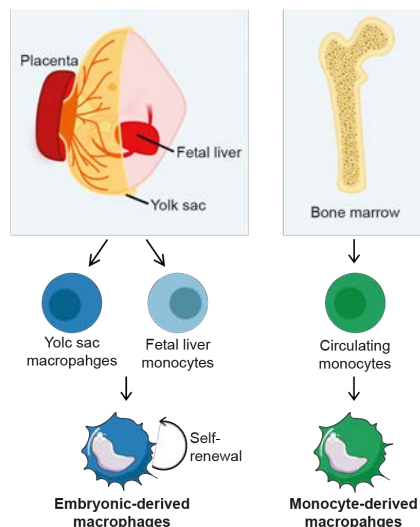

Figure 2. *Liver macrophages have distinct developmental origins. During embryonic development (embryogenesis), different waves of hematopoiesis in the yolk sac gives rise to both yolk sac macrophages and fetal liver monocytes. Both of which can differentiate into embryonic-derived tissue resident macrophages. At later timepoints, after the formation of the bone marrow, hematopoietic stem cells can also contribute to the macrophage pool by giving rise to peripheral monocytes in the circulation. These can infiltrate the liver and differentiate into monocyte-derived macrophages that acquires different phenotypes depending on the physiological context and signals from the tissue microenvironment.*

1.4.2 Liver macrophage dynamics

Recent advances in single cell and other advanced technologies have allowed for the high-resolution analysis of macrophage heterogeneity, plasticity and activation states at steady state and during disease pathogenesis. During steady state in mice, the majority of liver macrophages are represented by F4/80^{high} CD11b^{int} Kupffer cells defined by their expression of Clec4F, Tim4 and Vsig4. However, studies have also identified distinct populations of F4/80^{int} CD11b^{high} monocyte-derived macrophages (59, 68, 77-80) (**Figure 3**). Confocal imaging has for example identified a population of monocyte-derived macrophages residing in the hepatic liver capsule (so called capsular macrophages) that protect the liver against peritoneal infections (42). Moreover, single cell RNA sequencing has further revealed the presence of monocyte-derived Kupffer cells and monocyte-derived macrophages (59, 79, 81), where the latter has been shown to comprise both intrahepatic and capsular macrophages (43, 77, 79). Interestingly one study also identified the presence of so-called lipid-associated

macrophages at steady state (43), that have previously only been associated with the presence of metabolic disease (79, 82, 83).
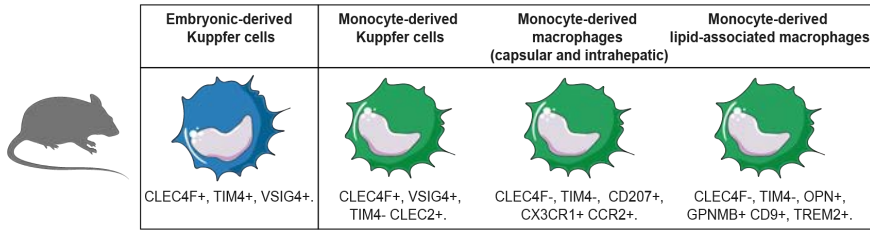

Embryonic-derived Kuppfer cells	Monocyte-derived Kuppfer cells	Monocyte-derived macrophages (capsular and intrahepatic)	Monocyte-derived lipid-associated macrophages
CLEC4F+, TIM4+, VSIG4+.	CLEC4F+, VSIG4+, TIM4- CLEC2+.	CLEC4F-, TIM4-, CD207+, CX3CR1+ CCR2+.	CLEC4F-, TIM4-, OPN+, GPNMB+ CD9+, TREM2+.

Figure 3. *Illustration of murine macrophage populations and markers defining each population in the steady state.*

Single cell RNA sequencing of whole human livers has revealed the presence of both tissue resident (*CD68+MARCO+*) and recruited macrophages (*CD68+MARCO-*) at steady state (**Figure 4**) (84, 85). The tissue resident macrophages were further characterized by a restorative phenotype, while the recruited macrophages were characterized by an inflammatory phenotype (57). These observations have further been confirmed by two other studies that revealed the presence of two phenotypically distinct liver macrophages with a similar profile as to the resident and recruited liver macrophages (86, 87). In addition, another study performing single cell RNA sequencing of human livers at steady state and in the context of liver cirrhosis further characterized subpopulations of the tissue resident and recruited macrophages, where tissue resident macrophages were separated into two populations (Kuppfer cell 1–2) and the recruited macrophages were divided into three distinct populations (Tissue monocytes 1–3) (88). This study further identified two small populations of *TREM2+CD9+* recruited macrophages (Scar–associated macrophage 1–2) that increased in proportion during cirrhosis, with a similar profile as lipid–associated macrophages in mice. This finding was further validated by another study that identified similar populations of cells (Mature lipid–associated macrophages and Immature lipid–associated macrophages) in human livers at steady state (43). Collectively, these identified populations of human tissue resident macrophages have been found to express markers shared with murine embryonic–derived Kuppfer cells (57, 84), while the populations of human recruited macrophages share genes with murine monocyte–derived macrophages. However, it is important to note that transcriptional analysis alone is not reliable to distinguish the ontogeny of human liver macrophage populations, as the local microenvironment can induce the expression of tissue–resident genes upon entry to the tissue.
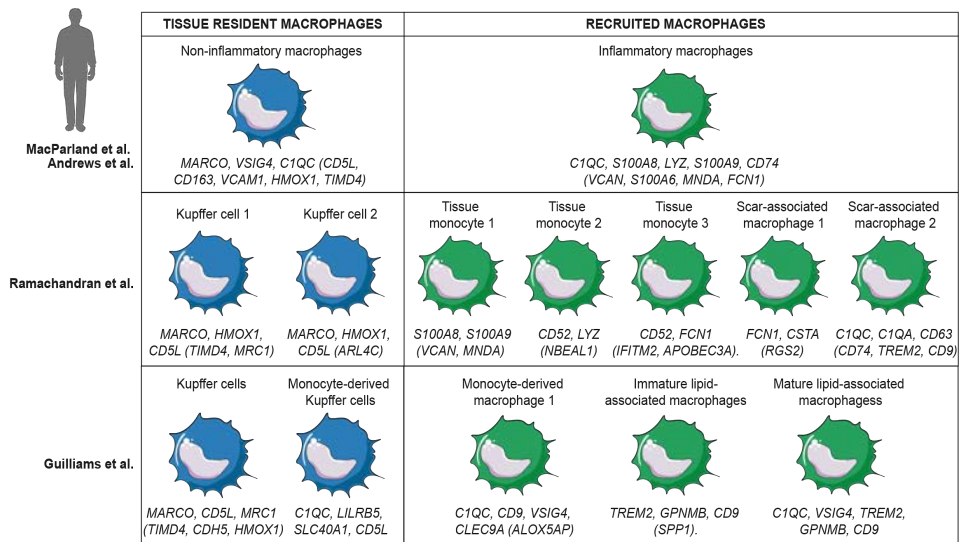
TISSUE RESIDENT MACROPHAGES		RECRUITED MACROPHAGES				
Non-inflammatory macrophages		**Inflammatory macrophages**				

MacParland et al. Andrews et al.

Non-inflammatory macrophages: *MARCO, VSIG4, C1QC (CD5L, CD163, VCAM1, HMOX1, TIMD4)*

Inflammatory macrophages: *C1QC, S100A8, LYZ, S100A9, CD74 (VCAN, S100A6, MNDA, FCN1)*

Ramachandran et al.

Kupffer cell 1	Kupffer cell 2	Tissue monocyte 1	Tissue monocyte 2	Tissue monocyte 3	Scar-associated macrophage 1	Scar-associated macrophage 2
MARCO, HMOX1, CD5L (TIMD4, MRC1)	*MARCO, HMOX1, CD5L (ARL4C)*	*S100A8, S100A9 (VCAN, MNDA)*	*CD52, LYZ (NBEAL1)*	*CD52, FCN1 (IFITM2, APOBEC3A).*	*FCN1, CSTA (RGS2)*	*C1QC, C1QA, CD63 (CD74, TREM2, CD9)*

Guilliams et al.

Kupffer cells	Monocyte-derived Kupffer cells	Monocyte-derived macrophage 1	Immature lipid-associated macrophages	Mature lipid-associated macrophagess
MARCO, CD5L, MRC1 (TIMD4, CDH5, HMOX1)	*C1QC, LILRB5, SLC40A1, CD5L*	*C1QC, CD9, VSIG4, CLEC9A (ALOX5AP)*	*TREM2, GPNMB, CD9 (SPP1).*	*C1QC, VSIG4, TREM2, GPNMB, CD9*

Figure 4. *Illustration of human macrophage populations present at steady state and differentially expressed markers genes as defined by single cell RNA sequencing studies. Parenthesis indicates genes that are specifically expressed by each population.*

1.4.3 Liver macrophage zonation

A remarkable feature of the liver is metabolic zonation, which refers to its spatial organization of key metabolic functions to distinct areas within the liver lobules (**Figure 5**) (89). Liver macrophages have previously been observed to reside more densely in periportal areas where they appear to be larger in size and have greater phagocytic activity than macrophages residing in midzonal and centrilobular regions in murine animal models (90–93). These findings have been further supported by a more recent study from 2021 where quantitative multiplex imaging was used to show that F4/80$^+$ macrophages concentrate around periportal regions in mouse livers to protect the liver against gut-derived pathogens (94). Macrophage zonation was demonstrated to be dependent on the sustained sensing of gut microbial products by liver sinusoidal endothelial cells, that in turn regulates the positioning of macrophages to the periportal regions by influencing chemoattractant gradients across the liver lobule. In addition, macrophages have also been found to reside in the liver capsule in both mice and humans (42, 43). These monocyte-derived macrophages, called liver capsular macrophages, have been suggested to protect the liver against pathogens derived from the peritoneum (42). In humans, studies using immunohistochemistry or spatial transcriptomics in combination with single-cell or single-nuclei RNA sequencing on healthy donor livers have observed increased expression of markers of tissue resident macrophages at periportal regions, whereas markers of recently recruited macrophages were expressed at higher levels closer to central veins (84, 85). Although a report from 2022 using

multiplex in situ analysis combining the detection of markers of individual macrophage subpopulations suggested that while tissue resident macrophages mainly accumulate in midzonal and centrilobular regions in human livers at the steady state, recruited macrophages distinctly localized to either the liver capsule, or surrounding the central and portal vein, or in close proximity to bile ducts (43).
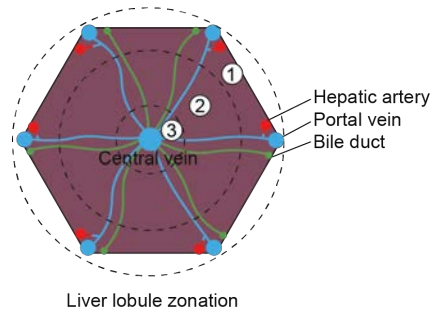

Liver lobule zonation

Figure 5. *Illustration of the liver lobule. The liver is composed of hexagonal–shaped units called liver lobules that are divided into three distinct liver zones: periportal (1), midzonal (2) and centrilobular (3) areas. Blood enters the liver lobule through the portal vein and hepatic artery and flows through the liver sinusoids to drain into the central vein. Conversely, bile flows from centrilobular areas and drains into bile ducts at periportal areas. Illustration is adapted from Barreby et al, Immunology 2022 (95).*

1.5   Liver macrophages in obesity and NAFLD pathogenesis

Macrophages have been shown to play an important role for the early development of NAFLD. Liver macrophages were first suggested to be important during the development of metabolic disease from studies in mice and rats, where depletion of liver macrophages was observed to prevent the development of steatosis and insulin resistance associated with obesity (96, 97). By responding to factors generated during obesity, such as gut–derived endotoxins, cholesterol and fatty acids, liver macrophages have been shown to induce the production of inflammatory cytokines (e.g., TNF and IL–1β). This further leads to a state of low–grade inflammation, that contributes to the development and progression of metabolic disease including NAFLD (96, 98–100). Conversely, macrophages have also shown to be protective during the pathogenesis of NAFLD and insulin resistance, by for example inducing apoptosis of pro–inflammatory macrophages to prevent their pathological effects during NAFLD (101–103). Meanwhile, during advanced stages of NAFLD and NASH, the activation of this anti–inflammatory and reparative phenotype in macrophages has instead been found to promote the development of fibrosis (104). However, the methods used in these studies have not been specific enough to only target tissue resident Kupffer cells. Furthermore, they also do not differentiate between different types of liver macrophages (e.g., tissue resident compared to

monocyte-derived macrophages), which could further explain why macrophages have been described as both beneficial and detrimental during the pathogenesis of obesity and NAFLD.

RNA sequencing of tissue resident Kupffer cells and monocyte-derived macrophages in mice has suggested that recruited macrophages, that infiltrate the liver during NAFLD and NASH, acquire a more distinct pro-inflammatory phenotype compared to tissue resident liver macrophages (81, 105). Recruited monocyte-derived macrophages have therefore been suggested to drive the development of inflammation in the liver, resulting in systemic insulin resistance (106). Nevertheless, these studies have not been performed at a single cell resolution and therefore does not fully reflect the diversity of liver macrophage populations and their specific contribution to metabolic disease associated with obesity.

1.5.1   Liver macrophage dynamics during NAFLD and NASH

Recent single cell RNA sequencing studies have investigated the distinct phenotypes of macrophages during the development of liver disease. The majority of such studies have been done using various mouse models of diet-induced NASH and have shown that the composition of liver macrophages dramatically changes during metabolic disease (59, 78-81, 83, 107). While embryonic-derived Kupffer cells have been shown to decrease in proportion due to impaired self-renewal and loss of Kupffer cell identity (59, 81, 108), these studies have confirmed the finding that populations of inflammatory monocyte-derived macrophages increase in proportion in the liver during NASH. In the context of NASH, recruited monocytes undergo different differentiation programs depending on signals derived from their local tissue environment in response to the NASH diet (59). A large proportion of monocytes develop into lipid-associated macrophages due to the altered regulation of the transcription factors LXR and ATF3 (59, 79, 83). This is associated with an increased expression of the genes *Cd9* and *Trem2*, along with an upregulation of genes associated with lipid metabolism (59). Interestingly, these lipid-associated macrophages have been found to be more mature and less inflammatory in livers with NASH compared to those from healthy livers, suggesting that this phenotype could be protective during the development of NASH (43, 109).

      Moreover, recruited monocytes can also develop into monocyte-derived Kupffer cells to replace the loss of embryonic-derived Kupffer cells in the liver during NASH (59, 79, 81). These monocyte-derived Kupffer cells appear to be less mature with reduced expression of Kupffer cell signature genes (e.g., *CD163* and *C6*) and have an altered metabolic phenotype compared to embryonic-derived Kupffer cells (59, 81). These monocyte-derived Kupffer have therefore been associated with increased liver damage (81). In summary, this indicates that macrophages with different origin could be functionally different during the development of liver disease. In addition, as these changes in macrophage composition have not been observed during the early development

of obesity and NAFLD in mice, this further suggests that infiltration of monocyte-derived macrophages into the liver is restricted to severe stages of NAFLD and NASH, as has been observed in patients with chronic liver disease (81, 110).

In mouse models of NASH, monocyte-derived lipid-associated macrophages have further been found to distinctly accumulate in periportal areas of the liver associated with liver inflammation and fibrosis (43, 59, 79, 83). In these fibrotic areas, lipid-associated macrophages have been proposed to aggregate into crown-like structures during NASH (83), or into so called syncytia's (large clusters of macrophages) during liver cirrhosis (108). In both conditions, the accumulation of monocyte-derived macrophages in the fibrotic niche have been suggested to be protective by either restricting liver fibrosis in the context of NASH, or by helping to compensate for the loss of integrity of liver sinusoidal vessels in response to cirrhosis and thereby protecting the liver from the increased number of blood-derived pathogens.

In humans, the proportion of scar-associated macrophages has also been shown to significantly increase in the liver during cirrhosis (88). Moreover, these scar-associated macrophages were found to accumulate in periportal areas associated with both fibrosis and cirrhosis, where they exhibited a pro-fibrotic phenotype expressing genes such as *SPP1*, *CCL2*, *LGALS3*, *PDGFB* and *VEGFA*. Comparable findings have been observed in patients with severe NASH and advanced fibrosis, where a study from 2023 used quantitative multiplex immunostaining and RNA sequencing to show that recruited ($IBA1^+CD16^{low}CD163^{low}$) macrophages accumulate in fibrotic and inflammatory areas of the liver, corresponding to periportal areas (110). The same population of recruited macrophages was observed to significantly decrease in proportion in non-fibrotic areas in the livers from patients with NASH and advanced fibrosis. Moreover, while monocyte-derived macrophages appeared to gradually accumulate in the liver during the progression of NAFLD, this accumulation was predominantly observed at severe stages of NASH and fibrosis (110). Interestingly, another study found that at earlier stages of NAFLD, lipid-associated macrophages were found to change their localization from the periportal areas (near bile ducts) at the steady state, to centrilobular areas of the liver that were associated with steatosis in a small cohort of patients with steatosis in more than 10% of hepatocytes (43). Finally, this suggests that during the progression of NAFLD, monocyte-derived macrophages are recruited to specific areas of the liver that are affected by the disease (e.g., areas of local inflammation and fibrosis).

2 Research aims

The overall aim of the work presented in this thesis was to explore the heterogeneity of liver macrophages during obesity-associated metabolic disease.

The specific aims addressed in the two papers were as follow:

- Reveal the subpopulations of macrophages in the liver
- Identify distinct phenotypes and functions of liver macrophage subpopulations
- Define the role of liver macrophage subpopulations during the development of obesity and metabolic disease

3 Methodological considerations

To address these research questions, we characterised the phenotype, turnover and function of liver macrophage subpopulations using various *ex vivo*, *in vivo* and *in vitro* methodologies discussed in this section.

3.1 Ethical considerations

3.1.1 Studies involving animals
The work performed in the two studies included in this thesis (**Paper I and II**) involves the use of animal models, and in particular mice. Animal models are necessary to understand the complex interplay between the immune system and systemic metabolism. In the work included in this thesis, our aim has been to study as many aspects of human disease as possible using *in vitro* cell based systems. However, there are currently no available *in vitro* system that can adequately recapitulate the complex integrative biology that exists in whole organisms. We have therefore used mice, since they are the most characterized model system when it comes to metabolism amongst vertebrates. Vertebrates are more developed compared to non-vertebrates such as fish and flies and therefore share many metabolic functions with humans. In addition, since mice can be genetically modified this allows us to perform more precise and refined experiments, which also limits the number of animals that needs to be used.

We have continuously strived to ensure the welfare of the animals, with special attention to the 3R principle. Our aim has therefore been to minimize the number of mice used for experiments by studying multiple metabolic parameters from different tissues and samples from the same animal. We have also performed all preliminary studies using *in vitro* cell-based systems so that mice are only used to test our strongest genetic targets for treating and investigating the development of metabolic disease, to avoid using more mice than necessary.

Moreover, to ensure adequate power of experiment we have whenever possible selected well-established models using inbred strains that have been acclimatized to the same environment. The health state of all animals has further been routinely monitored on a daily basis by experienced personnel. Finally, all studies involving mice have been carefully planned and designed in conjunction with the veterinary expert of the animal facility to minimize suffering of the animals during each procedure. All studies have been granted ethical approvals by the relevant ethical authority.

3.1.2 Studies involving human individuals
The second part of the thesis (**Paper II**) involves confirming the relevance of research findings from *in vitro* and *in vivo* studies in mice, with studies in humans. All the work involving human individuals has been carefully designed in regard to

ethical considerations and conducted with the outermost respect for the study participants, by weighing the benefits of the study against the burden of the research, the social value and the rights and interests of research participants. All study participants have provided oral and written informed consent, after receiving oral as well as written information about the study. Moreover, all participants were informed of their right to withdraw their participation from the study at any time. Finally, no vulnerable groups were included in the study and all participants were adults. Additionally, no compensation or any other form of payment was given to participants, to ensure that samples were donated out of free will.

All liver samples included in **Paper II** have been collected from patients already undergoing surgery. These include patients undergoing bariatric surgery (Roux-en-Y gastric bypass surgery), and patients scheduled for liver surgery or transplantation based on various clinical indications. For the collection of samples from patients undergoing gastric bypass surgery, clinical samples were collected during the surgery to minimize the risks associated with percutaneous biopsies. The procedure was also performed in the beginning of the surgery and after the gastric bypass had been completed the surgeon was carefully checking for any bleeding at the site of the biopsy. In addition, patient discomfort associated with the biopsy sampling was minimized since they were under general anesthesia. Patients with any known coagulation disorder was excluded from the study. For samples collected from individuals undergoing liver resection surgery, samples were collected from liver tissue that had already been surgically removed from the patients. After the resection, tissue that were not used for clinical assessment was collected for research and was therefore not considered to be associated with any risk for the patient. For individuals undergoing liver transplantation, liver biopsies were collected as per clinical routine for the pre- and post-transplantation clinical assessment and are therefore not considered as an additional risk for the patient. Post-transplantation biopsies were collected percutaneously with ultrasound guidance.

All data collected from the samples were confidential and stored coded and anonymously. All the studies involving human subjects were planned in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki) and have been granted ethical approvals by the Regional Ethical Review Board in Sweden.

3.2 Gene Expression Analysis

3.2.1 Bulk RNA sequencing
Bulk RNA sequencing is a powerful method to measure the average gene expression in a sample. In **Paper I** we performed bulk RNA sequencing to

compare the phenotype of the two identified Kupffer cell populations and to identify distinct transcriptomic signatures. With bulk RNA sequencing, mRNA is isolated from FACS-sorted cell populations and converted into cDNA libraries that are sequenced. While bulk RNA-sequencing does not provide information at the single cell level, it is a highly sensitive method that enables the detection of a large number of expressed genes and in particular genes with low expression.

In **Paper I** we also performed RiboTag sequencing (111) to be able to identify genes that are actively transcribed in the different cell populations. In this way, RiboTag sequencing provides a snapshot of the cellular transcriptional activities at a given moment, in comparison to bulk RNA-sequencing that detects the steady state levels of gene expression in a sample. Since macrophages are highly phagocytic cells, we further used this technique to exclude transcripts that could have been derived from the phagocytosis of other cells. In summary, so called RiboTag mice was used that express the ribosomal protein L22 (RPL22) together with an HA tag. Floxed RiboTag mice were crossed with Lyz2-Cre mice allowing for conditional tamoxifen-induced RPL22HA protein expression in Lyz2-expressing myeloid cells. The RPL22HA protein is incorporated into ribosomes, and ribosome-associated mRNA transcripts can then be isolated from FACS-sorted cell population using immunoprecipitation of the HA-tagged RPL22 protein. The isolated transcripts were then sequenced using RNA-sequencing, allowing for the characterisation of the two Kupffer cell populations by their actively transcribed gene expression signature (so called translatome).

3.2.2   Single cell RNA sequencing

Recent advances in single cell profiling have made single cell RNA sequencing a powerful tool to study the gene expression of individual cells. Single cell RNA sequencing therefore allows for the study of the heterogeneity of cell populations within a tissue and the identification of rare cell populations, which is crucial for understanding their contribution to the pathogenesis of several diseases. Although it is important to keep in mind that gene expression does not always result in protein expression, and it is therefore important to validate the identity and function of newly identified subpopulations using additional methods. Moreover, as most algorithms used to cluster cell populations requires the active decision of the number of clusters to infer on the data, it is important to use caution to avoid generating clusters based on biological variation or technical noise.

A variety of single cell RNA sequencing methods are currently available and differs in how RNA of single cells is isolated and converted into cDNA libraries for sequencing. In **Paper I** we have used both 10X Chromium and SMART-seq2 methods for single cell RNA sequencing. In **Paper II** we used a version of the SMART-seq2 method adapted for liver cells (112, 113). With single cell RNA sequencing using 10X Chromium, individual cells are captured into droplets (~1

nL) containing a unique barcode (unique molecular identifier; UMI) allowing for the high-throughput processing of thousands of cells. Moreover, SMART-seq2 is a highly sensitive technique where single cells are FACS sorted into microtiter plates containing lysis buffer. With SMART-seq2, the scale is limited to the number of plates that can be processed at the same time and this technique therefore have a lower throughput compared to the droplet-based 10X Chromium technique. However, SMART-seq2 has a full-transcript coverage and a high sensitivity that allows for the detection of a large number of genes and can be used to both detect genes with low expression or to study splice variants (114).

All murine liver cells included in **Paper I and II** have been sequenced freshly isolated, while human tissues have been collected and cryopreserved prior to sequencing. As human tissues were collected at different time points, samples were first cryopreserved and then typically sorted in groups of 3 samples per time point to avoid introducing bias to the index-sorting FACS data by differences generated by the FACS machine over time. Moreover, single cell transcriptomes have previously shown to be well conserved in cryopreserved tissue cells with minimal effects on the expression profiles compared to freshly prepared cells (87, 115). In addition, prior to library preparation (independent of the method used), viable cells were first enriched by FACS sorting with minimal mechanical force on the samples (e.g., low speed and large nozzle size), to remove debris and damaged or dead cells and/or to enrich for target cell populations.

3.3 Flow cytometric analysis

Flow cytometry is a powerful method that allows for the immunophenotyping of large number of cells based on their protein expression. In principle, single cells are stained with fluorescently labelled antibodies against surface or intracellular markers and fluorescence is measured at the single cell level by the flow cytometer. In both studies flow cytometry was primarily used for measuring the frequencies of immune cells including subpopulations of macrophages (**Paper I and II**) and evaluating the expression levels of cell surface and intracellular proteins (**Paper I**).

3.4 Mice models and functional studies in mice

3.4.1 Monocyte fate-mapping mouse model

To study the origin of liver macrophage populations we used the previously generated Ms4a3Cre x RosaTomato mouse model that efficiently and precisely fate maps murine monocyte-derived macrophages by tamoxifen-inducible heritable expression of Tomato fluorescent protein in Ms4a3-expressing monocytic progenitors (so called granulocyte-monocyte progenitors) (58).

3.4.2 Diet-induced obesity mouse model

To study the phenotype and functional role of macrophage populations in the context of obesity we used a mice model of diet-induced obesity where C57BL/6 wild type mice are fed a high fat diet (containing 60% fat) to induce obesity and metabolic disease. The C57BL/6 mouse strain is commonly used for the study of obesity since they are highly susceptible to high fat diet and develop severe obesity (116). Although, this strain does not consistently develop severe liver fibrosis and advanced NAFLD, they have a highly similar disease progression to human liver steatosis. On a high fat diet they develop obesity (117), liver steatosis, moderate insulin resistance and low-grade liver inflammation, that are all common metabolic and histological features of human NAFLD (116).

3.4.3 Wildling mice model

To study the effect of the environment on the composition of liver macrophage population in **Paper I** we used the wildling mice model. These wildling mice have been generated by transferring embryos from C57BL/6 laboratory mice into wild mice, resulting in a mice model that highly resembles wild mice (118). The wildling mice are therefore laboratory mice that have a natural microbiota consisting of a diverse range of microbes and have also been exposed to pathogen since birth. In this way, wildling mice are more similar to humans with coevolved microbes and pathogens compared to the typical specific-pathogen free laboratory mice.

3.4.4 KC2 depletion in mice

To specifically deplete KC2 but not KC1, we generated a mouse model of bone marrow chimeras in which KC2 cells expressed the human diphtheria toxin receptor (DTR), allowing for the depletion of KC2 by diphtheria toxin treatment. First, we crossed floxed RosaDTR mice with Cdh5-CreERT2 mice (119) allowing for conditional tamoxifen-induced DTR expression in KC2, as these cells express Cdh5 but not KC1. Secondly, to avoid affecting liver sinusoidal endothelial cells that also express Cdh5 we generated bone marrow chimeras where wild type mice (CD45.1) were depleted of liver macrophages by high-dose irradiation and then reconstituted with bone marrow from the Cdh5-CreERT2 x RosaDTR mice. This leads to the repopulation of liver macrophages by bone-marrow derived monocyte-derived Kupffer cells (64, 65, 120). 6 weeks after bone marrow reconstitution, mice were treated with 2 doses of tamoxifen followed by treatment with diphtheria toxin resulting in KC2-specific DTR-expression and depletion.

3.4.5 *Cd36* knock down in mice using the GeRP technology

To validate the transcriptomic data obtained in **Paper I** and to study the role of the macrophage subpopulation KC2 in the regulation of metabolism during obesity, we took advantage of a unique technique developed in our lab (121, 122). This technology, called glucan-encapsulated siRNA particles (GeRPs) enables gene manipulation in a cell and tissue specific manner. The technique is based

on glucan shells derived from baker's yeast, and by the use of an acidic buffer these can be loaded with siRNA. When injected intravenously in mice, the GeRPs can be administered to the liver and specifically taken up by liver macrophages through receptor recognition and phagocytosis. In **Paper I**, we used GeRPs to deliver silencing RNA (siRNA) to silence the expression of *Cd36* specifically in liver macrophages. The use of siRNA in combination with our delivery system *in vivo* thus represents a powerful tool that allows the silencing of genes specifically in liver macrophages at a given time point.

3.4.6   3D Liver cultures

NAFLD can be mimicked *in vitro* using liver spheroids (or organoids) (123, 124). These 3D cultures consist of a mix of human hepatocytes and non–parenchymal liver cells, where the primary human liver cells have been shown to maintain their proteomic and metabolic characteristics for several weeks in culture (125). To mimic the condition of obesity and NAFLD, the liver spheroids are treated with high concentrations of fatty acids, insulin and glucose (steatogenic media) that induce lipid accumulation and oxidative stress and reduce insulin sensitivity in the liver cultures (**Figure 6**). In this way, this human *ex vivo* model resembles the *in vivo* pathogenesis of human obesity and NAFLD.
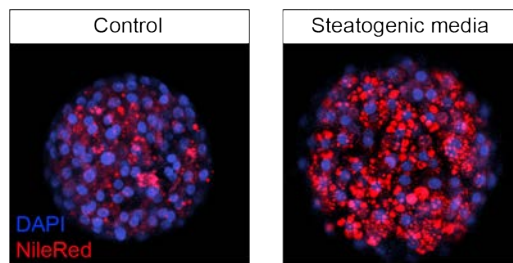

***Figure 6**. Immunofluorescence images of 3D liver cultures stained for lipids using NileRed.*

4 Results and Discussion

The work from the two papers included in this thesis discovered the existence of new subsets of liver macrophages with unique functions during the development of obesity–associated metabolic disease.

4.1 A subset of Kupffer cells regulates metabolism through the expression of CD36 (Paper I)

Macrophages are a heterogeneous cell population that regulate tissue homeostasis by responding to signals from their environment. Liver macrophages have previously been reported to also contribute to the metabolic impairments associated with obesity in mice and humans (126). Furthermore, recent studies using single cell RNA sequencing of liver cells have described the presence of both tissue resident Kupffer cells and several populations of monocyte–derived macrophages that expand in response to disease (43, 59, 77–80). Although tissue resident Kupffer cells have been shown to represent the majority (~90%) of liver macrophages at steady state (58, 59), little is known about different phenotypes of Kupffer cells and how these might contribute to obesity and insulin resistance.

In **Paper I** we focused on the tissue resident Kupffer cells and investigated their heterogeneity in the murine liver. Surprisingly, we identified two distinct populations of Kupffer cells using single cell RNA sequencing and single cell proteomics (**Figure 1**). The first population (KC1) expressed classical markers of Kupffer cells (e.g., Clec4F and Tim4) and the second (KC2) expressed both Kupffer cell and endothelial cell markers (e.g., CD31, CD206, ESAM and LYVE1) at both RNA and protein level (**Figure 4a–b, 5**). To confirm that the expression of endothelial cell markers in KC2 cells was not due to contamination of liver sinusoidal endothelial cells, that has previously been shown to tightly adhere to Kupffer cell after isolation (127), we analyzed the morphology of KC2 cells by scanning electron microscopy. We found that sorted KC2 cells lacked fenestrations on their surface, a typical characteristic of liver sinusoidal endothelial cells (**Figure 4d**). In addition, KC2 cells were shown to have a high phagocytic ability, similar to that of KC1 (**Figure 4f, S7f**). As liver sinusoidal endothelial cells are non–phagocytic cells, this further suggested the identity of KC2 as macrophages rather than endothelial cells.

To further characterize the phenotypes of the two Kupffer cell populations, we analyzed their proportion in the liver at steady state by flow cytometric analysis and immunofluorescence quantifications and found that KC1 represented the largest population (~85%) of Kupffer cells in murine livers, compared to KC2 (~15%) (**Figure 2a, 3d**). Moreover, we investigated the origin and turnover of KC1 and KC2 using the previously established monocyte fate–mapping Ms4a3Cre x RosaTomato mouse model, enabling lineage tracing of monocyte–derived

macrophages by tamoxifen-inducible expression of Tomato fluorescent protein in Ms4a3-expressing monocyte progenitors (58). Flow cytometric analysis of Tomato$^+$ (monocytic origin) and Tomato$^-$ (non-monocytic origin) Kupffer cells showed that neither KC1 nor KC2 originated from monocytic precursors at steady state (**Figure 2c**), further suggesting their identity as embryonic-derived Kupffer cells.

To then understand the role of KC1 and KC2 during the development of obesity, we used the diet-induced obesity model where mice are fed a high fat diet to induce obesity and metabolic disease including liver steatosis and insulin resistance. Interestingly, we observed that the proportion of KC2 cells increased with time on high fat diet (**Figure 6b**). Previous studies in mouse models of advanced stages of NAFLD and NASH, have revealed that monocyte-derived macrophages increase in proportion during the progression of NAFLD (59, 79–81, 83, 107). To therefore understand if the increase in KC2 with obesity was the result of infiltration of monocytes, we used flow cytometric analysis and observed that at early stages of obesity (9 weeks of high fat diet) the proportion of KC2 cells significantly increased in the liver, while the proprotein of monocyte-derived macrophages (F4/80$^+$CD64$^+$Tim4$^-$) or Ms4a3-expressing lineage traced KC2 macrophages remained unchanged (**Figure S6a–b**). Although, both expansion of monocyte-derived macrophages and increased proportions of Ms4a3-expressing lineage traced KC2 cells were observed at later stages of obesity (18 weeks of high fat diet) where liver inflammation is present (106), suggesting that monocytes contribute to the KC2 pool at more severe stages of metabolic disease (126).

To understand how the two KC populations respond in the context of metabolic disease, we performed bulk RNA-sequencing of isolated KC1 and KC2 from lean and obese mice after 8 weeks of high fat diet. This revealed an overall upregulation of metabolic genes with obesity in both KC populations (**Figure S6c–d**). Moreover, KC2 was observed to upregulate genes involved in lipid handling in the obese state, including the gene *Cd36*.

Given that the KC2 phenotype was the most affected by obesity and upregulated genes involved in lipid processing (**Figure 6c–e, S6c–d**), we focused on the role of the KC2 cells in obesity. We had identified Cdh5 as a specific marker for KC2 at both RNA and protein level, although it is also known to be expressed by liver sinusoidal endothelial cells. To specifically target KC2 but not KC1, we generated a mouse model of bone marrow chimeras in which KC2 cells expressed the human diphtheria toxin receptor (DTR) allowing for the depletion of KC2 by diphtheria toxin treatment. Surprisingly, mice in which KC2 had been specifically depleted did not gain weight after 6 weeks of high fat diet, compared to control mice that still had liver KC2 cells (**Figure 7a**). We further observed that depletion of KC2 reduced oxidative stress and lipid accumulation in the liver and improved insulin resistance upon high fat diet feeding (**Figure 7b–d**). Although, circulating lipids were found to increase with obesity in mice lacking KC2 cells

(**Figure 7e**). Together, these results suggests that KC2 might have detrimental effects on diet-induced obesity by contributing to both weight gain, lipid accumulation and oxidative stress in the liver during obesity.

Finally, we went on to further investigate the role of KC2 cells in regulating liver lipid metabolism and oxidative stress in obesity. Lipid accumulation in the liver during obesity results from the increase of both *de novo* lipogenesis (the synthesis of lipids) and lipid uptake. As the lipid transporter CD36 was found to be expressed and increased with obesity in KC2 cells we hypothesized that tissue resident KC2 cells contributeto liver oxidative stress induced by obesity through the upregulation of CD36. Moreover, several studies have described the role of CD36 in the formation of foam cells and induction of oxidative stress through the uptake of fatty acids and oxidized low-density lipoprotein (LDL) in mice (128, 129). We therefore used the glucan encapsulated RNAi Particle (GeRP) technology to silence the expression of *Cd36* in Kupffer cells of obese mice (121). Mice fed a HFD were treated with GeRPs loaded with siRNA targeting *Cd36* or with the non-targeting scrambled control. Specific silencing of *Cd36* in Kupffer cells lead to a significant decrease in oxidative stress in the liver independently of an effect on triglyceride content during early stages of obesity (9 weeks of high fat diet) (**Figure 7k, S7i, S7m**). These results were further confirmed by studying the transcriptomic profile of Kupffer cells following *Cd36* knockdown, that revealed the downregulation of genes involved in lipid metabolism and oxidative stress (**Figure 7j**). Moreover, decreasing oxidative stress led to an improvement of glucose homeostasis as assessed by fasting glycaemia and glucose tolerance, independently of an effect on body weight (**Figure 7i and S7k**). Considering that a reduction of oxidative stress in Kupffer cells can lead to a decreased release of reactive oxygen species (ROS), these results suggests that *Cd36* silencing in Kupffer cells could be sufficient to decrease the overall oxidative stress in the liver.

In conclusion, in **Paper I** we revealed the presence of two distinct Kupffer cell populations (KC1 and KC2) in healthy and obese animals. Furthermore, we demonstrated the unique functions of KC2 in obesity by contributing to oxidative stress and showed that this function can be therapeutically targeted by specific silencing of *Cd36* in mice.

4.2 Human resident liver myeloid cells protect against metabolic stress in obesity (Paper II)

Non-alcoholic fatty liver disease has become the major cause of chronic liver disease, being associated with an increasing prevalence of obesity and type 2 diabetes (17, 18). Macrophages have been shown to contribute to the development of these metabolic disorders in obesity. However, while humans with obesity and NAFLD are at great risk of developing NASH and other severe conditions including cirrhosis and cancer, little is known about the

characteristics and functions of distinct liver macrophage population during early stages of NAFLD pathogenesis.

In **Paper II**, we investigated the heterogeneity of liver myeloid (LM) cells in lean and obese humans at early stages of obesity and NAFLD. Using full-length single cell RNA sequencing at high sequencing depth we discovered four populations of liver myeloid cells (LM1-4) present in both lean and obese humans (**Figure 1, S1–S4**). Based on the most highly differentially expressed genes, we were able to identify unique markers of each individual cluster (**Table S3**). Moreover, we found that two of the subpopulations (LM1 and LM2) were characterized by a transcriptional signature of tissue resident macrophages, or Kupffer cells, while the other two (LM3 and LM4) had a less mature phenotype resembling monocyte-derived infiltrating macrophages (**Figure 1, S1–S4**). Interestingly, we identified a distinct population of myeloid cells (LM2) that expressed both macrophage (e.g., *CD68*, *CD14*, *CSF1R*, *VSIG4* and *MRC1*) and conventional dendritic cell markers (e.g., *CLEC10A*, *CD1C* and *FCER1A*) (**Figure S3a–k, Table S3–4**). However, more studies are needed to define the developmental origin and identity of this population as macrophages and conventional dendritic cells share many similarities. In addition, as recent studies have identified a population of conventional dendritic cells (DC3) with macrophage features, it could be possible that the LM2 cells represent this newly characterized DC3 population (130, 131).

Furthermore, we observed that the recruited liver myeloid cell population LM4 had a modest pro-inflammatory phenotype in comparison to the other liver myeloid cell subpopulations (**Figure S4g–h**). These cells had a similar transcriptional profile to previously described recruited pro-inflammatory macrophages (84, 85). However, this phenotype was mild and not exacerbated by obesity (**Figure S9e, Table S8**). Similarly, a recent study reported that both human liver macrophages with an inflammatory phenotype and tissue monocytes had significantly weaker pro-inflammatory responses compared to human circulating monocytes (132). This suggests that the LM4 population could be derived from inflammatory monocytes that upon entry into the liver adapts to the tolerogenic environment by decreasing their expression of these pro-inflammatory response genes.

Tissue resident Kupffer cells make up the largest population of liver macrophages in mice at steady state (58, 59). To understand if this was translatable to humans, we investigated the proportion of each of the identified human myeloid cell populations in the liver using flow cytometry. Of note, previous studies have shown that liver biopsies typically contain significant amounts of contaminating immune cells from the circulation due to that the fact that the liver is highly vascularized and contain large amount of blood (88, 132). We therefore compared liver samples that had been perfused *ex vivo* to remove all contaminating blood cells to samples that had been collected without liver perfusion, in order to distinguish between contaminating myeloid cells from the

blood and those resident in the liver tissue. We identified all four myeloid cell populations in both the perfused and non-perfused liver samples, confirming their identity as tissue resident cells (**Figure 2f–i**). In addition, we found that unlike in mice, human livers contain a mix of myeloid cells with both tissue-resident and monocyte-derived macrophage phenotypes, where the monocyte-derived LM4 cells represented the largest population. In addition, we further validated the presence of both tissue resident and recruited myeloid cells in human livers by immunofluorescence and spatial proteomics (**Figure 3e–h, S8**).

We hypothesized that the differences in proportion of myeloid cell populations in mice and humans could be due to differences in their immune system that has emerged throughout evolution (e.g., genetic or epigenetic differences). However, another likely explanation could be their different environmental conditions, since laboratory mice typically live in highly controlled and pathogen-free settings with little exposure to environmental antigens. In contrast, humans are continuously exposed to several environmental antigens and are typically infected by different viruses, bacteria and parasites, and humans might therefore have a more diverse microbiota compared to mice. Therefore, a high turnover of myeloid cells in human livers would likely be required to rapidly renew cells and adapt to the constant exposure to antigens and pathogens. To test this, we analyzed the proportion of liver macrophages in wildling mice that have a rich microbial exposure compared to laboratory mice (118, 133). We demonstrated that these wildling mice have lower proportions of tissue resident Kupffer cells and higher proportion of recruited monocyte-derived cells in comparison to specific pathogen free control mice (**Figure 2f–i**). This might further explain why the proportion of monocyte-derived macrophages is lower in laboratory mice compared to humans. Together, these data suggests that the composition of liver myeloid cell populations differs between humans and mice, where human livers contain comparably higher numbers of recruited myeloid cells.

While liver macrophage ontogeny has been extensively studied in mouse models, there have been few direct studies to characterize the turnover of distinct liver myeloid cell populations in humans. We therefore investigated the extent to which human myeloid cells were derived from monocytes from the circulation by examining the composition of liver myeloid cell populations in patients undergoing liver transplantation. We collected liver biopsies from the donor livers before and 6 hours after transplantation. By staining liver cells for the specific HLA-type of both the donor (transplanted liver) and the recipient, we were able to distinguish between donor-derived and recipient-derived cells. Using flow cytometric analysis, we demonstrated that a large proportion (~70%) of liver myeloid cells were rapidly renewed by infiltrating monocytes (recipient-derived) from the circulation after the transplantation (**Figure 3a–d, S7a–c**). One limitation is that the 6 hour time-point is relatively short and the results from these could be somewhat exaggerated since liver grafts could be mildly inflamed

immediately after transplantation (134). We therefore compared our data to a second cohort (71) and observed that while LM4 cells were quickly replaced by infiltrating monocyte–derived cells (hours post–transplantation), with time (several months to years) the majority of cells in all four populations had been replaced (**Figure S7d–f**). These results show that monocytes from the circulation can repopulate all four liver myeloid cell populations, where some populations are more rapidly replaced than others. Although whether infiltrating monocytes directly adopt the phenotype of a given subpopulation or if the rapidly recruited LM4 cells can further differentiate into other liver myeloid cell populations remains to be studied. Together with our single cell and flow cytometric data, this suggests that most myeloid cells in human livers have a monocytic origin.

We next investigated the effect of obesity on the transcriptomic profile of the myeloid cells and identified a population of tissue–resident myeloid cells (LM2) that switched their phenotype in response to obesity and upregulated genes involved in antioxidant response such as the antioxidant peroxiredoxin–2 (*PRDX2*) (**Figure 4a–b**). We observed that patients with obesity had lower frequencies of these LM2 cells, suggesting that this population might be less frequent in obese compared to lean individuals (**Figure 4c–g, S10g–i**) However, we did not detect altered levels of either apoptosis or proliferation with obesity, leading us to speculate that LM2 lose their ability to fully mature with obesity. In fact, LM2 cells displayed reduced levels of classical macrophage markers in obesity and *in silico* analysis revealed a less mature transcriptomic profile of LM2 cells in obesity. This could suggest that resident myeloid cells are unable to fully mature in obesity and metabolic disease as has been suggested in mice.

Although, the proportion of LM2 cells decreased, the *PRDX2* expression per cell was observed to significantly increase during obesity and insulin resistance (**Figure 4a–b**). We therefore hypothesized that the LM2 cells could be protective in obesity and investigated their function using human *in vitro* 3D liver cultures. From this, we observed that PRDX2 protein levels were increased in the media of liver cultures treated with steatogenic media, further suggesting that LM2 cells secrete PRDX2 into the liver during obesity. Furthermore, we observed that liver spheroids depleted of LM2 cells had significantly increased levels of both oxidative stress and lipid peroxidation (**Figure 5**), highlighting that LM2 cells might be protective by decreasing levels of oxidative stress in the liver during obesity.

In conclusion, in **Paper II** we show that a distinct population of tissue resident liver myeloid cells expresses antioxidant genes, including the antioxidant *PRDX2*. While more samples are needed to confirm that the reduction of PRDX2–expressing cells is a consistent event during the progression of obesity and NAFLD, our study suggests that this myeloid cell population could protect against oxidative stress associated with obesity.

5 Conclusion and Points of Perspectives

The recent advances with single cell technologies and in particular single cell RNA sequencing have revealed important changes in the composition, function, and turnover of liver macrophages during the pathogenesis of fatty liver disease. However, many of these studies have been done in the context of advanced NAFLD including NASH and liver cirrhosis. Therefore, little was known about the role of distinct populations of liver macrophages during the initiation and early development of NAFLD. With the studies included in this thesis, we characterised the heterogeneity of liver macrophages during health and at early stages of liver pathogenesis associated with obesity in animals and humans. Moreover, we unveiled the role of distinct populations of liver macrophages during the development of metabolic disease. However, several questions remain to be answered and are discussed here.

Liver macrophages have been strongly associated with the progression of early stages of NAFLD (e.g., steatosis) to NASH and cirrhosis, by inducing inflammation in the liver. More recently, liver inflammation has been shown to be linked to the recruitment of monocyte-derived macrophages during NASH, rather than the inflammatory activation of tissue resident macrophages (126). In alignment with this hypothesis, we did not observe a strong inflammatory phenotype in macrophage populations, neither in mice nor humans at early stages of NAFLD, where recruitment of monocyte-derived macrophages is limited. This further suggests that while liver inflammation is crucial for later stages of NAFLD including NASH, it might not represent the main driving factor for the initiation of disease. Furthermore, treatment with anti-inflammatory drugs in human patients have demonstrated limited effects against metabolic disease including insulin resistance and type 2 diabetes (135). While other attempts to either prevent monocyte recruitment to the liver or to block inflammatory activation of macrophages during NASH have only indicated somewhat promising effects against liver fibrosis, these have not been efficient in reversing or stopping the progression of NASH (36). This further highlights the strong need for a better understanding of the disease pathogenesis and the functional roles of different macrophage populations in the liver during metabolic disease and obesity. Moreover, as there are currently no approved drugs to treat NAFLD and precise and non-invasive diagnostic tools are so far lacking, there is a clear need to better understand the link between obesity and NAFLD. Studying early stages of the disease pathogenesis is therefore important. This could enable the identification of not only biomarkers to distinguish between NAFLD patients with high risk of developing severe disease and patients that will remain metabolically healthy, but also to identify therapeutic targets to treat or prevent the progression of NAFLD at an earlier stage.

In **Paper I**, we identified a tissue resident population of macrophages that contributed to metabolic disease in obesity, independently of inflammation, in a

mice model of obesity-induced insulin resistance and hepatic steatosis. We found that this population (KC2) was required for weight gain in response to diet-induced obesity in our model, and depletion of KC2 cells in mice protected against obesity independently of steatosis. Further studies are therefore needed to understand the precise role of KC2 in regulating systemic metabolism and could possibly reveal potential mechanisms controlling weight gain during obesity. Moreover, in **Paper I** we demonstrated that in obesity, KC2 contributes to the impairment of the antioxidant ability of the liver by driving the development of oxidative stress and insulin resistance via the lipid receptor CD36. As our study focused on the initial steps of NAFLD pathogenesis (obesity and hepatic steatosis), it would be important to analyse the roles of KC2, CD36 and oxidative stress at the various phases of fatty liver disease to understand the role of KC2 in the development of NASH and hepatocellular carcinoma.

In **Paper II**, we observed reduced levels of maturation in human tissue resident myeloid cells in livers during obesity compared to healthy livers. However, further studies are needed to functionally decipher whether these cells are lost due to impaired renewal in the context of metabolic disease. Moreover, as the local tissue environment have been shown to play an important role for the identity and maturation of liver macrophages, it would be highly plausible that changes in the phenotype of tissue resident macrophages are driven by the metabolically impaired liver environment during obesity. This requires additional functional studies in humans, by for example genetic manipulation of secreted factors from surrounding liver cells, to be able to define the contribution of the local microenvironment on macrophage maturation in obesity. In addition, it would also be interesting to study the effect of the liver microenvironment in the case of the murine Kupffer cell populations. As neither origin nor tissue localisation appeared to play a role in determining the phenotype of each individual population, it could be possible that differences in surrounding cell populations other than hepatocytes are responsible for their unique phenotypes. It would therefore be informative to study whether local differences in cell heterogeneity (e.g., of hepatic stellate cells or liver sinusoidal endothelial cells) result in local differences in secreted factors and/or metabolites that in turn induce these functionally distinct KC phenotypes.

In **Paper II** we also observed that LM2 cells expressed increased levels of antioxidant genes, including the antioxidant *PRDX2*, and were protective against oxidative stress associated by obesity. However, it is possible that LM2 cells could produce several other factors besides PRDX2 to help detoxify the liver from oxidative stress in obesity, and further studies are therefore needed to investigate the functional role of PRDX2 in obesity. One example would be to genetically manipulate the expression of *PRDX2* in LM2 cells (e.g., to knock it down) in 3D liver cultures, to study the specific effects of PRDX2 on the overall liver oxidative stress in the condition of obesity and metabolic disease.

Moreover, with these two studies (**Paper I and II**) we identified several discrepancies between the data from mice and humans such as differences in macrophage turnover and composition. Additionally, the majority of studies on the role of liver macrophages in metabolic disease have been performed on mice. It is therefore important to keep in mind that although diet-induced obesity mice models mimic many important aspects of human disease progression, many differences exist not only between different mice models but also to humans (36, 116). One important difference is the immune compartment, as mice and humans have inherently different composition of innate and adaptive immune cells. Humans are also constantly exposed to pathogens throughout their lives resulting in immunological memory and adaptations of immune cells, unlike laboratory mice that live in sterile environments. Moreover, few studies using mice have considered the effects of biological sex and other genetic differences on the pathogenesis of obesity and metabolic disease. Another aspect that differs between humans and mice is the disease duration. Humans have a longer life span where obesity typically develops at an early age and progress for years or even decades. In comparison, mice models of obesity typically have a rapid onset of obesity where mice remain obese for a relatively short period of time, and are therefore not chronically obese to the same extent as humans. It could thus be possible that the shorter lifespan and the relatively short duration of disease is not enough to recapitulate the full aspects of human obesity and might explain some of the differences observed between mice models and patients with obesity. Together, these differences are further highlighted by the current challenges in translating results from mice into clinical use for humans, where the majority of clinical trials for NASH in humans has failed (36). The field would therefore benefit from additional studies to investigating the biology and functional role of human macrophage populations, especially in the context of obesity and NAFLD. In addition, prospective studies using larger human cohorts would further help to define consistent and specific functions of different macrophage populations across different phases of NAFLD.

In conclusion, these two studies have contributed to the improved understanding of macrophage diversity in liver disease, further suggesting that modulating the phenotype of distinct macrophage populations could represent an interesting therapeutic approach for the treatment of metabolic disease associated with obesity.

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7 References

1. S. Gordon, A. Pluddemann, Tissue macrophages: heterogeneity and functions. BMC Biol 15, 53 (2017).
2. K. Liddiard, P. R. Taylor, Understanding local macrophage phenotypes in disease: shape-shifting macrophages. Nat Med 21, 119-120 (2015).
3. M. D. Park, A. Silvin, F. Ginhoux, M. Merad, Macrophages in health and disease. Cell 185, 4259-4279 (2022).
4. A. W. Roberts et al., Tissue-Resident Macrophages Are Locally Programmed for Silent Clearance of Apoptotic Cells. Immunity 47, 913-927 e916 (2017).
5. J. Park, T. S. Morley, M. Kim, D. J. Clegg, P. E. Scherer, Obesity and cancer--mechanisms underlying tumour progression and recurrence. Nat Rev Endocrinol 10, 455-465 (2014).
6. S. Klein, A. Gastaldelli, H. Yki-Jarvinen, P. E. Scherer, Why does obesity cause diabetes? Cell Metab 34, 11-20 (2022).
7. P. L. Valenzuela et al., Obesity and the risk of cardiometabolic diseases. Nat Rev Cardiol 20, 475-494 (2023).
8. F. Rubino et al., Joint international consensus statement for ending stigma of obesity. Nat Med 26, 485-497 (2020).
9. R. L. Batterham, Weight stigma in healthcare settings is detrimental to health and must be eradicated. Nat Rev Endocrinol 18, 387-388 (2022).
10. M. Bluher, Obesity: global epidemiology and pathogenesis. Nat Rev Endocrinol 15, 288-298 (2019).
11. N. C. D. R. F. Collaboration, Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. Lancet 390, 2627-2642 (2017).
12. G. B. D. O. Collaborators et al., Health Effects of Overweight and Obesity in 195 Countries over 25 Years. N Engl J Med 377, 13-27 (2017).
13. The Public Health Agency of Sweden. Nationella folkhälsoenkäten "Hälsa på lika villkor", <https://www.folkhalsomyndigheten.se/livsvillkor-levnadsvanor/mat-fysisk-aktivitet-overvikt-och-fetma/overvikt-och-fetma/statistik-om-overvikt-och-fetma/overvikt-och-fetma-hos-vuxna/> (2022).
14. L. Vishvanath et al., Pdgfrbeta+ Mural Preadipocytes Contribute to Adipocyte Hyperplasia Induced by High-Fat-Diet Feeding and Prolonged Cold Exposure in Adult Mice. Cell Metab 23, 350-359 (2016).
15. Q. A. Wang, C. Tao, R. K. Gupta, P. E. Scherer, Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nat Med 19, 1338-1344 (2013).
16. A. M. Cypess, Reassessing Human Adipose Tissue. N Engl J Med 386, 768-779 (2022).
17. Z. M. Younossi et al., The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. J Hepatol 71, 793-801 (2019).
18. Z. M. Younossi et al., Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 64, 73-84 (2016).
19. S. Albhaisi, A. Chowdhury, A. J. Sanyal, Non-alcoholic fatty liver disease in lean individuals. JHEP Rep 1, 329-341 (2019).

20. M. Eslam, A. J. Sanyal, J. George, P. International Consensus, MAFLD: A Consensus-Driven Proposed Nomenclature for Metabolic Associated Fatty Liver Disease. Gastroenterology 158, 1999–2014 e1991 (2020).
21. P. S. Dulai et al., Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. Hepatology 65, 1557–1565 (2017).
22. D. Q. Huang et al., Global epidemiology of cirrhosis - aetiology, trends and predictions. Nat Rev Gastroenterol Hepatol 20, 388–398 (2023).
23. J. M. Llovet et al., Nonalcoholic steatohepatitis-related hepatocellular carcinoma: pathogenesis and treatment. Nat Rev Gastroenterol Hepatol 20, 487–503 (2023).
24. R. Loomba, S. L. Friedman, G. I. Shulman, Mechanisms and disease consequences of nonalcoholic fatty liver disease. Cell 184, 2537–2564 (2021).
25. K. L. Donnelly et al., Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 115, 1343–1351 (2005).
26. L. P. Bechmann et al., The interaction of hepatic lipid and glucose metabolism in liver diseases. J Hepatol 56, 952–964 (2012).
27. S. Fu, S. M. Watkins, G. S. Hotamisligil, The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. Cell Metab 15, 623–634 (2012).
28. M. E. Ertunc, G. S. Hotamisligil, Lipid signaling and lipotoxicity in metaflammation: indications for metabolic disease pathogenesis and treatment. J Lipid Res 57, 2099–2114 (2016).
29. T. Tsuchida, S. L. Friedman, Mechanisms of hepatic stellate cell activation. Nat Rev Gastroenterol Hepatol 14, 397–411 (2017).
30. H. Yki-Jarvinen, Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol 2, 901–910 (2014).
31. G. Marchesini et al., Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 107, 450–455 (1999).
32. S. Tamura, I. Shimomura, Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease. J Clin Invest 115, 1139–1142 (2005).
33. D. E. Kleiner et al., Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41, 1313–1321 (2005).
34. Z. M. Younossi, S. Zelber-Sagi, L. Henry, L. H. Gerber, Lifestyle interventions in nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 20, 708–722 (2023).
35. S. A. Harrison, A. M. Allen, J. Dubourg, M. Noureddin, N. Alkhouri, Challenges and opportunities in NASH drug development. Nat Med 29, 562–573 (2023).
36. P. N. Brennan et al., Antifibrotic therapy in nonalcoholic steatohepatitis: time for a human-centric approach. Nat Rev Gastroenterol Hepatol 20, 679–688 (2023).
37. I. N. Crispe, Liver antigen-presenting cells. J Hepatol 54, 357–365 (2011).
38. V. Racanelli, B. Rehermann, The liver as an immunological organ. Hepatology 43, S54–62 (2006).
39. A. W. Thomson, P. A. Knolle, Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol 10, 753–766 (2010).
40. O. Krenkel, F. Tacke, Liver macrophages in tissue homeostasis and disease. Nat Rev Immunol 17, 306–321 (2017).
41. J. Bonnardel et al., Stellate Cells, Hepatocytes, and Endothelial Cells Imprint the Kupffer Cell Identity on Monocytes Colonizing the Liver Macrophage Niche. Immunity 51, 638–654 e639 (2019).

42. F. Sierro et al., A Liver Capsular Network of Monocyte-Derived Macrophages Restricts Hepatic Dissemination of Intraperitoneal Bacteria by Neutrophil Recruitment. Immunity 47, 374–388 e376 (2017).

43. M. Guilliams et al., Spatial proteogenomics reveals distinct and evolutionarily conserved hepatic macrophage niches. Cell 185, 379–396 e338 (2022).

44. K. S. Carpentier et al., Discrete viral E2 lysine residues and scavenger receptor MARCO are required for clearance of circulating alphaviruses. Elife 8, (2019).

45. D. Sun et al., Fungal dissemination is limited by liver macrophage filtration of the blood. Nat Commun 10, 4566 (2019).

46. K. Y. Helmy et al., CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. Cell 124, 915–927 (2006).

47. G. Liu et al., CRIg plays an essential role in intravascular clearance of bloodborne parasites by interacting with complement. Proc Natl Acad Sci U S A 116, 24214–24220 (2019).

48. Z. Zeng et al., CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. Cell host & microbe 20, 99–106 (2016).

49. F. Heymann et al., Liver inflammation abrogates immunological tolerance induced by Kupffer cells. Hepatology (Baltimore, Md.) 62, 279–291 (2015).

50. Q. You, L. Cheng, R. M. Kedl, C. Ju, Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. Hepatology (Baltimore, Md.) 48, 978–990 (2008).

51. Y. Wang et al., Plasma cholesteryl ester transfer protein is predominantly derived from Kupffer cells. Hepatology (Baltimore, Md.) 62, 1710–1722 (2015).

52. C. L. Scott, M. Guilliams, The role of Kupffer cells in hepatic iron and lipid metabolism. Journal of hepatology 69, 1197–1199 (2018).

53. V. Terpstra, T. J. C. van Berkel, Scavenger receptors on liver Kupffer cells mediate the in vivo uptake of oxidatively damaged red blood cells in mice. Blood 95, 2157–2163 (2000).

54. S. K. Moestrup et al., Identification of the haemoglobin scavenger receptor. Nature (London) 409, 198–201 (2001).

55. I. Theurl et al., On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. Nature medicine 22, 945–951 (2016).

56. B. A. David et al., Combination of Mass Cytometry and Imaging Analysis Reveals Origin, Location, and Functional Repopulation of Liver Myeloid Cells in Mice. Gastroenterology 151, 1176–1191 (2016).

57. C. L. Scott et al., Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. Nat Commun 7, 10321 (2016).

58. Z. Liu et al., Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells. Cell 178, 1509–1525 e1519 (2019).

59. J. S. Seidman et al., Niche-Specific Reprogramming of Epigenetic Landscapes Drives Myeloid Cell Diversity in Nonalcoholic Steatohepatitis. Immunity 52, 1057–1074 e1057 (2020).

60. E. Gomez Perdiguero et al., Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. Nature 518, 547–551 (2015).

61. S. Yona et al., Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity 38, 79-91 (2013).

62. F. Ginhoux, M. Guilliams, Tissue-Resident Macrophage Ontogeny and Homeostasis. Immunity 44, 439–449 (2016).

63. C. Bleriot et al., Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. Immunity 42, 145–158 (2015).
64. Y. Lavin et al., Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell 159, 1312–1326 (2014).
65. L. Beattie et al., Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions. J Hepatol 65, 758–768 (2016).
66. M. Sakai et al., Liver-Derived Signals Sequentially Reprogram Myeloid Enhancers to Initiate and Maintain Kupffer Cell Identity. Immunity 51, 655–670 e658 (2019).
67. E. Mass et al., Specification of tissue-resident macrophages during organogenesis. Science 353, (2016).
68. C. L. Scott et al., The Transcription Factor ZEB2 Is Required to Maintain the Tissue-Specific Identities of Macrophages. Immunity 49, 312–325 e315 (2018).
69. H. Bennett, T. D. Troutman, M. Sakai, C. K. Glass, Epigenetic Regulation of Kupffer Cell Function in Health and Disease. Front Immunol 11, 609618 (2020).
70. I. Bittmann et al., The role of graft-resident Kupffer cells and lymphocytes of donor type during the time course after liver transplantation--a clinico-pathological study. Virchows Arch 443, 541–548 (2003).
71. L. J. Pallett et al., Longevity and replenishment of human liver-resident memory T cells and mononuclear phagocytes. J Exp Med 217, (2020).
72. D. M. Popescu et al., Decoding human fetal liver haematopoiesis. Nature 574, 365–371 (2019).
73. Z. Bian et al., Deciphering human macrophage development at single-cell resolution. Nature 582, 571–576 (2020).
74. A. Sharma et al., Onco-fetal Reprogramming of Endothelial Cells Drives Immunosuppressive Macrophages in Hepatocellular Carcinoma. Cell 183, 377–394 e321 (2020).
75. C. Suo et al., Mapping the developing human immune system across organs. Science 376, eabo0510 (2022).
76. Y. Zeng et al., Tracing the first hematopoietic stem cell generation in human embryo by single-cell RNA sequencing. Cell Res 29, 881–894 (2019).
77. K. B. Halpern et al., Paired-cell sequencing enables spatial gene expression mapping of liver endothelial cells. Nat Biotechnol 36, 962–970 (2018).
78. O. Krenkel et al., Myeloid cells in liver and bone marrow acquire a functionally distinct inflammatory phenotype during obesity-related steatohepatitis. Gut 69, 551–563 (2020).
79. A. Remmerie et al., Osteopontin Expression Identifies a Subset of Recruited Macrophages Distinct from Kupffer Cells in the Fatty Liver. Immunity 53, 641–657 e614 (2020).
80. X. Xiong et al., Landscape of Intercellular Crosstalk in Healthy and NASH Liver Revealed by Single-Cell Secretome Gene Analysis. Mol Cell 75, 644–660 e645 (2019).
81. S. Tran et al., Impaired Kupffer Cell Self-Renewal Alters the Liver Response to Lipid Overload during Non-alcoholic Steatohepatitis. Immunity 53, 627–640 e625 (2020).
82. D. A. Jaitin et al., Lipid-Associated Macrophages Control Metabolic Homeostasis in a Trem2-Dependent Manner. Cell 178, 686–698 e614 (2019).
83. S. Daemen et al., Dynamic Shifts in the Composition of Resident and Recruited Macrophages Influence Tissue Remodeling in NASH. Cell Rep 34, 108626 (2021).

84. S. A. MacParland et al., Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. Nat Commun 9, 4383 (2018).
85. T. S. Andrews et al., Single-Cell, Single-Nucleus, and Spatial RNA Sequencing of the Human Liver Identifies Cholangiocyte and Mesenchymal Heterogeneity. Hepatol Commun 6, 821-840 (2022).
86. X. Wu et al., Human Liver Macrophage Subsets Defined by CD32. Front Immunol 11, 2108 (2020).
87. N. Aizarani et al., A human liver cell atlas reveals heterogeneity and epithelial progenitors. Nature 572, 199-204 (2019).
88. P. Ramachandran et al., Resolving the fibrotic niche of human liver cirrhosis at single-cell level. Nature 575, 512-518 (2019).
89. J. Paris, N. C. Henderson, Liver zonation, revisited. Hepatology 76, 1219-1230 (2022).
90. R. S. McCuskey, P. A. McCuskey, R. Urbaschek, B. Urbaschek, Species differences in Kupffer cells and endotoxin sensitivity. Infect Immun 45, 278-280 (1984).
91. E. C. Sleyster, D. L. Knook, Relation between localization and function of rat liver Kupffer cells. Lab Invest 47, 484-490 (1982).
92. I. Bykov, P. Ylipaasto, L. Eerola, K. O. Lindros, Functional Differences between Periportal and Perivenous Kupffer Cells Isolated by Digitonin-Collagenase Perfusion. Comp Hepatol 3 Suppl 1, S34 (2004).
93. L. Bouwens, M. Baekeland, R. De Zanger, E. Wisse, Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. Hepatology 6, 718-722 (1986).
94. A. Gola et al., Commensal-driven immune zonation of the liver promotes host defence. Nature 589, 131-136 (2021).
95. E. Barreby, M. Aouadi, To be or not to be a hepatic niche macrophage. Immunity (Cambridge, Mass.) 55, 198-200 (2022).
96. W. Huang et al., Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 59, 347-357 (2010).
97. A. M. Neyrinck et al., Critical role of Kupffer cells in the management of diet-induced diabetes and obesity. Biochem Biophys Res Commun 385, 351-356 (2009).
98. A. Asghar, N. Sheikh, Role of immune cells in obesity induced low grade inflammation and insulin resistance. Cell Immunol 315, 18-26 (2017).
99. A. Guilherme, J. V. Virbasius, V. Puri, M. P. Czech, Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol 9, 367-377 (2008).
100. A. C. Tosello-Trampont, S. G. Landes, V. Nguyen, T. I. Novobrantseva, Y. S. Hahn, Kupffer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-alpha production. J Biol Chem 287, 40161-40172 (2012).
101. J. I. Odegaard et al., Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. Cell Metab 7, 496-507 (2008).
102. J. Wan et al., M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology 59, 130-142 (2014).
103. Z. Papackova et al., Kupffer cells ameliorate hepatic insulin resistance induced by high-fat diet rich in monounsaturated fatty acids: the evidence for the involvement of alternatively activated macrophages. Nutr Metab (Lond) 9, 22 (2012).
104. S. S. Rensen et al., Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. Am J Pathol 175, 1473-1482 (2009).

105. H. Morinaga et al., Characterization of distinct subpopulations of hepatic macrophages in HFD/obese mice. Diabetes 64, 1120-1130 (2015).
106. C. Morgantini et al., Liver macrophages regulate systemic metabolism through non-inflammatory factors. Nat Metab 1, 445-459 (2019).
107. A. Deczkowska et al., XCR1(+) type 1 conventional dendritic cells drive liver pathology in non-alcoholic steatohepatitis. Nat Med 27, 1043-1054 (2021).
108. M. Peiseler et al., Kupffer cell-like syncytia replenish resident macrophage function in the fibrotic liver. Science 381, eabq5202 (2023).
109. T. Hendrikx et al., Soluble TREM2 levels reflect the recruitment and expansion of TREM2(+) macrophages that localize to fibrotic areas and limit NASH. J Hepatol 77, 1373-1385 (2022).
110. A. Guillot et al., Mapping the hepatic immune landscape identifies monocytic macrophages as key drivers of steatohepatitis and cholangiopathy progression. Hepatology 78, 150-166 (2023).
111. E. Sanz et al., Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. Proc Natl Acad Sci U S A 106, 13939-13944 (2009).
112. S. Picelli et al., Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods 10, 1096-1098 (2013).
113. S. Picelli et al., Full-length RNA-seq from single cells using Smart-seq2. Nat Protoc 9, 171-181 (2014).
114. E. Mereu et al., Benchmarking single-cell RNA-sequencing protocols for cell atlas projects. Nat Biotechnol 38, 747-755 (2020).
115. A. Guillaumet-Adkins et al., Single-cell transcriptome conservation in cryopreserved cells and tissues. Genome Biol 18, 45 (2017).
116. D. Jahn, S. Kircher, H. M. Hermanns, A. Geier, Animal models of NAFLD from a hepatologist's point of view. Biochim Biophys Acta Mol Basis Dis 1865, 943-953 (2019).
117. M. Charlton et al., Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. Am J Physiol Gastrointest Liver Physiol 301, G825-834 (2011).
118. S. P. Rosshart et al., Laboratory mice born to wild mice have natural microbiota and model human immune responses. Science 365, 461 (2019).
119. R. Gentek et al., Hemogenic Endothelial Fate Mapping Reveals Dual Developmental Origin of Mast Cells. Immunity 48, 1160-1171 e1165 (2018).
120. C. L. Scott et al., Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. Nat Commun 7, 10321 (2016).
121. M. Aouadi et al., Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation. Nature 458, 1180-1184 (2009).
122. G. J. Tesz et al., Glucan particles for selective delivery of siRNA to phagocytic cells in mice. Biochem J 436, 351-362 (2011).
123. C. C. Bell et al., Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. Sci Rep 6, 25187 (2016).
124. M. Kozyra et al., Human hepatic 3D spheroids as a model for steatosis and insulin resistance. Sci Rep 8, 14297 (2018).
125. S. U. Vorrink et al., Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. FASEB J 31, 2696-2708 (2017).
126. E. Barreby, P. Chen, M. Aouadi, Macrophage functional diversity in NAFLD - more than inflammation. Nat Rev Endocrinol 18, 461-472 (2022).

127. R. W. Lynch et al., An efficient method to isolate Kupffer cells eliminating endothelial cell contamination and selective bias. J Leukoc Biol 104, 579–586 (2018).
128. Y. Chen et al., Mitochondrial Metabolic Reprogramming by CD36 Signaling Drives Macrophage Inflammatory Responses. Circ Res 125, 1087-1102 (2019).
129. S. Spahis, E. Delvin, J. M. Borys, E. Levy, Oxidative Stress as a Critical Factor in Nonalcoholic Fatty Liver Disease Pathogenesis. Antioxid Redox Signal 26, 519–541 (2017).
130. K. Mulder et al., Cross-tissue single-cell landscape of human monocytes and macrophages in health and disease. Immunity 54, 1883-1900 e1885 (2021).
131. C. A. Dutertre et al., Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells. Immunity 51, 573–589 e578 (2019).
132. J. Zhao et al., Single-cell RNA sequencing reveals the heterogeneity of liver-resident immune cells in human. Cell Discov 6, 22 (2020).
133. J. Ma et al., Laboratory mice with a wild microbiota generate strong allergic immune responses. Science immunology 8, eadf7702–eadf7702 (2023).
134. I. Borozan et al., Gene expression profiling of acute liver stress during living donor liver transplantation. Am J Transplant 6, 806–824 (2006).
135. M. Y. Donath, Targeting inflammation in the treatment of type 2 diabetes: time to start. Nat Rev Drug Discov 13, 465–476 (2014).