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Kupffer Cell Maintenance in Tissue Repair and Ageing

Kémy Adé

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Kupffer Cell Maintenance in Tissue Repair and Ageing

Doctoral thesis in Immunology,
Ecole doctorale Complexité du Vivant, Sorbonne Université

Presented by
Kémy Adé
Macrophages and Endothelial Cells
Department of Developmental and Stem Cell Biology, Department of Immunology
Institut Pasteur

Directed by Elisa Gomez Perdiguero

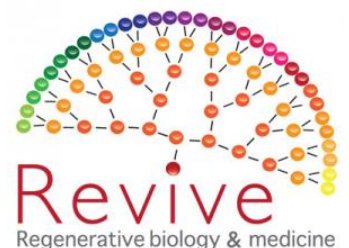
Publicly presented and defended on 16 December 2021
in front of a jury composed of:

Dr. Ana-Maria Lennon-Duménil
Dr. Alexandre Boissonnas
Prof. Dr. Christian Schulz
Prof. Dr. Charlotte L. Scott
Dr. Elisa Gomez Perdiguero

Examiner
Examiner
Reviewer
Reviewer
PhD Supervisor



European
Research
Council



To my son, Louis-Andréa

*« dans le calme des nuits, je cherche les mots, le poème
qui répandront dans ton esprit une vive lumière,
pour te révéler enfin le profond secret des choses. »*
Lucreèce¹

¹, Lucretius, T. C., & Kany-Turpin, J. (1997). De la nature, I, 142-145. Flammarion.

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ABBREVIATIONS

AAP	Acetaminophen
AGM	Aorta-Gonad-Mesonephros
AILI	Acetaminophen Induced Liver Injury
ALD	Alcohol induced Liver Disease
AMR	Ashwell Morel Receptor
APP	Acute Phase Proteins
BMP	Bone Morphogenetic Protein
CCL	Chemokine Ligand
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CDP	Common Dendritic cell Progenitor
CHOP	C/EBP Homologous Protein
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CSF	Colony Stimulating Factor
CX3CR1	C-X3-C Motif Chemokine Receptor 1 (Fractalkine)
DAMP	Damage Associated Molecular Pattern
DT	Diphtheria Toxin
DTR	Diphtheria Toxin Receptor
ECM	Extracellular Matrix
EMP	Erythro-myeloid Progenitor
Flt3	Fms-like Tyrosine Kinase
GMP	Granulocyte Monocyte Progenitor
GSH	Glutathione
HMGB1	High Mobility Group Box 1
HSC	Haematopoietic Stem Cell
IL	Interleukin
IM	Infiltrating Macrophage
KC	Kupffer Cell
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LSEC	Liver Sinusoidal Endothelial Cell
MEP	Megakaryocyte Erythrocyte Progenitor
MGL	Macrophage Galactose Lectin
MSR1	Macrophage Scavenger Receptor I
MtB	Mycobacterium tuberculosis

NAFLD	Non Alcoholic Fatty Liver Disease
NAPQI	N-acetyl-p-benzoquinone imine
NASH	Non-Alcoholic Steatohepatitis
NK	Natural Killer Cell
OH-TAM	Hydroxy Tamoxifen
PAMP	Pathogen Associated Molecular Pattern
PLX	Plexxikon
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
SAM	Scar Associated Macrophages
SASP	Senescence Associated Secretory Phenotype
TAM	Tamoxifen
TGF	Transforming Growth Factor
TICAM	Tlr domain Containing Adaptator Molecule
TLR	Toll-Like Receptor
YFP	Yellow Fluorescent Protein

INTRODUCTION

Macrophages are phagocytic innate immune cells that contribute to the first line of defence against pathogens and other tissue damages. They are present in all solid organs of the body, where they perform functions specific to their tissue. They were first described by Elie Metchnikoff in 1883, and he coined their name big (macro) eaters (phages)(Gordon, 2016)). Although they have been known for about 150 years now, they have not yielded all their secrets yet. During my PhD I focused on liver resident macrophages, the Kupffer cells. I worked on unveiling their mechanisms of maintenance in different physiopathological conditions, and their functional implications.

1. Macrophages, swiss army knives of the innate immune system

1.1. The innate immune system

The immune system is the network of cells and organs by which our body defends against external invaders (virus, bacteria) but also internal disorders like cancer. This system of defence is composed of (i) cells that attack pathogens and “abnormal” cells and (ii) secreted molecules that modulate the action of the cells (cytokines) or directly bind pathogens to facilitate their elimination (complement and antibodies). In order to efficiently protect the body, the immune system must distinguish threats from harmless elements: it learns to tolerate beneficial external elements like nutrients and recognize potential dangers.

The immune system can be divided into two main components: innate and adaptive. Innate immunity is the first line of defence composed of cells that rapidly and non-specifically recognize pathogens and other danger signals. They act to clear pathogens and dead cell debris. They are also able to recruit the adaptive immune response, that is slower to mount but more specific, with a memory of previous infections.

At the centre of the innate immunity are phagocytes: neutrophils, macrophages, and dendritic cells. They engulf and degrade pathogens or dead cells and can subsequently present elements of these threats at their surface in order to activate the adaptive immunity (Figure1).

1.2. The origins of Macrophages

From birth, the bone marrow is the main source of blood cells. There, multipotent haematopoietic stem cells (HSCs) continuously give rise to progenitors, while maintaining their pool, through proliferation. Studies on the origins of the haematopoietic system stemmed from observations made on victims of the Hiroshima and Nagasaki bombings. Scientists remarked that some survivors died long after exposure because of a compromised immune system that did not allow them to fight infections. Subsequent irradiation

experiments in mice helped to refine our understanding. The seminal work that exposed the nature of HSCs was performed by James Till & Ernest MacCulloch in the early 60s. They subjected mice to high doses of radiation and then injected them with bone marrow cells from inbred, non-irradiated counterparts. A couple of weeks later, nodules were observed in the spleen, and were shown to be clusters of cells that they called Colony Forming Units in spleen (CFU-S) (Steensma & Kyle, 2021). These were later identified as pluripotent haematopoietic progenitors with two defining characteristics: the ability to differentiate into all blood cells (multipotency) and the ability for self-renewal. More experiments were needed to refine the embryological origins of these progenitors. It is now well characterized that all Haematopoietic Stem Cells (HSC) present in the adult bone marrow were generated exclusively during embryonic development, in the aorta-gonad-mesonephros (AGM) region and other major arteries (vitelline and placental) (Cumano et al., 1996).

Since the 1960s, our understanding of the origin of macrophages is based on the mononuclear phagocyte system model established by Zanvil A. Cohn and Ralph van Furth in the late 60s when they used thymidine labelling to trace monocytes in bone marrow and peripheral blood (van Furth & Cohn, 1968). In this model, monocytes come from a cascade of differentiation that starts when HSCs give rise to the Common Myeloid Progenitor (CMP), which in turn differentiates into the Granulocyte Monocyte Progenitor (GMP). Once GMPs give rise to monocytes, they enter the circulation and can, depending on the cues provided by their environment, differentiate into macrophages or dendritic cells (Figure 1). In this model, macrophages are terminally differentiated cells that do not proliferate and are sustained by continuous recruitment of their precursors, the blood monocytes.

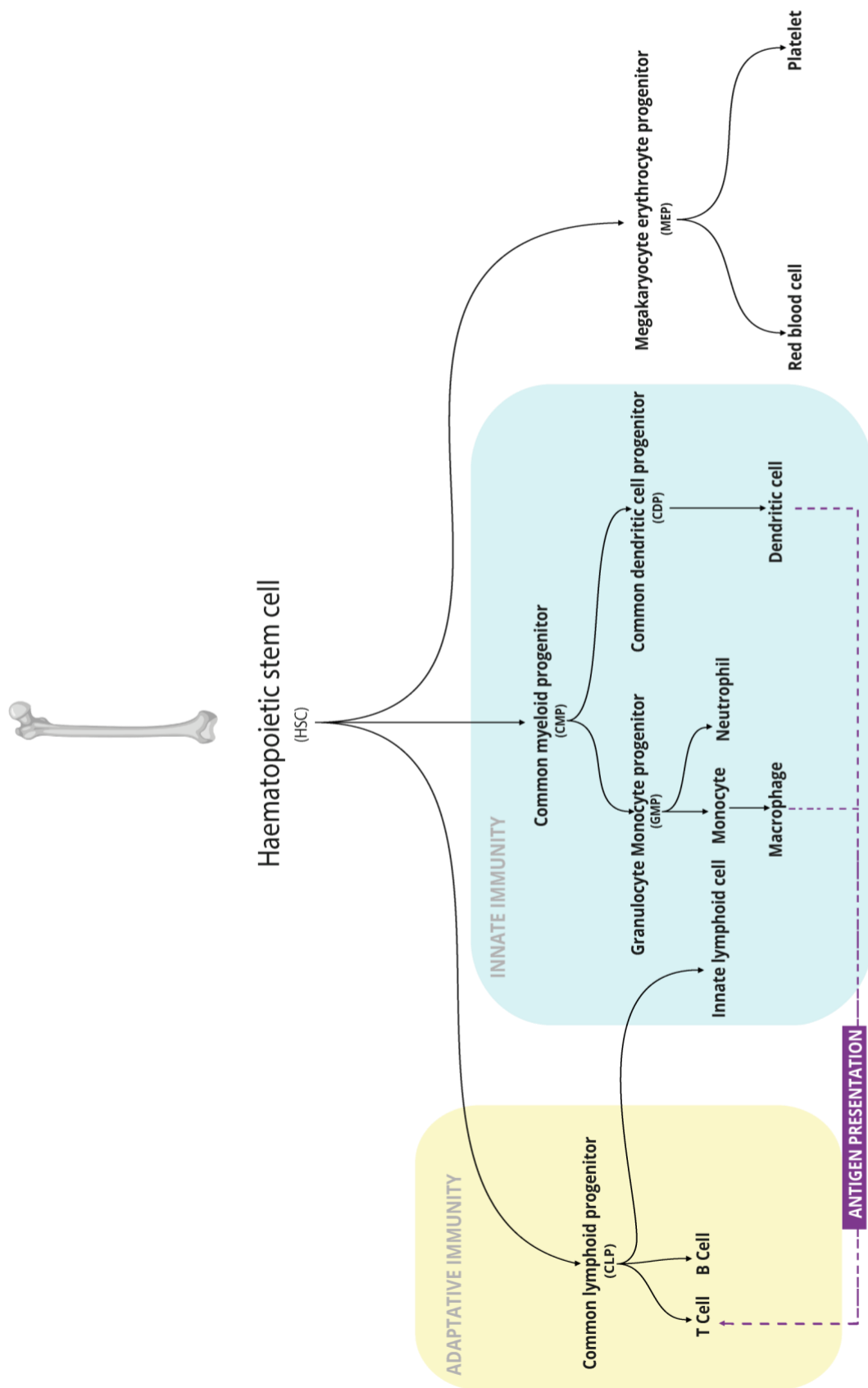


Figure 1 Simplified and schematic representation of the adult bone marrow haematopoiesis

Although this model accurately describes the generation of macrophages that are transiently present in the tissues (such as the macrophages in intestinal lamina propria (Bain et al., 2014) and those recruited after injury), it has been recently demonstrated that, in steady-state, macrophages that reside in many different organs do not originate from HSCs and are not maintained by the differentiation of monocytes (D. Hashimoto et al., 2013; Schulz et al., 2012; Yona et al., 2013).

These macrophages are sessile, often radio-resistant and long-lived and they have been thus termed resident. They arise from transient progenitors named Erythro Myeloid Progenitors (EMP) that emerge in the embryo from the extra-embryonic yolk sac prior to HSC emergence (Gomez Perdiguero et al., 2015; McGrath et al., 2015). EMP are independent from HSC and they differentiate locally in the yolk sac niche into macrophages and megakaryocytes (Iturri et al., 2021) and then into granulocytes, monocytes, mast cells and red blood cells in the fetal liver niche (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015). EMP-derived macrophages and/or Macrophage precursors are found in fetal circulation and colonize all tissues before birth (Mass et al., 2016; Stremmel et al., 2018), where they can persist there during adulthood, depending on the tissue.

In the adult liver, most macrophages that are present in steady state come from this origin. Indeed, the pool of macrophages established in these early embryonic days self maintains in the liver by local proliferation in steady state. In case of inflammation, they are transiently joined by monocyte-derived macrophages from the circulation (Shi & Pamer, 2011), as described by the mononuclear phagocyte system. This elicits the question of whether, over time, these monocyte-derived macrophages that can be generated regularly from the bone marrow in great numbers, can replace the resident macrophages established at birth, as the latter's proliferative capability wanes (Figure 2).

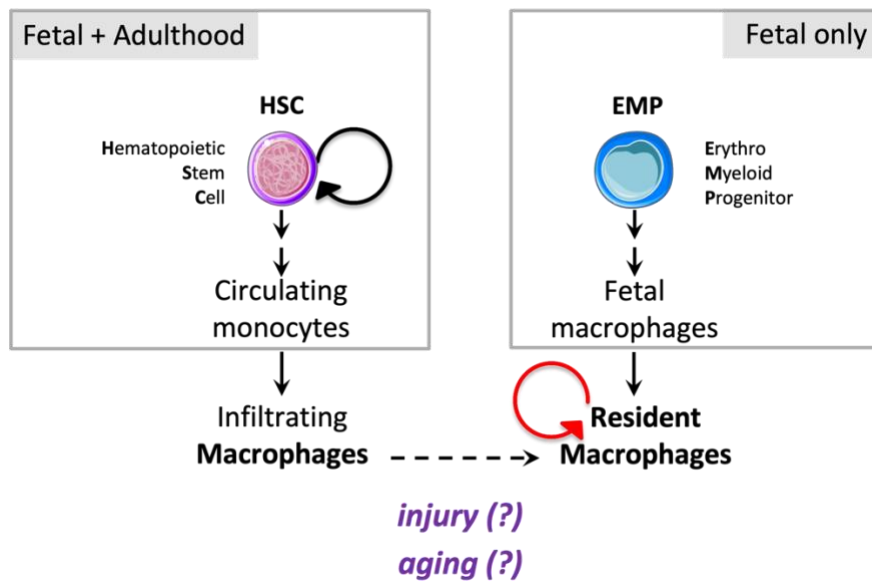


Figure 2 Two lineages of macrophages coexist in the liver, with resident macrophages been the predominant subset in homeostasis and infiltrating macrophages expanding during inflammation

1.3. Inflammation

Inflammation is the initial reaction to tissue injury or infection mediated by the innate immune system. It is triggered by harmful stimuli (tissue damage or the intrusion of a pathogen), sensed by local immune cells like resident macrophages but also by non-immune cells present at barriers, like epithelial cells and intestinal enterocytes. To sense these Danger Associated Molecular Patterns (DAMPs, endogenous molecules that are released by damaged or dying cells) and Pathogen Associated Molecular Patterns (PAMPs, conserved molecular structures produced by microorganisms), they possess a variety of cell surface receptors called Pattern Recognition Receptors (PRRs) (reviewed in (Takeuchi & Akira, 2010)). The activation of these receptors induces a cascade of reactions that culminate with the production of cytokines and chemokines. In a very schematic and simplistic manner, the main pathways leading to cytokine gene expression from PRR activation are the NF κ B pathway and the inflammasome pathway. In the latter, a cascade of protein cleavages after activation of the inflammasome complex leads notably to the production of IL1 β together with pyroptosis of the immune cell, a form of inflammatory programmed cell death (Guo et al., 2015).

Cytokines and chemokines in turn contribute to the recruitment and activation of more immune effectors to clear the menace. The effects of cytokines are not restricted to the immune cell compartment, as they can also modify the permeability of the local endothelium to promote immune cell infiltration in the tissue (Marcus et al., 1996; Skaria et al., 2016).

Upon arrival onsite, the recruited immune cells will help eliminate the pathogens and debris and promote the repair of damaged tissues. Once the menace is cleared, inflammation must be resolved, to prevent further damage to the tissue either by destruction of sound cells or excessive scarring (fibrosis). Resolution can be promoted through the removal of the trigger, through the inhibition of proinflammatory cytokine production or inhibition of their action, and the apoptosis of recruited immune effectors and their removal (deCathelineau & Henson, 2003; Feehan & Gilroy, 2019) .

1.4. Ageing and the innate immune system

At the cellular level, and across all body systems, nine hallmarks of ageing were identified : genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, impaired sensing of nutrients, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and impaired intercellular communication.(López-Otín et al., 2013).

The collective effects of ageing on the immune system are called immunosenescence. Hallmarks of this process are low grade systemic inflammation, a general higher susceptibility to infections and a less efficient tissue repair process (Sadighi Akha, 2018). In the elderly, adaptive immune responses are impaired, as evidenced by poor vaccination success.

In aged mice, higher levels of C-reactive protein (CRP) and inflammatory cytokines have been measured. This general inflammation, termed Inflammageing, may be the result of repeated activations of the immune system by infections combined with the longer duration of the inflammatory episodes because of a less efficient immune response. Moreover, with time, there is an accumulation of dead cells and other debris that the body fails to clear, and these can trigger an innate immune response (Allaire & Gilgenkrantz, 2020).

Regarding the production of immune cells, a general bias toward myelopoiesis has been observed in the bone marrow stem cell pool (Rossi et al., 2005). Several cell-intrinsic regulators of HSC dysfunction in aged animals have been identified (genomic instability associated with DNA repair deficiency (Mohrin et al., 2010; Rossi et al., 2007) or replication stress (Flach et al., 2014) autophagy and metabolic deregulation (Ho et al., 2017) mitochondrial stress (Luo et al., 2019; Mohrin et al., 2015), epigenetic drift linked to replicative lifespan (Beerman et al., 2013; Sun et al., 2014). There has also been widespread interest recently in the concept of “rejuvenating” stem cells in aged animals, either by blood born components or by rejuvenating their niche. However, limited benefit was observed when transplanting old HSC into young BM (Kuribayashi et al., 2021), in heterochronic parabiosis or in caloric restriction settings (Ho et al., 2021).

Some studies have suggested that macrophage functions were altered with ageing, with impaired phagocytosis, and decrease ROS and cytokine production. But these results have not been recapitulated in other works (Gomez et al., 2008).

2. The liver

2.1. General considerations

The liver is, in mass, the biggest solid organ of the body. It participates in vital metabolic, endocrine, detoxifying and clearance functions.

2.1.1. Development

In the mouse, cells from the definitive endoderm form a tube at embryonic day (E)7.5 that can be divided in three domains: the foregut, midgut and hindgut. The liver starts developing from the endoderm as early as E8.5. Liver budding occurs from E9.5 onwards, when the ventral part of the foregut directly adjacent to the cardiac mesoderm starts thickening, and form a monolayer of cells that turns into a multilayer of hepatoblasts. These then separate from their basal lamina and proliferate to invade the septum transversum and form the liver bud, enveloping the umbilical and vitelline vessels. From E10, the liver acts as a site of haematopoiesis, while hepatoblasts are actively proliferating and driving liver growth. Hepatoblasts and haematopoietic progenitors thus interact throughout embryo development and influence each other. From E13.5 until birth, hepatoblasts then undergo several rounds of differentiations to form hepatocytes and cholangiocytes (the epithelial cells that line the bile duct)(Gordillo et al., 2015).

2.1.2. General anatomy

The liver is at a central place in the mouse and human body metabolism: it sits at the junction between the intestinal portal vessels and the general circulation (Figure 2) (Prin et al., 2015). This provides it with a unique position to sample all nutrients derived from the intestinal absorption but also to act as a first line of defence against pathogens, microbial-derived products and drugs derived from the gut.

The adult mouse liver is divided into four main lobes: a central one to which the gall bladder is attached, two lateral (left and right) and caudal one that is leaf shaped (Frith et al., 1981). The liver is coated by a thick membrane called the capsule. It is formed of two layers: an external, thin serous layer that comes from the peritoneum and an internal thick and fibrous layer called the Glisson capsule (Lee et al., 2008).

The liver is irrigated by the hepatic artery and portal vein that give rise to multiple branches and respectively bring oxygenated and deoxygenated blood. This inflow passes through a unique network of polarized sinusoids. Its deoxygenated “filtered” blood in turn flows out through the hepatic veins to the inferior vena cava (Elsayes et al., 2017).

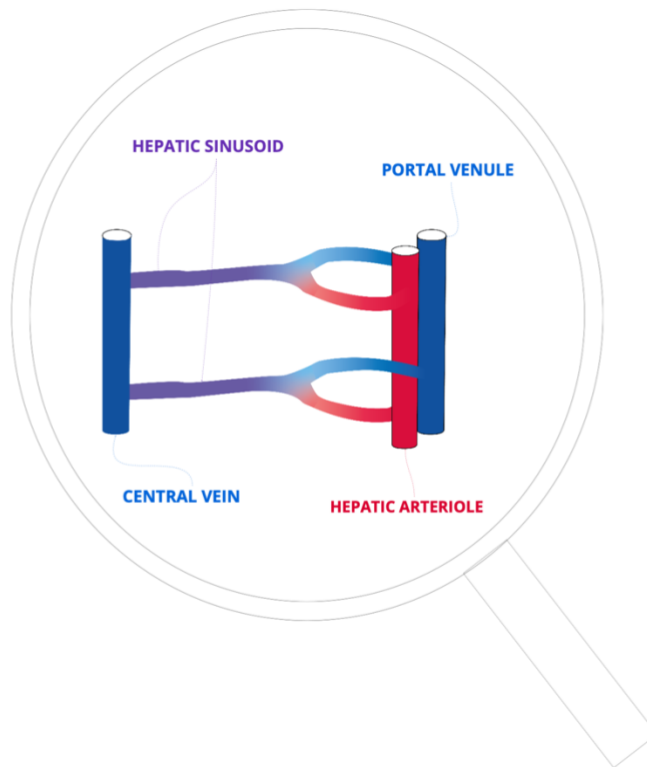
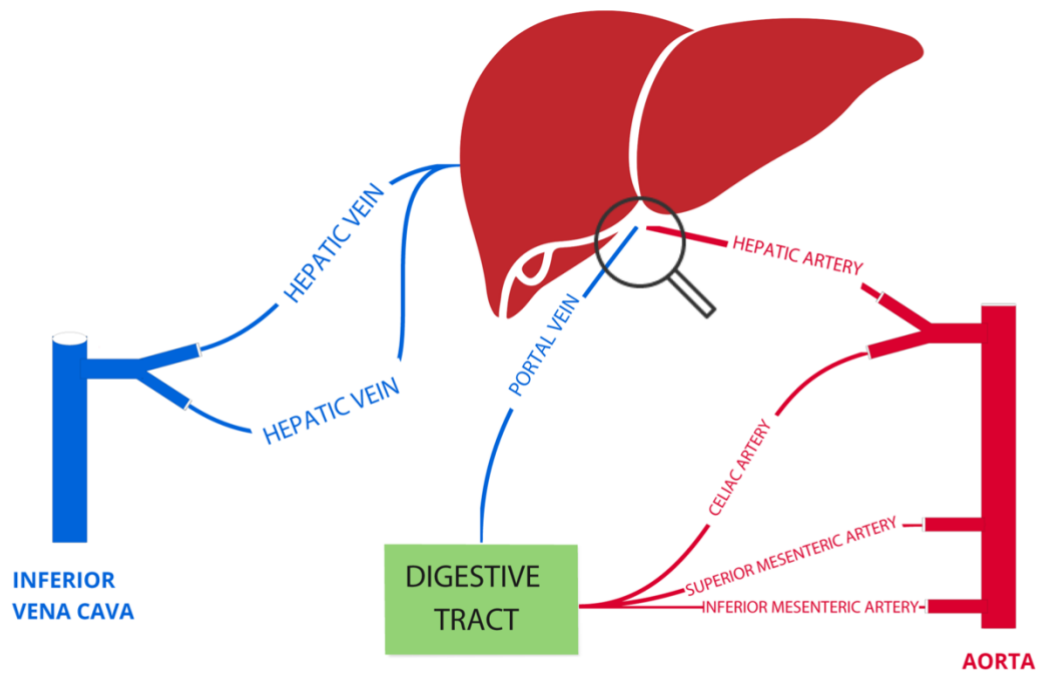


Figure 3 : The liver is at the centre of the splanchnic circulation, adapted from Prin et al., 2015

The functional unit of the liver is the lobule. It is a polygonal structure that consists of cords of cells organized around the central vein and delimited by portal triads. These triads are composed by a portal vein, a bile duct, and a hepatic artery. The lobule functions are compartmentalized: there is a gradient of oxygen and nutrients from the portal vein to the central one. This creates a functional polarization where hepatocytes from different parts of the lobule express different genes and present different metabolisms (Droin et al., 2021): this is the liver zonation (Figure 4).

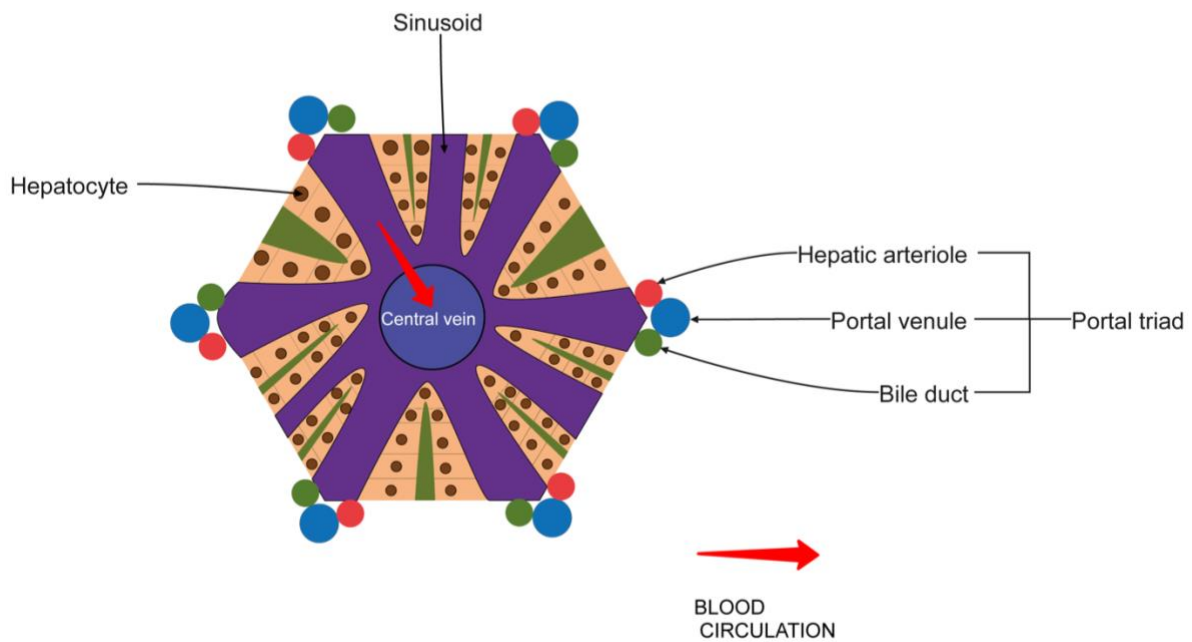


Figure 4 : Cross section of the liver lobule

Apart from the hepatic mesenchyme constituted by hepatocytes and cholangiocytes, the liver lobule presents two other spaces: a network of fenestrated sinusoids and the space between this network and the hepatocytes, called space of Disse.

2.1.3. Functions

The liver is one of the key players of glucose homeostasis. Upon feeding, there is an increase in the blood levels insulin, a pancreatic hormone that stimulates glucose uptake by hepatocytes and its storage in the form of glycogen. Conversely, in periods of fasting, high levels of glucagon ensure that glycogen is catabolised, and glucose is liberated in the circulation to provide energy to the different tissues.

The liver also plays a central role in the metabolism of lipids. Its production of bile allows the breaking down and uptake of fats. After intestinal absorption, these digested lipids can provide energy for the liver itself or other organs. Lipids can be stored as triglycerides in the

hepatocytes, and then transported with low-density lipoproteins (LDLs) to other tissues: the muscles to provide energy, or the adipose tissue for long-term storage. Cholesterol can be extracted from the diet or synthesized de novo by the liver.

The liver is also the major producer of proteins in the body. Most of them are albumin, present in the blood to limit its volume and carry macromolecules. Other proteins produced by the liver are immune modulators (Acute Phase Proteins, Complement) and growth factors. The liver also catabolizes proteins to provide amino acids (Trefts et al., 2017).

Finally, the liver acts as a detoxifying organ that clears the bloodstream of drugs, infectious agents, ammonia, and bilirubin. It is notably able to convert lipophilic elements into metabolites that can be excreted into urine.

2.2. Liver cell types

2.2.1. The hepatocytes

The effectors of liver primary functions are the hepatocytes, which constitute around 80% of the liver mass. They are large polygonal polarized epithelial cells with one or two round nuclei. Hepatocytes can either interact with sinusoids (the liver vessels) or other hepatocytes (Figure 5). They harbour many mitochondria, which shows the importance of their metabolic activity. They also present large networks of endoplasmic reticulum that is an indication of their intense synthesis activity.

Hepatocytes also play a prominent role in the modulation of the innate immune response (Zhou et al., 2016). In response to inflammatory stimuli, hepatocytes rapidly produce a variety of immune proteins called the Acute Phase Proteins (APPs). APPs can either directly act to kill pathogens or activate other components of the immune system. For instance, hepatocytes are the main producers of CRP. A homopentameric protein, CRP binds to polysaccharides found at the surface of bacteria and activates the complement reaction cascade. This cascade of protein cleavages helps in the clearance of pathogens through opsonisation (the marking of cell targets to facilitate their elimination), direct lysis, or recruitment of adaptive immune effectors. Hepatocytes produce most of the elements of the complement reaction.

The epithelial cells that line the bile ducts are called cholangiocytes and won't be discussed further here.

2.2.2. The hepatic stellate cells

The liver also comprises a population of star-shaped cells located in the space of Disse called stellate cells (Figure 5). They were first described by von Kupffer as black star shaped cells (Starzellen) on a red background when stained with gold chloride (Senoo et al., 2013). They have long processes that they project through the basal membranes to wrap around endothelial cells tubes. On their other side, they contact hepatocytes.

In steady state, hepatic stellate cells are quiescent and store Vitamin A in lipid droplets, they are the main site of storage of this vitamin in the body. In inflammation, stellate cells also play a role in the mounting of an immune response by producing cytokines. (Friedman, 2008). Upon activation, stellate cells can also transdifferentiate into fibrogenic myofibroblasts. By producing components of the extracellular matrix such as glycoprotein and collagen, myofibroblasts play a central role in the onset of fibrosis, a condition characterised by the excessive deposition of scarring tissue (reviewed in (Tsuchida & Friedman, 2017)

2.2.3. The Liver Sinusoidal Endothelial Cells (LSECs)

LSECs are specialized endothelial cells that form the liver unique fenestrated, basal membrane-lacking endothelium: the sinusoids. LSECs are present at the interface between the blood flow and the liver parenchyma. Their fenestrae are regrouped in structures called sieve plates that actively regulate blood filtration and maximize the contact between hepatocytes and the content of the circulation (Figure 5).

Their origin has been subject to intense scrutiny and debate, and it is now believed that they come from a dual origin: some LSECs derive from the hemangioblast a progenitor for blood and endothelial cells and others from the endocardium (Poisson et al., 2017).

LSECs form a selective permeable frontier and are also the effectors of several biological functions. They are involved in the maintenance of a low blood pressure in the portal veins. This low blood flow is critical as it slows down antigens and particles, thus allowing their efficient uptake and clearance.

Through their high endocytic activity, LSECs also play a role in the clearance of particles and molecules from the blood, together with Kupffer Cells (Deleve et al., 2008). This endocytic activity is part of their immune role, as they identify and engulf viral particles. They notably present many members of the Pattern Recognition Receptors (PRRs), such as the Toll Like Receptors (TLRs) that allow them to identify Pathogen Associated Molecular Patterns (PAMPs). They further modulate the immune response through the activation of their inflammasome to produce cytokines. They also take part in the inhibition of fibrogenesis by maintaining hepatic stellate cells in a quiescent state. Before the initiation of fibrosis, LSECs lose their fenestrations in a process called capillarization. It has been shown that in this capillarized state, LSECs are unable to control the activation of stellate cells (Deleve et al., 2008).

2.2.4. Hepatic stem cells

Hepatic stem cells have been hypothesized to exist in the liver, taking part in its regeneration after injury. In rat, “oval cells” were observed in injury where hepatocytes did not proliferate. It was proposed that, in certain contexts, these stem cells would differentiate into new hepatocytes when hepatocyte proliferation was not sufficient. But their existence has not been formally proven, and some extensive studies failed to find any evidence of their presence (Grompe, 2014; Yanger et al., 2014). In the mouse, it is now demonstrated by lineage tracing that the hepatocyte epithelium is capable of robust regeneration. When hepatocyte proliferation is experimentally impaired during regeneration, cells originating from the biliary epithelium (cholangiocytes) can act as Hepatic stem cells and take over hepatocyte regeneration, in particular in chronic liver injury (Raven et al., 2017).

2.2.5. Innate immune cells

With its unique position at the centre of the digestive circulation, the liver plays a major role in detecting and fighting infections. To help prevent the onset of systemic infections and maintain its own integrity, the liver harbours a variety of immune cells that are primarily located in the sinusoids. In steady state, these cells have been known to exert a tolerogenic activity to prevent the liver from overreacting to the abundance of antigens it encounters daily (Jenne & Kubes, 2013).

	Phenotype	In steady state
Neutrophils	CD45+ CD11b+ Ly-6G+	rare
Dendritic Cells	CD45+ CD11b+ CD11c+ MHCII+	present
NK cells	CD122 ⁺ CD3 ⁻ CD19 ⁻ NK1.1 ⁺	abundant
Kupffer cells	CD45+ CD11b+ F4-80+ TIM4+ CLEC4F+	abundant
Capsular Macrophages	CD45+ F4-80+ CD11b ^{lo} CD11c ^{lo} TIM4 ^{neg}	present
Monocytes	CD45+ CD11b+ Ly-6C ⁺ or -	rare
Infiltrating Macrophages	CD45+ CD11b+ F4-80+ TIM4 ^{neg} CLEC4F ^{neg}	rare

Table 1: Innate Immune Cells of the Liver and their main cell surface markers

2.2.5.1. Kupffer cells

The liver harbours the most abundant population of resident macrophages: the Kupffer cells (KCs). Described in 1876 by Karl Wilhelm von Kupffer, these sessile macrophages are defined as F4-80+ TIM4+ CLEC4F+ cells that reside in the fenestrated sinusoids of the liver (Figure 5), directly in contact with the circulation. Through their long projections, they interact with other cell types like dendritic and stellate cells. Kupffer Cell identity is different from other resident macrophages in the body and is instructed by signals from their niche. Their terminal differentiation is mediated by specific transcription factors like LXR α , SPIC and ID3 (Sakai et al., 2019).

They are reviewed in more detail page 32.

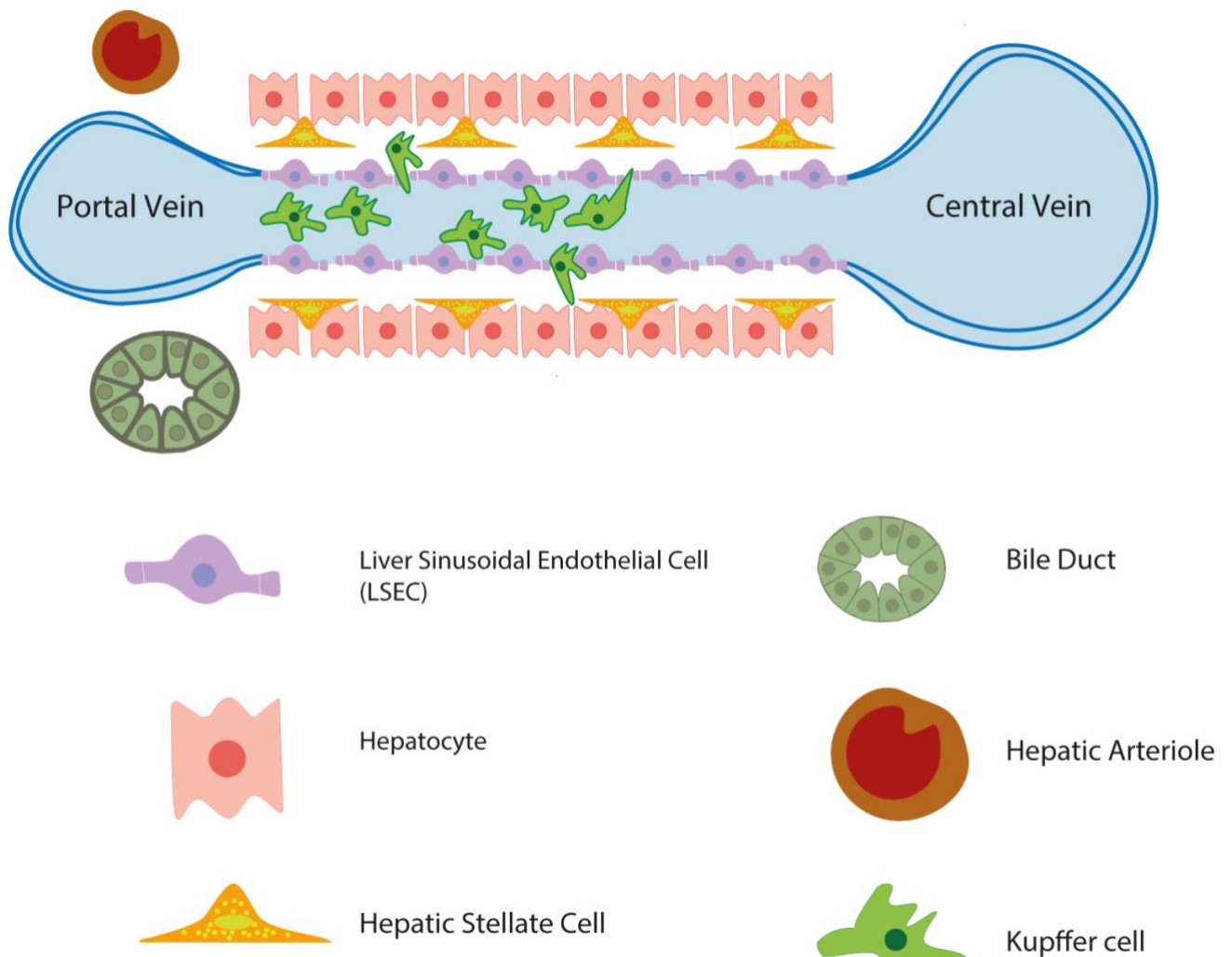


Figure 5 : Main cell types found in the steady state liver

2.2.5.2. Liver Capsular Macrophages (LCMs)

In the late 80s, a population of MHCII positive cells was described and ascribed to the dendritic cell family (Prickett et al., 1988). In the following years, different papers described these populations under different names from KCs to stellate cells, and their real identity remained elusive. Recently, they have been described as a population of macrophages distinct from the KC pool (S. A. Park et al., 2018; Sierro et al., 2017). The authors propose that they are paramount to the defence of the liver against pathogens derived from the peritoneum as opposed to KCs that take charge of blood borne intrusions

They express key macrophage markers (F4-80, CX3CR1, CSF1R, MHCII) but express low levels of CD11b and the DC CD11c marker and lack TIM4. Irradiation followed by bone marrow transplantation, together with liver transplants and parabiosis, showed that these LCMs received significant contribution from the circulation in the absence of inflammation. Intravital microscopy also showed LCM ability to sense bacterial antigens. LCM depletion in the context of infection evidenced their role in controlling bacterial influx through the recruitment of neutrophils (Sierro et al., 2017). These intriguing findings are important for our comprehension of liver macrophages heterogeneity but only a couple of papers have characterised these macrophages so far.

2.2.5.3. Neutrophils

While they are the most common granulocytes found in mouse and human blood, very few neutrophils are present in the steady state liver. They pass through the circulation and are regularly slowed down at the surface of the endothelium through selectin-integrin interactions. They can sense chemokines produced by the inflamed liver, which will cause their arrest, and they will transmigrate into the tissue (Rossaint & Zarbock, 2013). There, they can phagocytose bacteria or debris, release cytotoxic granules containing Reactive Oxygen Species (ROS) and form Neutrophil Extracellular Traps (NETs) (Rosales, 2020). In certain injury contexts, like ischemia reperfusion, it has been reported that neutrophils can aggravate the damage by further destroying hepatocytes through their indiscriminate cytotoxic action. (Huang et al., 2015). Recently, it has also been reported that they help regulate hepatic metabolism by patrolling the liver along a circadian pattern and producing elastase to activate proteins of the fat storage pathway (Crespo et al., 2020).

2.2.5.4. Dendritic Cells (DCs)

DCs in the liver represent a heterogeneous population that derives from the circulation. They are usually characterised as CD11c⁺ MHCII⁺, although some studies have suggested that a subset of macrophages are also able to express CD11c (Itoh et al., 2017; Lloyd et al., 2008). Hepatic DCs are considered “immature” compared to other DCs in the body, as they express low levels of MHCII and costimulatory proteins (Dou et al., 2018). Hepatic DCs take part in the induction of tolerance by producing the prostaglandin E2, and when stimulated, they produce

lower levels of inflammatory cytokines compared to DCs found in the spleen, for example (Castellaneta et al., 2009).

2.2.5.5. Natural Killer (NK) cells and other innate lymphoid cells(?)

The liver harbours the largest population of NK cells in the body. They are lymphocytes with a cytotoxic activity that, contrary to CD8 T cells, do not need prior activation to perform their functions. As such, NK cells are now considered as the cytotoxic effector branch of the family of innate lymphoid cells (ILCs). NK cells were first identified in the liver by electron microscopy observation of rat liver, in the mid 1970s (Wisse et al., 1976). They were described as “pit cells” of irregular shape, found attached to the endothelium. They were found in the sinusoidal space, in close vicinity with the Kupffer cells, with which they often interacted. Their cytoplasm presented numerous small granules. These cells did not appear phagocytic and were not proliferating, except in certain pathological contexts, such as regeneration after partial hepatectomy (Peng et al., 2016). Although NK cells are rare in circulation, they represent up to half of the liver lymphocytes. In the liver, it has been shown that two lineages coexist: one that is dependent on the transcription factor Eomes and can be found in circulation, and another that is under the control of T-bet and is restricted to the liver sinusoid (Daussy et al., 2014). Together, they take part in the maintenance of a tolerogenic environment in the liver environment and in the response to microbial infections.

2.2.5.6. Monocytes

Monocytes are leukocytes produced in the bone marrow and found circulating in the blood stream. Two subsets have been identified in mice.

- Ly-6C^{hi} CX3CR1^{low} monocytes are efficiently recruited to tissues in case of inflammation and they are attracted by CCL2 and CCL7, ligands of the CCR2 receptor they express in large quantities. They will then roll at the surface of blood vessels, be immobilized, transmigrate in tissues and migrate towards the site of inflammation through a process that is not completely described (Figure 6) (Ed Rainger et al., 2015). Once in tissues, they can differentiate into macrophages and dendritic cells (Chomarat et al., 2000; Jakubzick et al., 2017).

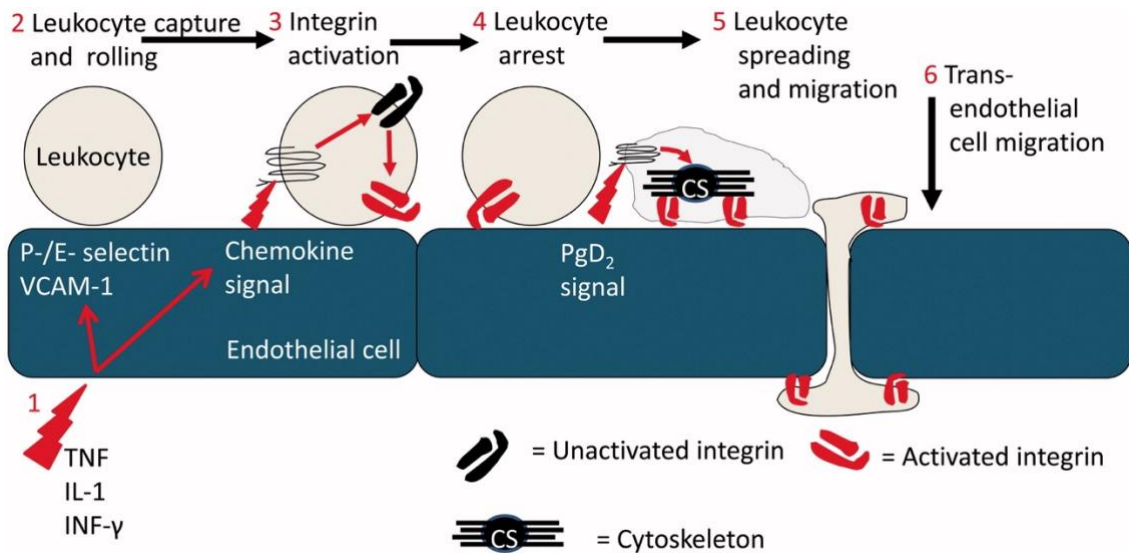


Figure 6 Canonical model of Leukocyte Adhesion and transmigration © Source : Rainger et al., Platelets, 2015

- Ly-6C^{low} CX3CR1^{hi} monocytes, also known as non-classical or patrolling monocytes, are less prevalent in the circulation but patrol the blood vessels surface by crawling on the luminal side of endothelial cells. They maintain vascular homeostasis by clearing dying or infected endothelial cells (Carlin et al., 2013). They can also be recruited to tissues, to a lesser extent than their Ly-6C^{hi} counterparts.(Shi & Pamer, 2011). It has been reported that in the tissue, they can promote the resolution of fibrosis (Ramachandran et al., 2012).

2.2.5.7. Infiltrating Macrophages

There is no consensual definition of monocyte-derived macrophages in tissues, in part due to their great heterogeneity and plasticity, depending on the tissue and physiopathological context. Great efforts are currently devoted to better define monocyte-derived macrophage subsets and to understand their functions (for example in the liver (Remmerie et al., 2020)). I chose to use the term "Infiltrating Macrophages (IM)" to describe macrophages that are the result of the differentiation of recruited monocytes, particularly Ly-6C^{hi} ones. They express common macrophage markers but lack TIM-4 and CLEC4-F expression, markers specific for KCs.

While they do not contribute to the pool of KCs in steady state, they can become the most abundant macrophage source in several disease models (Devisscher et al., 2017). Upon differentiation, they adopt a very heterogeneous transcriptomic profile that is adapted to the pathological context (Remmerie et al., 2020).

It has also been shown that monocyte-derived macrophages can start to upregulate KC markers, under the influence of the microenvironment, and adopt a transcriptomic signature

similar to KC when KC are depleted by Diphtheria toxin (DT)-based strategies. DT-mediated depletion involves both apoptosis and necroptosis of the targeted cells. In this case, an activation of neighbouring cells was observed (hepatocytes and hepatic stellate cells), that led to the release of monocyte-chemoattractants, their recruitment, persistence and acquisition of a KC phenotype (Bonnardel et al., 2019; Sakai et al., 2019) .

2.3. Liver diseases

The liver is susceptible to a wide array of diseases, both acute and chronic.

2.3.1. Acute injury and liver failure

As a central detoxifying organ, the liver must metabolise nutrients but also drugs found in the circulation. If they are present in excessive amounts, this can cause extensive damage, cell death and ultimately organ failure.

The first cause of acute liver failure in the developing world is overdose of acetaminophen (paracetamol) (Larrey & Pageaux, 2005). Upon entry in the liver, most acetaminophen is metabolised in non-toxic products that can then be excreted in the urine. A small portion is converted by the cytochrome P450 into N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive compound that must be detoxified through conjugation with glutathione (GSH). In case of overdose, there is an excess of NAPQI compared to the available GSH, and NAPQI starts conjugating with mitochondrial proteins. The resulting oxidative stress disrupts mitochondrial function and leads to massive hepatocyte death (Yan et al., 2018). Liver injury can also be caused by infectious agents, like in viral hepatitis, or insufficient perfusion of the liver (ischemic hepatitis).

Necrotic hepatocytes then release their contents, in what is recognized by adjacent cells as Danger Associated Molecular Patterns (DAMPs). Hepatic Stellate Cells and KCs are activated, and inflammation is initiated (Lafoz et al., 2020). Recruited immune effectors clear the debris and initiate the repair process. Although the purpose of inflammation is to recruit effectors that will help limit the damage, it has been proposed that inflammation itself contribute to the worsening of the injury, as recruited neutrophils for example, may further destroy the liver parenchyma (Kruger et al., 2015).

In mild injuries, the liver regenerates itself by clearing the debris and replacing the necrosed hepatocytes by local proliferation of surviving hepatocytes. But if the damage is too extensive, it can lead to rapid loss of liver function and result in life threatening liver failure.

2.3.2. Fibrosis and cirrhosis

Fibrosis is the result of unregulated wound repair. It is characterised by an excessive scarring with accumulation of extracellular matrix components. Hepatic stellate cells are key players of fibrogenesis in the liver. In short-term injury, the activated stellate cells either reverse to

quiescence or are cleared by apoptosis. But if the injury persists or is repeated, inflammation is sustained. This maintains stellate cells in their active state, stimulates their proliferation, and they continue to produce large amounts of extra cellular matrix (ECM), notably collagen fibres. This scarring tissue takes over in lieu of hepatocytes, and liver function is impaired. Advanced fibrosis is termed cirrhosis (Roehlen et al., 2020).

The aetiology of fibrosis is diverse: alcohol abuse, cholestasis, metabolic syndrome (with accumulation of glucose and lipids) can all induce repeated inflammatory episodes that can progress into fibrosis. In these diseases, fibrosis is a strong prognosis indicator.

Alcohol is the second cause of chronic liver disease after Hepatitis C. It accounts for 25% of the cases. The first step of alcohol induced liver disease (ALD) is “fatty liver” or steatosis. Only a minority of patients will further develop fibrosis and cirrhosis. Alcohol also aggravates liver injury from other causes. The liver metabolizes most of the consumed alcohol, through oxidative and non-oxidative pathways. The oxidative pathways can produce intermediate products like acetaldehyde that can damage the cells. When alcohol consumption is frequent and heavy, the mitochondrial cytochrome P450 takes part in its oxidation and produces Reactive Oxygen Species (ROS), which stimulates inflammation. In non-oxidative pathways, there is also production of a fatty acid which can evolve into steatosis (Seth et al., 2011).

Non-alcoholic fatty liver disease is another leading cause of chronic liver injury (Non-Alcoholic SteatoHepatitis, NASH). Often associated with obesity and diabetes, it involves a complex combination of lipotoxicity, perturbation of the gut microbiome, hepatocyte death and resulting permanent inflammation.(Maurice & Manousou, 2018). These processes are recapitulated in the ‘multiple hit’ model that postulates that multiple injury episodes on a genetically predisposed terrain leads to sustained inflammation and fibrosis. A diet rich in saturated fat and carbohydrates is one of the leading drivers of the disease: it increases the level of circulating fatty acids, promotes insulin resistance and *in fine* fatty acid and triglyceride accumulation in the liver. This activates TLR receptors, induces oxidative stress and the production of TNF alpha, triggering hepatocyte apoptosis and inflammation. (Bechmann et al., 2012). High fat diet also alters the gut microbiome and increases intestinal permeability. This liberates toxins and other PAMPs (like LPS) in the circulation. Uptaken by the liver, they lead to TLR activation and inflammation. Inflammatory cascades further increase hepatic lipid content. This can be further aggravated by subsequent liver injury, when cytokines produced by the affected liver stimulate lipolysis in the adipose tissue. The progression of the disease is also influenced by genetic predisposition (Wree et al., 2013).

Macrophages play multiple roles in the control of fibrogenesis. In early phases of injury, KCs and stellate cells mutually stimulate each other: KCs produce cytokines to activate stellate cells (Seki et al., 2007) which in turn produce IL-6 and CSF1 (Roehlen et al., 2020). As injury progresses, KCs provide stellate cells with TGF β to sustain their activation and stimulate the production of ECM (Cai et al., 2018). But some liver macrophages start expressing

metalloproteases to digest the extra cellular matrix and promote the resolution of fibrosis (Fallowfield et al., 2007).

In NAFLD specifically, transcriptomic analyses in the fatty liver highlighted changes in the macrophage pool, with a reduction of KCs and their replacement by a heterogenous set of IMs : a KC-like population that resides in the unaffected areas, and a CLEC4Fneg Osteopontin+ population found in the fibrotic area. This latter population may be implicated in the progression of fibrosis through the osteopontin-mediated activation of stellate cells. In contrast, KC and KC-like IMs showed no disease-related inflammatory response (Remmerie et al., 2020).

2.4. Liver Ageing

Overall, with age, the liver is affected by an increased oxidative stress burden that is associated with functional deficiencies: decreased ability for detoxification, altered production of proteins and a decrease regenerative potential after drug- or infection-induced injury (Cheng et al., 2017). Two hallmarks of liver ageing in the mouse are the accumulation of senescent cells and the increased contents of lipids.

Age has also been associated with a dysregulation of the liver microcirculation, with a reduction of the overall blood flow and the loss of the endothelial fenestrations (Maeso-Díaz et al., 2018). It has been proposed that this pseudocapillarisation creates a barrier to the free diffusion of metabolites, especially oxygen. This would lead to hypoxia in hepatocytes, which in turn may explain or contribute to their decreased function (Le COUTEUR et al., 2007). An accumulation of senescent LSECs with reduced endocytic and metabolic activity has also been reported (Grosse & Bulavin, 2020).

The aged liver presents a higher susceptibility to fibrosis: a higher prevalence of fibrotic diseases has been reported in the elderly, and some experimental results suggest it is not just the reflect of the culmination of long-term developing diseases. The aged liver is in fact more susceptible to profibrotic cues like High Fat Diet (Kim et al., 2016).

It also presents a less efficient tissue regeneration after partial hepatectomy (Pibiri, 2018).

As KCs play a large array of immune and non-immune functions in the liver, we hypothesized that any change in their biology could participate in the overall decline of liver function.

We used flow cytometry, histology and RNA sequencing to elucidate the fate of ageing Kupffer cells: their number, and the potential alteration of their functions. Our findings are recapitulated in the paper presented page 45, that is currently in submission.

3. Kupffer Cells

3.1. First and foremost, Phagocytes

As innate immune sentinels, KCs sample their environment and the bloodstream through a wide range of cell surface receptors, notably PRRs such as the TLRs and Complement receptors. When activated, KCs initiate the inflammatory response by producing and releasing cytokines to recruit effector cells like neutrophils and monocytes. Their phagocytic ability, in particular their ability to phagocytose very large particles from the bloodstream, is at the core of their functions. This is probably best exemplified by their role in the continuous clearance of senescent red blood cells (a process called efferocytosis). Phagocytosis of bacteria also leads to their activation, and in the case of *Listeria monocytogenes*, it leads to their cell death by necroptosis, which in turn releases mediators that will recruit monocytes to the site of infection (Blériot et al., 2015). It is also through phagocytosis that KCs internalise, degrade and then present antigens to activate T cells. Through phagocytosis, Kupffer cells can thus clear the consequences of tissue damage and help fight microbial infections. The timely clearance of these various debris is critical for the proper functioning of the liver and the rapid resolution of inflammation episodes.

While infiltrating or monocyte-derived macrophages need “find me” signals, chemoattractants that will guide them towards their target, for mostly sessile KCs, these signals act as early indicators of apoptosis, sensed in a paracrine manner. A diverse range of “find me” molecules have been identified. Triphosphate nucleotides (ATP, UTP) can be released through a regulated, caspase dependent process by apoptotic cells. These nucleotides can be sensed by KCs harbouring purinergic receptors such as P2X4 (Besnard et al., 2016; Elliott et al., 2009). The chemokine CX3CL1 is another well studied “find me” signal ((S.-Y. Park & Kim, 2017; Truman et al., 2008).

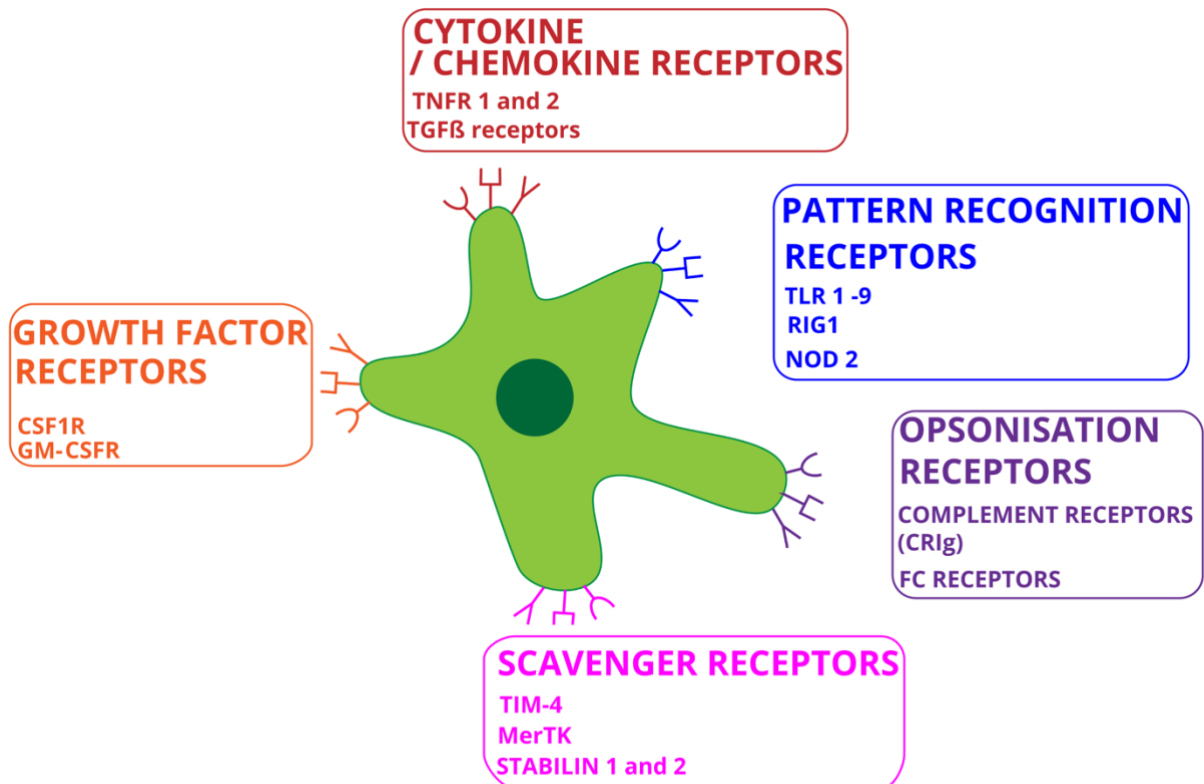


Figure 7 Examples of receptors found at the surface of KCs. Many of these receptors are related to phagocytosis (PRRs, Opsonisation receptors, Scavenger receptors).

On their surface, Kupffer Cells harbour a great variety of phagocytosis related receptors (Figure 7) with sometimes redundant functions, that are specialized in recognising different “objects” to be phagocytised.

PRRs are such receptors: this class of receptors include Toll-Like Receptors (TLRs), NOD-like receptors, RIG-I-like receptors and C-type lectin receptors (Takeuchi & Akira, 2010). Mouse KCs harbour LR 1 to 9, and can express RIG1 and NOD-2 (Woltman et al., 2014). Foreign antigens can be directly recognised by PRRs but clearance of pathogens can also occur through a process called opsonisation. Opsonisation describes the marking of phagocytosis targets by antibodies or Complement components. The Complement is a system of plasma and cell surface proteins, that are activated by a proteolytic cascade. Its activation leads to the coating of pathogens and the secretion of inflammatory mediators (Dunkelberger & Song, 2010). KCs express both receptors for the Fc fragment of antibodies (Fc Receptors) and Complement receptors such as CR1g (Helmy et al., 2006). Recognised pathogens are endocytosed, the endocytic vesicle then fuses with a lysosome that progressively acidifies, leading to the degradation of the phagocytosed object (S.-Y. Park & Kim, 2017).

However, some reports indicate that KCs may not play such a major role in the clearance of bacteria, and that their main task during infection would be the removal of bactericidal neutrophils to allow the resolution of inflammation (Gregory & Wing, 2002).

Indeed, KCs efficiently remove dying cells, process called efferocytosis. KCs recognise “eat me” signals exhibited on the surface of these cells. This recognition will trigger the engulfment and

subsequent phagolysosomal degradation. Most apoptotic cell will start exhibiting Phosphatidylserine, a phospholipid that is usually present inside the cells, on their outer membrane. This exposed phosphatidylserine can then be recognized through receptors such as TIM4, Stablin-1 and MerTK, the latter through an the Gas6 and Pros1 adapter proteins. (Horst et al., 2019; Lemke, 2017).

Phagocytosis profoundly remodels macrophages (Z. Li & Weinman, 2018). First, the newly engulfed cells provide the macrophage with a new burden of nutrients and the metabolism of the cell must be significantly enhanced in response. Moreover, sensing of these additional metabolites by intracellular receptors induces the production of cytokines such as IL-10 and TGF- β to promote the resolution of the inflammation (Figure 8) (Elliott et al., 2017).

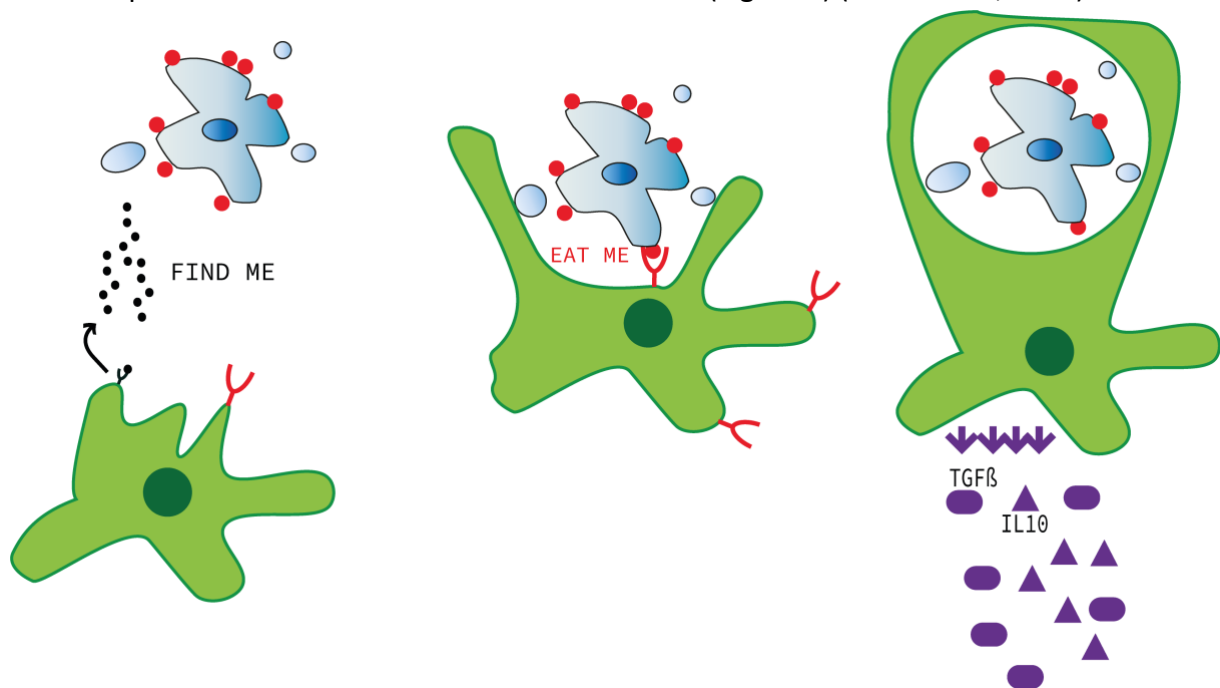


Figure 8 : Phagocytosis of apoptotic cells regulates KC cytokine production. Apoptotic cells first express paracrine “find me” signals (like nucleotides) to orient KCs in their direction. KCs can then sense the “eat me” signals (like phosphatidylserine) these cells exhibit at their surface. This will trigger the engulfment of the cell in an endosomal vesicle that will fuse with a lysosome, progressively acidify and degrade the cargo. TGF β : Transforming Growth Factor β , IL10: Interleukin 10

3.2. Transcriptional control of KC identity

Resident macrophages throughout the body share a core transcriptional programme that defines the macrophage identity. This transcriptional signature is acquired as soon as macrophages start to differentiate from EMP derived progenitors during development (Mass et al., 2016). It includes transcription factors like Maf and Batf3, but also receptors like CSF1R and CX3CR1. This core programme further includes the expression of a large array of cytokine receptors and antibody (Fc) receptors (T'Jonck et al., 2018).

The well-studied PU.1 is the master regulator of macrophage development. It is upregulated during macrophage differentiation and controls the expression of many genes, notably the CSF1R receptor. (Ross et al., 1998; Zhang et al., 1994). After tissue seeding, macrophages start to express transcriptional programs specific to their tissue of residency. DNA binding protein inhibitor (Id3) and LXRA have been specifically associated with liver macrophages. LXRA expression is itself under the control of ZEB2, a transcription factor involved in the maintenance of tissue specific macrophage identity (Scott et al., 2018). A deficiency in Id3 resulted in a reduction in Kupffer cell numbers while other macrophage populations appeared unaffected. In these mice an upregulation of Id1, which is co-expressed with Id3 was observed (Mass et al., 2016). As *Id3* and *Id1* are under the control of TGF β , at least in tumour contexts, this cytokine could play a role in establishing KC identity (Strong et al., 2013).

Interestingly, most of the KC-specific signature was only observed once the premacrophages had colonised the liver and differentiated into macrophages, and not at the precursor stage, suggesting that their tissue specialisation was not predetermined but emerged in contact with the tissue environment (Figure 9). This is reinforced by the important transcriptomic changes detected in macrophages after birth and even during weaning, when major modifications of the organ function and environment occurs. Analysis of the chromatin landscape of different adult tissue macrophage populations captured these tissue specific regulatory signatures and correlated with the functions they entail. CLEC4F expression was notably specific of KC while SpiC, a transcription factor involved in red blood cell efferocytosis was highly active in spleen red pulp macrophages and to a lesser extent KCs. Irradiation followed by bone marrow transplant experiments further supported a role of the microenvironment in shaping the chromatin landscape and the transcriptome (Lavin et al., 2014).

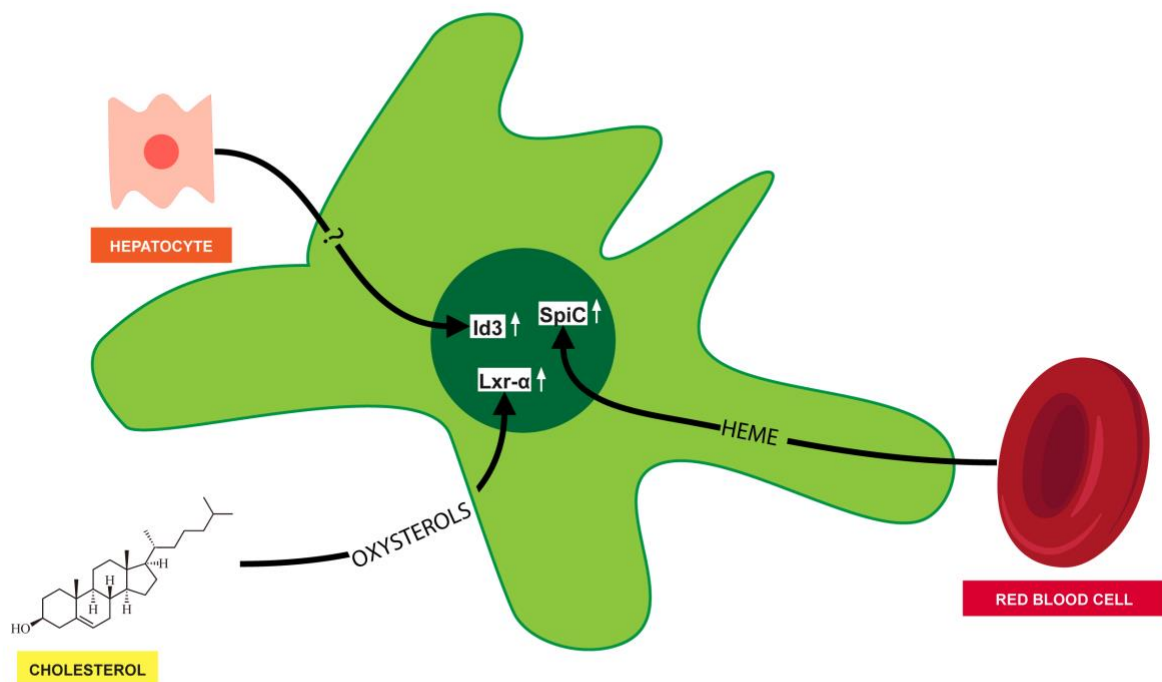


Figure 9 : Cues from the liver microenvironment help establish the KC identity by inducing the upregulation of KC specific genes.

3.3. The Kupffer Cell Niche

The niche is defined as the specific microenvironment that surrounds a certain cell type and provides the conditions for its maintenance. The niche is thus an anatomical location, an ensemble of supporting cells, and the chemical cues they produce.

In the liver, endothelial cells, hepatocytes and stellate cells directly interact with KCs. Stellate cells are the main producers of CSF1, which is the key cytokine regulating macrophage maintenance and proliferation. In experimental depletion of KCs followed by massive infiltration of monocytes, hepatocytes have been shown to induce the upregulation of Id3 in infiltrating monocytes. Moreover, the production of DLL/Notch by LSECs induced the upregulation of other core KC genes such as CLEC4F. This suggests that these three cell types constitute the niche required for acquisition of the KC identity, at the transcriptomic level (Bonnardel et al., 2019) (Figure 10).

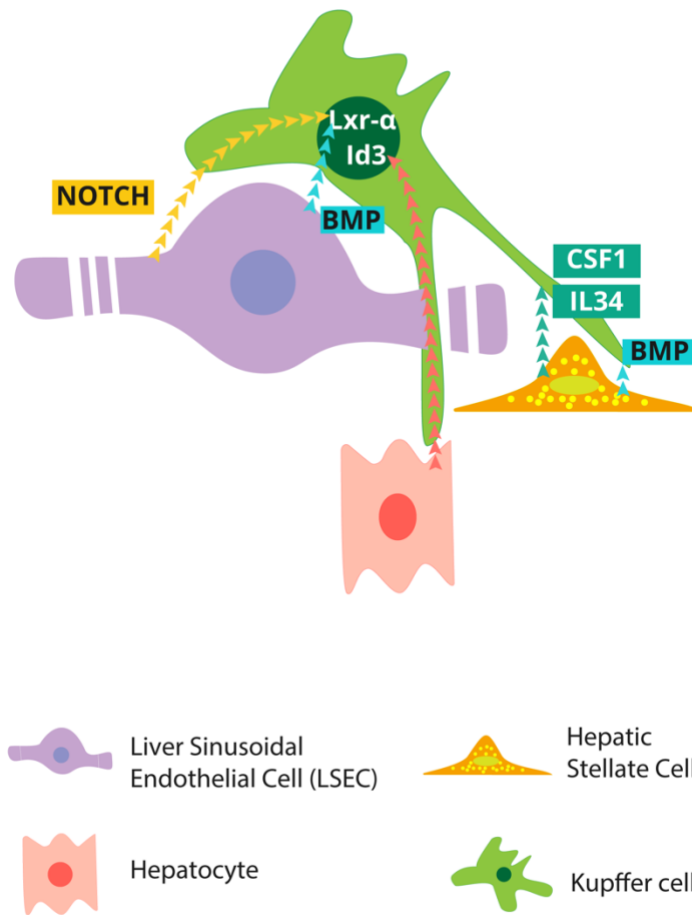


Figure 10 The Kupffer Cell Niche instructs and maintains KC identity. BMP : Bone Morphogenetic Protein, CSF1 : Colony Stimulating Factor 1, IL-34: Interleukin 34.

3.4. CSF1 signalling

Colony Stimulating Factor 1 (CSF1) is one of the main trophic factors for KCs and other tissue macrophages. It regulates their survival, proliferation and differentiation (Chitu & Stanley, 2006). It is produced both systemically and locally. CSF1 exists in 3 different forms: a membrane bound glycoprotein, and a soluble proteoglycan that acts locally, and a soluble glycoprotein that is found in circulation and can act systemically.

CSF1R is the only known receptor for this growth factor. It is a Receptor Tyrosine Kinase, like Flt3 and Kit.

Mice deficient for *Csf1* (*Csf1^{op/op}*) are osteopetrotic, due to the impairment of osteoclasts, the bone macrophage like-resorbing cells. They present neural, dermal, and fertility defects. They have decreased resident macrophages in all tissues (Cecchini et al., 1994). In the skin, only a slight and transient reduction in the number of Langerhans cells was observed, and microglia is moderately affected, and only in certain regions of the brain (Wang & Colonna, 2014). In adulthood, these two tissues rely on IL34, the only other known ligand of CSF1R (Wang et al., 2012). Consequently, CSF1R deficient mice present more severe phenotypes and increased circulating levels of CSF1 (Dai et al., 2002). Indeed, upon fixation to its receptor, the complex is internalised and degraded, thus regulating the levels of circulating CSF1.

As KC sit within the blood circulation, it could rely on systemic CSF1 in the serum and/or locally presented CSF1. Analysis of *op/op* neonates from *op/+* or *op/op* mothers shed light on the importance of serum CSF1 for KC development: compensation by circulating, maternal CSF-1 is tissue-specific and most prominent in liver (Roth et al., 1998). This was further investigated by the analysis of *op/op* animals rescued by the expression of the cell surface *Csf1* isoform (Dai et al., 2004). Expression of the membrane-bound *Csf1* isoform rescued liver macrophage deficiency at birth but failed to rescue the lack of adult KC observed in the *op/op* background.

CSF1R activation induces its dimerization and its autophosphorylation on some specific tyrosine residues. This creates binding sites for different adaptor proteins that will further activate a cascade of other tyrosine kinases to produce pleiotropic effects. Erk1/2, Akt and Stat3 are among the pathways activated, and they modulate the expression of many target genes. *Bcl-xl* and *Cyclin D1*, which inhibit apoptosis and promote proliferation, respectively, are among the identified gene targets (Hamilton, 1997).

Upon internalisation, CSF1R is not directly degraded but continues to signal from the endosome. The signalling there activates additional pathways. This may be one of the mechanisms explaining the vast perimeter of CSF1 action (Huynh et al., 2012).

3.5. Non-immune functions

3.5.1. Clearance of dead and senescent cells

KCs routinely perform the removal of aged cells from the liver and the circulation. They are notably involved in the removal of aged and/or damaged erythrocytes and platelets. The mechanisms of platelet removal have recently been elucidated, shedding light on the essential role of Macrophage Galactose Lectin (MGL) and the Ashwell Morell Receptor (AMR). Platelets continuously circulate through the liver sinusoids, and transiently contact KCs before exiting. But as they age, they lose their sialic residues, and tend to get stuck on KCs. They are then phagocytosed. This process seems to be of importance for clotting efficiency throughout the body, as impairment of this removal leads to an accumulation of desialylated platelets and an increase duration of bleeding. (Deppermann et al., 2020).

In the liver itself, senescent cells, especially hepatocytes, may accumulate, in physiological ageing or over the course of chronic diseases. Senescent cells are in irreversible cell cycle arrest, and they express proteins such as p16, p21 and p53, that prevent cell cycle progression while also inhibiting programmed cell death (apoptosis). Senescence can be triggered by DNA damages, ROS induced lesions, or extensive DNA damage (Stahl et al., 2018). It has been proposed to be a defence mechanism to prevent the proliferation of damaged cells that could turn into cancer. Senescent cells excrete a variety of molecules called Senescence Associated Secretory Phenotype (SASP). Senescent cells help initiate tissue remodelling : produced during development, they help regulate embryogenesis (Davaapil et al., 2017; Muñoz-Espín et al., 2013) and in injury contexts they promote tissue repair (DiRocco et al., 2014). But their abnormal accumulation has been involved in the onset of age-related diseases (Muñoz-Espín & Serrano, 2014). Their timely removal is thus required to maintain tissue homeostasis and KCs have been shown to participate in this process after senescent cells where targeted by NK cells in the context of liver cancer (Xue et al., 2007).

3.5.2. Iron recycling

Aged and damaged erythrocytes are sequestered in two main sites in the body: the liver and the spleen. There, they are engulfed by the resident macrophages of these tissues. As KCs engulf dying red cells, they degrade their components and recycle the iron they contain. This iron is exported through their membrane ferroportin and released in circulation to be used in newly formed red blood cells (Slusarczyk & Mleczko-Sanecka, 2021).

3.5.3. Lipid removal

KCs are also involved in lipid metabolism. Like all macrophages, they are equipped to process the lipids derived from the cells they phagocyte. But KCs present a distinct transcriptional signature enhanced with elements of lipid metabolism (Remmerie & Scott, 2018). Recently, a subset of KCs expressing the lipid transporter CD36 have been identified and linked to the regulation of glucose and lipid metabolism. Kupffer cells uptake oxidised Low-Density Lipoproteins, through the combined action of CD36 and Macrophage Scavenger Receptor I (MSR1). When lipids are present in excess, like in steatosis, KCs turn into “foam cells”, bloated macrophages filled with lipids, as observed in atherosclerotic plaques (Bieghs et al., 2012; Blériot et al., 2021).

3.6. Functions in tissue repair

As the immune sentinels of the liver, Kupffer cells are among the first to sense damage. Upon recognition of dead cells or pathogens, they can induce inflammation to recruit other effectors on the site of injury. They actively take part in the clearance of the damage but also help orchestrate tissue repair. Both deleterious and beneficial effects of KC interventions have been reported in injury contexts. In acetaminophen induced liver injury for example, it has been shown that the massive death of hepatocytes leads to the release of DAMPs, among which High Mobility Group Box 1 (HMGB1). This protein strongly stimulates the activation of KC and their production of cytokines and chemokines. This amplifies the recruitment of neutrophils that may further extend the damage. Some studies have reported that KC depletion with gadolinium chloride or clodronate improved survival after intraperitoneal injection of high doses of acetaminophen (Fisher et al., 2013). But in other works, KC depletion with clodronate was associated with more extensive damage and delayed tissue repair, and KCs were deemed protective (Holt et al., 2010; Ju et al., 2002). Furthermore MerTK+ KCs have been involved in the clearance of neutrophils to limit damages in Acetaminophen Induced Liver Injury (AILI) (Triantafyllou et al., 2018). Similarly in fibrosis, which results from chronic inflammation, opposing roles have been attributed to KCs: they can induce fibrosis after inflammation by producing TGF β , an activator of stellate cell differentiation into myofibroblasts. But their production of metalloproteases also helps resolve fibrosis by removing excess matrix (Feng et al., 2018).

The very diverse and sometimes opposing roles attributed to liver macrophages in injury contexts can be explained by:

- their plasticity: KCs functions are modulated by signals in their microenvironment that vary over the course of the injury. In response to them, KCs can modulate their metabolism and their production of cytokines and growth factors. Classically, KCs would produce inflammatory cytokines at the onset of liver injury before switching to a more restorative phenotype and producing cytokines that dampen inflammation.
- their heterogeneity: now that we understand the two lineages that coexist in the tissue in case of injury, this can inform our understanding of their respective functions.

3.7. Tools to study KC maintenance

The recent paradigm shift in the way we understand macrophage ontogeny prompted new questions: first, does the pool of KCs established during development and maintained in adulthood persist into old age? Does the local proliferation potential that sustains them in the long-term exhaust itself in ageing, thereby leading to increased contribution of monocyte-derived macrophages with age?

A second axis of investigation concerns whether ontogeny bears functional importance: in inflammation, when the two lineages coexist in the liver, are their functions segregated by origins? Does this functional heterogeneity persist after resolution of the insult?

3.7.1. Fate mapping

While phenotypic markers can help distinguish recruited macrophages in short term models of inflammation, they are not sufficient in the long term when these recruited cells have the potential to upregulate KC markers and *in fine* resemble resident KCs. Fate mapping models are thus needed to track their respective contribution.

These genetically engineered mouse lines allow us to label a population of progenitors and to follow their descendance. These “ontogeny-“reporters can either be constitutively active (*Flt3-Cre Rosa26^{YFP}*) or inducible.

3.7.2. *Flt3^{Cre} Rosa26^{YFP}*

The *Flt3^{Cre} Rosa26^{YFP}* model is obtained by crossing *Flt3-Cre* mice (where the Cre recombinase is under the control of the Fms-like Tyrosine Kinase 3 (*Flt3*) promoter) with *Rosa^{YFP}* mice (where the YFP gene is integrated into the ubiquitous *Rosa* locus preceded by a STOP cassette flanked by LoxP sites). *Flt3* belongs to the Type III family of Tyrosine Kinase receptors and is expressed in multipotent hematopoietic progenitors, but also in several committed progenitors (CLP, CDP).

To produce this line, the Cre recombinase was inserted into the exon 1 of the *Flt3* gene on a bacterial artificial chromosome. After injection of the BAC in embryonic stem cells, the successfully recombined ones are injected in a blastocyst and transferred to a pseudo pregnant mouse that will give birth to the genetically modified mice. In these mice, homologous recombination allows the random insertion of the Cre transgene into the genome. In our *Flt3-Cre* line, the insertion occurred in the Y chromosome. (Benz et al., 2008). In the cells that express the *Flt3* promoter, the recombinase will recognize the loxP sites to remove the floxed STOP cassette in front of the YFP gene and allow its expression. It is a robust

model for the tracking of bone marrow-derived macrophages, but it does not allow a time dependent control. Further, as the transgene is located on the Y chromosome, we can only analyse males with this model.

3.7.3. $Csf1^{MeriCreMer} Rosa26^{Tomato}$

To pulse label our cells of interest, we used inducible Cre lines : $Csf1^{MeriCreMer} Rosa26^{Tomato}$ (Qian et al., 2011), and $CCR2^{CreERT2} Rosa26^{Tomato}$ (Croxford et al., 2015; Lacerda Mariano et al., 2020). In these mice, the Cre is only active for a short period of time after the injection of tamoxifen. Thus, the cells that retain the labelling in the long term after the injection are the very same cells (or the descendants) that were pulsed. Mer-iCre-Mer is a double fusion protein between the murine mutant estrogen receptor (mer) and the improved Cre recombinase (Shimshek et al., 2002). CRE-ERT2 is a fusion protein that combines the cre recombinase with a mutated human estrogen receptor (Qian et al., 2011; Zhao et al., 2006).

In the absence of the estrogen analog Tamoxifen, these fusion proteins are restricted to the cytoplasm by the HSP90 chaperon protein. Upon injection of tamoxifen, the interaction between them is disrupted, and the cre recombinase can enter the nucleus to recognize the floxed regions it can act on.

CSF1R is the receptor for Colony Stimulating Factor 1 (CSF1) also known as MCSF (Macrophage Colony Stimulating Factor). It is expressed in macrophages. By injecting tamoxifen in steady state, when Kupffer cells are the only macrophages in the liver, we selectively label them. Monocytes are also labelled, but their labelling efficiency decreases rapidly several days after pulse, due to their short half-life. This model can then be used to look at the maintenance of Kupffer cells throughout time or injury as any dilution of the proportion of KC labelled by the reporter would suggest the contribution of newly arrived, non-labelled cells.

3.7.4. $CCR2^{CreERT2} Rosa26^{Tomato}$

In these mice, the Cre recombinase is under the control of the CCR2 promoter. Upon hydroxytamoxifen injection, monocytes are efficiently labelled (around 80-90% are tomato positive). This labelling then rapidly decreases, as these cells are short lived. Pulse-labelling these cells before a challenge (KC depletion or liver injury) allows to track their contribution to the maintenance or recovery of the KC pool. Monocyte contribution will translate into an increase proportion of labelled cells among KCs.

3.7.5. Selective depletion

3.7.5.1. Plexxikon

As detailed above, CSF1 is the cytokine responsible for KC survival, differentiation, and maintenance. It is produced by different cells of the KC niche, but also present in the circulation. CSF1 is recognized by cell surface CSF1 receptors (CSF1R) harboured by KCs. The blocking of these receptors by the chemical PLX5622 affects macrophage viability.

PLX was first developed to target microglia. The first generation (PLX3397) showed 99% depletion of microglia without much effect on peripheral macrophage populations after three weeks of treatment. As these cells were identified by surface markers, the authors checked if the apparent depletion of these cells was not in fact a dedifferentiation where they lost their defining markers while remaining present in the brain. They found evidence of microglia death by apoptosis after treatment. This confirms that CSF1R blocking impairs macrophage viability. (Elmore et al., 2014).

We used PLX5622, an optimised version of the compound that presents a better cell permeability and better selectivity than the original PLX3397 (Spangenberg et al., 2019). A 3-day course of treatment was sufficient to induce an efficient depletion of KCs.

3.7.5.2. Genetic models

We are currently developing a model of macrophage depletion based on the CD64-DTR strain. CD64 is a Fc receptor, also called FCYRI, expressed on resident macrophage populations (Gautier et al., 2012). CD64-DTR mice express the diphtheria toxin receptor under the control of the CD64 promoter active in resident macrophages across multiple tissues (Baranska et al., 2018). Injection of diphtheria toxin will allow the depletion of these populations. However, this depletion will likely be systemic, compared to the liver specific CLEC4F-DTR (Scott et al., 2016a). CLEC4F expression starts at E11.5, in the fetal liver (Yang et al., 2013) while CD64 can be detected as early as E10.5 (Mass et al., 2016).

Both CD64-DTR and CLEC4F-DTR could thus be amenable for the depletion of resident macrophages during development, in order to understand the contribution of ontogeny to resident macrophage functions. However, to prevent the compensation of liver macrophage depletion by the production of circulating macrophages during this embryonic window, the body-wide depletion with CD64-DTR may be more appropriate.

AIMS AND OBJECTIVES

Since the discovery that macrophages come from two distinct origins and that resident macrophage pools can be maintained into adulthood without significant input from the circulation, new questions have emerged regarding the extent of this maintenance potential into old age and the functional implications of these two ontogenies. Although it is established that KCs can locally proliferate, it is unclear whether this potential can get exhausted over time. Moreover, the ability of circulating monocytes to efficiently replace KCs has been studied in depletion contexts, but it remains to assess the extent to which this process occurs physiologically, notably in ageing. Notably, the long-term effects of inflammation on liver macrophage heterogeneity require more investigation.

Using a combination of flow cytometry, fate mapping and immunofluorescence, this project aims to study the fate of KCs in ageing and sterile inflammation contexts and the extent of circulating cell contribution to KCs during these challenges.

RESULTS

1. Kupffer Cell Maintenance in Ageing

Our characterisation of Kupffer cell maintenance in ageing is reported in the following manuscript, currently in submission.

Main Findings

Ageing is accompanied by profound alterations of the immune system, including low grade, systemic inflammation. However, the effects of age on tissue resident macrophages are not fully understood. More specifically, the fate of embryonic macrophages into old age and their interplay with infiltrating macrophages in age-related diseases are still under investigation. Kupffer cells (KC), the liver resident macrophages, are produced during embryonic life, seed their organ of residency during development and persist there during adulthood. In young adult mice, the KC pool receives no significant contribution from the recruitment and differentiation of monocytes in steady state. KCs play key roles in maintaining liver homeostasis: they monitor tissue damage and infection, clear debris, orchestrate immune responses and participate in liver metabolism.

We thus sought to elucidate the fate of KCs in ageing, regarding their maintenance from HSC-independent macrophages and/or the contribution of HSC-derived circulating monocytes, and their place in the aged liver physiology.

We first evaluated the density of KCs (cells per g of tissue) and other liver myeloid cells by flow cytometry. We found that KC density decreased with age. This decrease was not compensated by the recruitment and differentiation of monocytes, as evidenced by the number of YFP⁺ KCs in old *Flt3^{Cre} Rosa^{YFP}* mice. We also observed an increase in liver neutrophil density, indicative of inflammation. Old KC had increased size and granularity. But RNA sequencing of young and old KCS found minimal differences. Only about 80 genes were differentially expressed. Pathway analysis indicated that aged KCs presented an increase in inflammation-related genes and decrease in metabolism. Old KCs seemed to retain their phagocytic activity in bead injection assays, however, further assessment of this function in pathologic contexts will be needed. KC proliferation rate, in steady-state and after withdrawal of CSF1R inhibition, was not altered with age, as shown by EdU incorporation and Ki67 immunostaining on sections. This suggested that increased cell death underlie KC loss during ageing, which was confirmed by Tunel quantifications on sections. We identified repeated inflammation as a possible driver of this phenotype. We performed repeated poly (I:C) injections in 8-week-old mice to mimic successive infection-induced inflammation events, which reproduced the ageing phenotype. Conversely, the analysis of old *Trif^{Lps2}* mice, in which the inflammation response through TLR signalling is impaired, showed no decrease of KC numbers with age, although systemic inflammation was still present. Hepatic Stellate cells play key trophic functions for KCs, through the production of CSF1R ligands, CSF1 and IL34. Tissue section staining for hepatic

stellate cells (desmin) and CSF1/IL34 did not allow us to identify age-related changes that could account for the loss of KCs or the lack of macrophage differentiation from monocytes. Finally, the loss of KCs correlated with increased senescent cells and lipid accumulation, measured by SA- β Gal and Oil-Red-O staining, respectively. This phenotype was mitigated in *Trif^{flps2}* mutants.

My findings on age-related changes in KCs (and tissue neutrophils) could be generalised to other tissue resident macrophages, except for the brain and kidney. Altogether these data suggest that inflammation-driven loss of KCs in ageing may contribute to age related liver dysfunctions.

Inflammation drives age-induced loss of tissue resident macrophages

Kémy Adé^{1,2,§}, Javier Saenz Coronilla^{1,§}, Caroline Kaiser¹, Sébastien Mella³, Cheng Chen⁴, Lida Katsimpardi⁵, Catherine Werts⁶, Han Li⁴, Pascal Dardenne¹, Yvan Lallemand¹ and Elisa Gomez Perdiguero^{1,*}

1- Institut Pasteur, Macrophages and endothelial cells, Department of Developmental and Stem Cell Biology, UMR3738 CNRS, F-75015 Paris, France

2 - Sorbonne Université, Collège Doctoral, F-75005 Paris, France

3- Institut Pasteur, Department of Developmental and Stem Cell Biology, UMR3738 CNRS, F-75015 Paris, France

4- Institut Pasteur, Cellular plasticity and disease modelling, Department of Developmental and Stem Cell Biology, UMR3738 CNRS, F-75015 Paris, France

5- Institut Pasteur, Perception and Memory Lab, Neuroscience Department, F-75015 Paris, France

6- Institut Pasteur, Biology and genetic of the bacterial cell wall unit, F-75015 Paris, France

[§] These authors contributed equally to this work

* corresponding author

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Abstract: Low-grade chronic systemic inflammation, or inflammageing, is a hallmark of ageing and a risk factor for both morbidity and mortality in elderly people. Among immune cells that participate to age-associated tissue dysfunction, resident macrophages emerge as potential key contributors as they are tissue homeostasis sentinels that are embedded in their tissue of residence since embryonic development, thus been exposed to cumulative tissue insults throughout life. Contrary to what is currently postulated, we demonstrate here that the pool of embryo-derived resident macrophages exhibits an age-dependent depletion in liver, and other solid organs and that they are not replaced by Hematopoietic Stem Cell (HSCs)-derived monocytes throughout life. Further, we demonstrate that gradual, cumulative inflammation during ageing induces this specific loss of tissue resident macrophages. Preserving a “youthful” density of resident macrophages prevents the development of classic hallmarks of liver age-associated dysfunction.

Introductory paragraph

Ageing is associated with excessive innate immune cell production, defective adaptive immune responses, and systemic low grade chronic inflammation^{1,2}. While the frequency of circulating innate immune cells is increased in aged animals, there are conflicting results regarding tissue-specific myeloid cells³⁻⁵. Among them, two lineages of macrophages coexist in most adult tissues: embryo-derived Hematopoietic Stem Cell (HSC)-independent “resident” macrophages and HSC-derived monocytes/macrophages⁶. Pathological situations have shed light on the impact of ontogeny on tissue macrophage functions⁷⁻¹⁰. However, the homeostatic maintenance and functions of resident macrophages are still poorly understood, in particular in the context of ageing. Whether HSC-derived macrophages can compensate for resident macrophage loss is a subject of intense investigation^{11,12}. Given that the progressive replacement of heart resident macrophages with HSC-derived cells over time^{13,14} has been linked to age-associated cardiac fibrosis and deleterious outcomes following cardiac injury^{15,16}, a shift in ontogeny among tissue resident macrophages could contribute to age-related changes, such as impaired tissue repair.

The liver comprises the largest population of resident macrophages in the body. These Kupffer cells (KCs) sit in the liver fenestrated sinusoidal endothelial cells, where they are in close contact with the circulation. In the aged rodent liver, alteration of the microvasculature¹⁷ activation of stromal cells and decline in hepatocyte function have been observed, along with general inflammation. Moreover, a higher prevalence of inflammation related chronic liver diseases (cirrhosis, non-alcoholic steatohepatitis) has been observed in aged individuals¹⁸. Recently, the Covid-19 pandemic, which disproportionately affect elderlies and people with chronic metabolic pathologies, has highlighted the need to disentangle the interplay between inflammation, ageing and tissue repair. KCs are established key players of the inflammation and tissue repair processes. A better understanding of their maintenance and functions in physiological ageing will thus prove critical for the treatment of age-related pathologies.

Because embryo-derived macrophages are usually thought to be replaced by HSC-derived cells that can adopt a similar phenotype, in particular after acute macrophage depletion^{11,12,19}, we expected to observe with age an increased contribution of HSC-derived cells to tissue resident macrophages without changes in their overall tissue density. Here we show that resident macrophage density decreases 2-fold during ageing in the liver and other solid organs, and this resident macrophage loss is associated with liver tissue dysfunction. The net contribution of HSC-derived cells to resident macrophage populations is constant over time and no compensatory recruitment of monocyte-derived cells was observed. This loss was specific to KCs, when compared to other innate immune cells present in the liver and was not due to proliferative exhaustion. Furthermore, sustained inflammation is sufficient to drive resident macrophage loss while impaired inflammation sensing rescues the age-associated phenotype. Our study demonstrates that increased niche availability, defined here as available space due to a decrease in macrophage density in combination with available CSF1 growth factor presentation by Hepatic Stellate cells, is not sufficient to elicit the recruitment and local differentiation of HSC-derived cells. These data support a model where inflammation triggers

the net loss of resident macrophages by modifying the homeostatic proliferation/death balance, thereby leading to a cumulative loss of resident macrophages over time without compensation from HSC-derived cells. Moreover, preventing liver resident macrophage sparseness improved age-associated tissue dysfunction. Thus, further understanding the factors regulating resident macrophage homeostasis and the signals mediating cell recruitment in the context of ageing will lead to new strategies to improve aged tissue homeostasis and repair.

Results

We scored phenotypic resident macrophages in the liver, named Kupffer cells (KCs), defined here by the expression of the cell-surface markers F4/80, CD11b and Tim4. Four age groups were studied: Young mice (2-4 months-old, mO) were compared with three groups of aged mice (16-18mO, 20-25mO and 29-30mO). To our surprise, phenotypic resident macrophage density started to decrease at 16-18 months, with an average 2-fold-reduction at 20-25 months ([Figure 1A](#)). These results were confirmed by counting F4/80+ macrophages *in situ* in liver sections ([Extended Data 2A](#)). The reduction in density of macrophages with age was specific to resident macrophages, as the density of other liver innate immune populations, such as neutrophils and MHC-II⁺ CD11b^{high} F4/80^{-/low} cells, were increased at 20-25 months and 29-30mO, respectively ([Figure 1B](#) and [Extended Data 2B](#)). Given the increased proportion of neutrophils among circulating cells in peripheral blood at 20-25 months of age ([Figure 1C](#)), the increase in liver neutrophil density could be the reflection of increased circulating cells and represent an increase of neutrophils within the liver sinusoids. To better assess neutrophil localisation (circulation vs intra-tissue), we performed an intravenous injection of anti-CD45 antibody. We evaluated the proportion of liver monocytes and neutrophils that were labelled by this antibody and normalised them to the labelling observed on their circulating counterparts. We found that in young and aged animals, ly-6C^{hi} monocytes were equally labelled in the blood and in the liver. In contrast, neutrophil labelling was decreased with age in the liver when compared to circulating cells. This suggests a transmigration of neutrophils from the liver circulation to the liver parenchyma ([Extended Data 2C](#)). Histological examination confirmed the increase in tissue MPO⁺ neutrophils ([Extended Data 2D](#)) and demonstrated that the proportion of neutrophils that were intra-tissular (not located within CD31⁺ blood sinusoid vessels) was increased in aged livers ([Extended Data 2E](#)). Collectively, these results suggest that the density of neutrophils within the liver could be a read-out of the low-grade sustained inflammation that is postulated to occur in aged tissues.

Recent literature suggested that the contribution of HSC-derived cells would increase over time, and the reduction in the total number of macrophages does not preclude this possibility. We thus sought to investigate the contribution of HSC-derived cells to phenotypic resident macrophages during ageing by using the *Flt3^{Cre} Rosa^{YFP}* fate mapping model^{20,21}. HSC-derived cells are efficiently labelled in *Flt3^{Cre} Rosa^{YFP}* mice, with 80% of circulating monocytes been YFP⁺. The proportion of HSC-derived (*Flt3^{Cre}*)-YFP⁺ cells among resident macrophages increased with time in the liver ([Figure 1D](#)). However, because the population size changed

over time as demonstrated in Figure 1A, we examined the density of (*Flt3^{Cre}*)-YFP⁺ and -YFP^{neg} cells among resident macrophages. While there was no change in HSC-derived (*Flt3^{Cre}*)-YFP⁺ cells, we observed a specific loss of (*Flt3^{Cre}*)-YFP^{neg} cells (2-fold reduction at 20-25mO when compared to Young) (Figure 1E). Thus, the observed reduction of resident macrophage density was specific to HSC-independent macrophages and was not compensated by increased recruitment and/or differentiation of HSC-derived cells. This result was confirmed using a complementary approach, where macrophages were pulsed-labelled in Young and Old mice expressing the inducible Cre recombinase (MeriCreMer) under the control of the macrophage growth factor receptor (Colony stimulating factor receptor 1, *Csf1r*) promoter. Pulse-labelling of macrophages in Young and Old *Csf1r^{MeriCreMer} Rosa^{tdTomato}* mice led to efficient recombination (evidenced by a Tomato labelling efficiency of 60%) in liver resident macrophages three days after a single dose of tamoxifen (Figure 1F). Contribution of (non-labelled) HSC-derived cells to the resident macrophage population would lead to a decline in Tomato labelling over time. There was no significant difference in macrophage Tomato labelling between day 3 and day 72 post tamoxifen injection between Old and Young, suggesting that macrophage self-maintenance or half-life in aged livers was not affected when compared to young livers (Figure 1F). Collectively, this indicates that there was no switch in the ontogeny of liver resident macrophages with ageing. In contrast to listeria infection, whole body-irradiation or genetic ablation of resident macrophages^{11,12,19,22}, HSC-derived cells were either not recruited or failed to differentiate into tissue macrophages when liver macrophage density was reduced with age.

We next investigated whether liver resident macrophages (KCs) were functionally affected with ageing. A functional feature of liver KCs is their ability to phagocytose large particles directly from the bloodstream, due to their localization within the liver sinusoids. KC phagocytosis of 2µm PE-latex beads was unaffected in Old animals when compared to Young controls (Figure 2A). We observed morphological changes in old liver resident macrophages. Old KCs were larger in size and had increased granularity (Extended data Figure 3A and B). Old KCs also had increased cell-surface expression of the macrophage marker F4/80 and the myeloid integrin CD11b as measured by the median fluorescent intensity. To take into account the increased cell size, F4/80 and CD11b expression were normalized to cell size (FSC-A). While normalized F4/80 expression was not changed between Young and Old, the increase in CD11b expression could not be explained alone by the increased cell size (Extended data Figure 2C). An exploration of the Tabula Muris Senis Consortium RNA sequencing repository²³ showed an increase in the number of reads in KCs, a feature traditionally associated with increased cell size. Conversely, the hepatocytes seemed to decrease in size with age (Extended Data Figure 2D).

Whole transcriptomic analysis of Old (22-23 mO) versus Young (2-4 mO) KCs showed only 37 up-regulated genes and 47 down-regulated genes (Figure 2B). Gene set enrichment analysis revealed an up regulation for pathways related to inflammatory response, leukocyte

migration and LPS-stimulated macrophages in old KCs, while pathways for peroxisome, fatty acid and bile acid metabolism were significantly up regulated in young KCs (*Figure 2B*). We also took advantage of our *Flt3^{Cre} Rosa^{YFP}* model and compared young and old macrophages; both (*Flt3^{Cre}*)-YFP^{neg} and (*Flt3^{Cre}*)-YFP⁺ Kupffer Cells. Here too, we found only minimal differences (*Figure 2C*), in accordance the importance of tissue environment in instructing resident macrophage phenotype and transcriptome ²⁴.

Thus, age minimally changed the liver resident macrophage transcriptome (as recently show for aged neural stem cells²⁵) and old KCs appeared to retain their self-maintenance and phagocytic abilities.

Resident macrophages are maintained in adult tissues by local proliferation^{26–31} and we could not detect any changes in the proliferation of liver macrophages (measured by the proportion of Ki67⁺ cells among F4/80⁺ macrophages in liver cryosections) between Young and Old animals (*Figure 3A*). We refined our analysis by measuring proliferation using two complementary approaches among HSC-independent (*Flt3^{Cre}*)-YFP^{neg} macrophages and we found a similar proportion of proliferating (*Flt3^{Cre}*)-YFP^{neg} resident macrophages in both young and aged livers (*Figure 3B*). This suggested that proliferative exhaustion was not responsible for the reduction in resident macrophage density. Although basal levels of proliferation were unaffected, old KCs could still fail to mount a proliferative burst in response to injury or challenge. A classical model to induce a proliferative burst in macrophages is withdrawal of CSF1R inhibition. CSF1 is the main growth factor that stimulates the maintenance and proliferation of most adult macrophages, including Kupffer Cells. We targeted the CSF1 receptor (CSF1R) with the blocking compound Plexikkon 5622 (PLX)³² and inhibited CSF1R signalling for three days. We then measured KC proliferation by a single EdU pulse two hours before tissue harvest. Both young and old KCs had similar proliferation levels, confirming that age did not affect KC proliferative potential nor their ability to respond to CSF1 stimulation (*Figure 3C*). The old KC proliferative potential was also evident when analysing the replenishment of the KC compartment after CSF1R inhibition. CSF1R inhibition for three days led to a depletion of KC in both Old and Young. When analysed 9 days after withdrawal of CSF1R inhibition, both young and Old had increased their KC numbers at comparable levels (*Figure 3B*), suggesting similar proliferative potential. Further, analysis of YFP labelling efficiency in *Flt3^{Cre} Rosa^{YFP}* after PLX recovery demonstrated no significant input from bone marrow progenitors or circulating cells to the repopulated KC pool (*Figure 3D*).

The age-associated reduction of resident macrophage numbers was observed in other major solid organs, with the exception of the kidney (*Extended Figure 4*). This reduction was also specific to resident macrophages, as other resident immune populations (dendritic epidermal T cells, DETCs) and other myeloid populations were stable until at least 20-25mO (e.g. F4/80^{bright} CD64⁺ CD11b⁺ interstitial lung macrophages, Siglec-F⁺ F4/80^{low} CD11b⁺ lung eosinophils and spleen Ly-6C⁺ CD11b^{high} F4/80^{low} cells) (not shown). There was also no age-associated switch in the ontogeny of resident macrophages in the epidermis (Langerhans

cells), lung (alveolar macrophages), spleen (red pulp macrophages), and brain (microglia) with ageing, as evidenced by the specific loss of (*Flt3^{Cre}*)-YFP^{neg} cells in 20-25mO *Flt3^{Cre} Rosa^{YFP}* mice. In contrast, the number of (*Flt3^{Cre}*)-YFP⁺ macrophages seemed increased at 20-25mO in the kidney (*Extended Figure 4B*). It thus appeared that kidney resident macrophage density was in part maintained during ageing by an increased recruitment and differentiation of HSC-derived cells. Altogether, the density of most resident macrophages, with the exception of kidney macrophages, declined with age without proliferative exhaustion and without compensation from HSC-derived cells.

Since proliferative capacity was impaired in aged KC, we next investigated whether increased cell death could be responsible for the observed KC loss in ageing. In aged livers, we observed a 5-fold increase in the proportion of TUNEL positive Kupffer Cells compared to young, indicating that a gradual and cumulative increase in KC cell death could be driving the aged phenotype (*Figure 4A*). We then decided to investigate if inflammation could be driving KC cell death over time, for two main reasons: inflammation has been shown to drive macrophage cell death³³ and, in humans, age is associated with low-grade, sustained inflammation, as evidenced by the increased serum levels of pro-inflammatory cytokines. Common viruses have been proposed to play a major role, given the prevalence of sub-clinical viral infections in the elderly (reviewed in ³⁴). Given that we found an up-regulation of pro-inflammatory pathways in Old resident macrophages in the liver (*Figure 2B*) and a similar up-regulation was recently reported in aged microglia cells²⁵, we thus investigated whether sustained inflammation with a viral mimic, Poly(I:C), could drive resident macrophage loss. First, acute Poly(I:C) challenge did not affect resident macrophage density in the liver, nor any other organ studied (*Extended data Figure 5*). Sustained stimulation was performed by injecting every other day Poly(I:C) (5mg/kg) or saline for a month (*Figure 4B*). Two weeks after the end of the Poly(I:C) treatment, resident macrophage density was decreased 2-fold in the liver (*figure 4C*). This loss of KC was not transient, as it was still observed one month later (6 weeks post last injection, pli). In order to investigate whether sustained inflammation was driving the specific loss of HSC-independent resident macrophages as shown during ageing, we next examined *Flt3^{Cre} Rosa^{YFP}* mice subjected to sustained Poly(I:C) and analysed 2 weeks and 6 weeks pli (*Figure 4B*). As observed during ageing, there was a specific loss of HSC-independent (*Flt3^{Cre}*)-YFP^{neg} cells in liver while HSC-derived (*Flt3^{Cre}*)-YFP⁺ cells remained at comparable levels (*Figure 4D*). Sustained Poly(I:C) only elicited a transient neutrophil density increase 48h pli in the liver that resolved by 2 weeks pli (*Extended data Fig. 6A*). Thus, sustained inflammation with Poly(I:C) did not fully recapitulate the ageing phenotype in regard to the neutrophils. Further, these results evidenced that inflammation stimuli was not sufficient to induce monocyte recruitment and differentiation into macrophages, in contrast to what is observed after 'total' depletion of KC ^{11,12}. All tissues examined showed a similar decrease of resident macrophage density after sustained Poly (I:C) (1.2-fold decrease in epidermis 1.7-fold in spleen, 2.3-fold in lung and 2.6-fold in kidney), except in the brain (*Extended data Figure 6*). Interestingly, in spleen and kidney, both macrophage populations were affected by sustained

inflammation and the return to steady-state macrophage density at 6 weeks pli in the spleen led to a similar proportion of HSC-derived (*Flt3^{Cre}*)-YFP⁺ cells and HSC-independent (*Flt3^{Cre}*)-YFP^{neg} cells than the one observed in steady-state (*Extended data Fig. 6B*). Further, TUNEL staining on liver sections showed an increase in the proportion of TUNEL+ positive Kupffer cells two weeks post Poly (I:C) treatment, a phenotype that was maintained 6 weeks post treatment (*Figure 4E*). As in aged mice, sustained inflammation induced KC cell death that led to a decreased KC density, without compensation (by either monocyte recruitment or local proliferation).

To investigate whether systemic gradual, cumulative inflammation could be responsible for the resident macrophage loss observed during ageing, we next analysed mutant mice where inflammation sensing is defective, but not abolished. We took advantage of *Ticam^{Lps2}* mutant mice that have a point mutation in the signalling protein TICAM/TRIF that is downstream of TLR signalling³⁵. *Ticam^{Lps2}* mutant mice lack cytokine responses to Poly(I:C) and have severely impaired responses to LPS. 21-month-old *Ticam^{Lps2}* mutant mice had increased blood and liver neutrophils when compared to young mutant mice (*Figure 4 F-G*), as observed in *wild-type* ageing. However, *Ticam^{Lps2}* mutation rescued the ageing phenotype observed in KCs (*Figure 4H*). The same was observed in resident macrophages in all tissues, as differences in macrophage density were no longer observed between Young and Old *Ticam^{Lps2}* mutant mice (*Extended Figure 7B*). Moreover, the 5-fold increase in dying (TUNEL+) KC in old WT animals was absent in old *Ticam^{Lps2}* mice (*Figure 4I*).

Therefore, impaired inflammation signalling specifically rescued the age-associated resident macrophage loss in all tissues examined. In combination with the sustained inflammation challenge, our data shows that cumulative inflammation drives resident macrophage cell death, which in turn leads to the age-associated reduction in resident macrophage density. Further, our results indicate that the decrease in macrophage density is not required to induce the accumulation of tissue neutrophils and conversely, that the neutrophil infiltration is not sufficient to induce macrophage loss.

The loss of resident macrophages combined with the inaptitude for HSC-derived cells to infiltrate the liver tissue and replace them, may reflect a change in the liver environment that renders it less supportive of macrophage survival, proliferation and/or differentiation. Stellate cells, hepatocytes and endothelial cells have been proposed as the key actors responsible for Kupffer cell identity and maintenance¹². In particular, Hepatic Stellate Cells have been shown to support survival and proliferation of KCs by producing Interleukin 34 (IL34) and Colony Stimulating Factor 1 (CSF-1), the two ligands of CSF1R. We thus investigated whether changes in hepatic stellate cells with age could contribute to the KC phenotype. First, we quantified the number of desmin+ Hepatic Stellate cells and found no significant change that could correlate with the loss of KCs with time. We then investigated whether aged stellate cells had impaired CSF1 and/or IL34 presentation at their cell surface (*Figure 5A*). In WT mice we found an increase in the proportion of CSF1-expressing stellate cells that could reflect an

increased production of CSF1 to help sustain a dwindling pool of macrophages. Ageing did not differentially affect *Ticam*^{Lps2} stellate cells compared to WT, suggesting that this increase in CSF1 production may not be solely due in response to KC decreased density (Figure 5B). In young mice that received repeated injections of Poly(I:C), the number of Desmin+ cells remained stable up to 6 weeks post last injection, but the proportion of CSF1 and IL34 producing cells dropped at 2 weeks (Figure 5C) before returning to initial levels. This suggests a specific effect of Poly (I:C) on stellate cells. Altogether, diminished availability of CSF1 and of hepatic stellate cells is not responsible for the lack of monocyte-derived macrophage engraftment in old livers.

Finally, to characterize the functional relevance of resident macrophage density during ageing, we investigated key features of liver age-associated dysfunction: increased lipid content and senescent cell accumulation, in both wild-type and *Ticam*^{Lps2} mutants. As reported previously³⁶, we observed a massive increase in senescent cell and lipid contents accumulation in the aged liver (Figure 6). Importantly, when macrophage density is maintained in old *Ticam*^{Lps2} mice, senescent cells (Figure 6A-B) and lipid droplets failed to accumulate with age (Figure 6C). Thus, maintaining a youthful resident macrophage density seems to be critical to prevent key features of liver dysfunction with age.

Conclusion and Perspectives

Collectively, our findings demonstrate that there is a specific loss of HSC-independent tissue resident macrophages during ageing in the liver and most other tissues examined. Loss of HSC-independent resident macrophages was not explained by proliferative exhaustion and was not compensated by increased local recruitment and/or differentiation of HSC-derived cells. Instead, we found that sustained inflammation is sufficient to induce the age-associated macrophage phenotype in the liver while lack of inflammation sensing during ageing abolished specifically the resident macrophage loss.

These data support a model where inflammation specifically impacts HSC-independent resident macrophages by triggering an imbalance in their proliferation/death homeostasis, thereby leading to their cumulative loss over time without compensation from HSC-derived cells. While HSC-derived cells can compensate for the loss of HSC-independent resident macrophages in very specific experimental paradigms as recently illustrated for the liver^{19,22}, we did not observe this phenomenon during physiological ageing or sustained inflammation in the liver, which raises the question of why HSC-derived cells fail to infiltrate and differentiate into tissue macrophages in aged tissues. Among the different liver cells that provide supportive signals to KCs, liver sinusoid cells undergo profound changes and remodelling during ageing, in a process called pseudo-capillarisation, and could play a significant but yet under-recognized role in this process. Thus, further work is needed to identify the factors regulating resident macrophage homeostasis and the signals mediating cell recruitment and survival in different physio-pathological conditions.

It has been theorized that accumulation of gradual sub-clinical tissue damage (in response to sterile inflammation or infection) increases the burden of tissue repair with ageing, thereby leading to the increased inflammation observed in the elderly³⁴. In accordance, increased longevity has been reported in germ free mice³⁷. Here we report both a decreased resident macrophage density and an increased neutrophil density with age. These changes during ageing in innate immune populations could be a contributing factor that may negatively affect tissue repair outcome in old mice, as evidenced by the attenuation of ageing hallmarks in liver that presented “youthful” resident macrophage density. Since resident macrophages have critical functions during tissue homeostasis and repair, in part through the swift removal of apoptotic cells and the secretion of cytokines and growth factors³⁸, the reduction in resident macrophage numbers associated with an increased neutrophil infiltration could contribute to increased neutrophil activation within tissues, thereby leading to deleterious effects on tissue integrity³⁹ and exacerbating age-induced tissue dysfunction.

Methods

Animals

Csf1r^{MeriCreMer} (ref ⁴⁰), *Flt3*^{Cre} (ref ⁴¹), *Ticam*^{Lps2} (ref ³⁵), *Rosa26*^{YFP} (ref ⁴²) and *Rosa26*^{tdTomato} reporter (ref ⁴³) mice have been previously described. *Csf1r*^{MeriCreMer} and *Csf1r*^{iCre} mice were on FVB background, other mice were on C57BL/6 (CD45.2) background. *Csf1r*^{MeriCreMer} mice were generated by J. W. Pollard. *Flt3*^{Cre} mice were generated by C. Bleul and provided by T. Boehm and S. E. Jacobsen. *Rosa26*^{tdTomato} (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze) and *Rosa26*^{YFP} (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J) were provided by S. Tajbaksh and G. Eberl, respectively. Aged and Young C57BL/6JRj were purchased from Janvier labs.

No randomization method was used and the investigators were blinded to the genotype of the animals during the experimental procedure. Results are displayed as mean ± s.e.m. All experiments included littermate controls and the minimum sample size used was 3. No statistical method was used to predetermine sample size.

Animal procedures were performed in accordance with the Care and Use Committee of the Institut Pasteur (CETEA) guidelines and with their approval.

Genotyping

PCR genotyping of *Ticam*^{Lps2} (ref ³⁵), *Csf1r*^{MeriCreMer} and *Flt3*^{Cre} (ref ²⁰) mice was performed according to protocols described previously.

Fate-mapping of *Flt3*⁺ haematopoietic progenitors

For fate-mapping analysis of *Flt3*⁺ precursors, *Flt3*^{Cre} males (the transgene is located on the Y chromosome) were crossed to homozygous *Rosa26*^{YFP} reporter females. *Flt3*^{Cre} *Rosa26*^{YFP} males were blood phenotyped. Animals with YFP labelling efficiency above 60% in the lymphocytes, monocytes and granulocytes were used for experiments and female littermates were used as Cre-negative controls.

Pulse labelling of adult macrophages

For pulse labelling experiment of *Csf1r*⁺ macrophages in Young and Old mice, *Csf1r*^{MeriCreMer} females were crossed to homozygous *Rosa26*^{tdTomato} reporter males. Tamoxifen powder (Sigma-Aldrich) was resuspended in 100% Ethanol at 10mg/100μl and further dissolved in corn oil at a concentration of 20mg/ml by shaking overnight at 37°C. *Csf1r*^{MeriCreMer} *Rosa26*^{tomato} mice were injected with 70 mg/kg of body weight tamoxifen intraperitoneally and Cre-negative littermates were used as controls.

LPS and Poly (I:C) acute and sustained inflammation experiments

For sustained inflammation experiments, mice were injected intraperitoneally with 5 mg/kg of saline, LPS (Sigma) or Poly (I:C)(Novus biologicals) every other day for one month. Mice were then sacrificed 48 hours, 2 weeks or 6 weeks after the last injection. For acute inflammation experiments, mice were injected intraperitoneally with 5 mg/kg of saline, LPS or Poly (I:C) received a total of 4 injections, two injections on alternate days, one week rest and two more injections on alternate days. Mice were then sacrificed 48 hours, 2 weeks (Poly(I:C)) or 3 weeks (LPS) after the last injection. Organs were harvested for flow cytometry and histology analyses.

Processing of tissues for flow cytometry

Mice were killed by cervical dislocation and perfused by gentle intracardiac injection of 10 ml prewarmed (37°C) 1× PBS. Organs were weighted after harvesting. After being cut in small pieces, they were digested for 30 minutes at 37°C in PBS 1X containing 1mg/mL Collagenase D (Roche), 100 U/mL DNase I (Sigma), 2.4 mg/mL Dispase (Invitrogen) and 3% FBS (Invitrogen). Epidermal sheets were separated from the dermis after incubation for 45 min at 37°C in 2.4 mg/ml of Dispase and 3% FBS and the epidermis was further digested for 30 min in PBS containing 1 mg/ml collagenase D, 100 U/ml DNase I, 2.4 mg/ml of Dispase and 3% FBS at 37°C. Tissues were then mechanically disrupted with a 2mL-syringe piston on top of 100 μm filters to obtain a single cell suspension. For liver, hepatocyte depletion was performed by centrifugation of the whole liver cell suspension at 50g for 3 minutes. The supernatant was then collected for further staining of non-parenchymal liver cells. For liver, spleen and lung, red blood cell lysis was performed as previously described⁴⁴.

Flow cytometric analysis of adult tissues

Cells were centrifuged at 320g for 7 min, resuspended in 4°C FACS Buffer (PBS 1X, 2mM EDTA, 0.5% BSA), plated in multi-well round-bottom plates and immunolabelled for FACS analysis. After 15 min incubation with purified anti-CD16/32 (FcγRIII/II) diluted 1/50, antibody mixes were added and incubated for 30 min. When appropriate, cells were further incubated with streptavidin conjugates for 20 min. Samples were acquired using a Beckman Coulter Cytoflex S or a BD Fortessa cell analyser. All data was analysed using FlowJo 10.5.3 (TreeStar). Cell density was calculated using the following formula: Cells/g of tissue = $\left(\frac{\text{count}}{V_{\text{acq}}} \times V_{\text{tube}} \times \frac{V_{\text{resuspension}}}{V_{\text{plated}}}\right) / \text{Weight}$, with V_{acq} : volume acquired; $V_{\text{resuspension}}$: volume added to the cell pellet after first centrifugation; V_{plated} : volume of cells plated;

Vtube: total final volume; Weight: weight of the piece of tissue used for single-cell suspension preparation.

Table1: Antibodies used for flow cytometry

Antibody	clone	conjugates
CD45.2	104	APC-CY7
CD11b	M1/70	PE-Cy7
F4-80	BM8	BV421
TIM-4	RMT4-54	AF647or PE-Cy7
CD64	X54-5/7.1 FC	AF647 or PE-Cy7
Ly-6C	HK1.4/AL-21	APC, BV605 or FITC
Ly-6G	1A8	PE, or BV711
Gr-1	RB6-8C5	PE or PerCP Cy5.5
MHC-II (IA/IE)	M5/114.15.2	AF647
CD115 (Csf1r)	AF598	AF647 or PE
CD3e	145.2C11	FITC or PE
CD19	1D3	eF450

RNA sequencing of Young and Old KCs

For RNA sequencing of Young (4 months) and Old (22-23 months) KCs, livers were processed as described before and KCs were then sorted with a BD FACS Aria III based on F4/80 and Tim4 expression. Labelling of cells was performed as described above using the following antibodies: CD45-APC-Cy7, CD11b-PE-Cy7, F4/80-BV421 and Tim4-APC. Gates were defined using unstained, single stained and fluorescence minus one (FMO) stained cells. 10 000 cells were directly sorted in 350 μ L of RLT buffer (Qiagen), vigorously vortexed for disruption and spun down. RNA was subsequently extracted using the Qiagen RNeasy micro kit. Purified RNA was quantified and the RNA quality assessed using an Agilent Bioanalyzer (Agilent). Selected samples had an RNA Integrity Number > 6.2. Library preparation was performed using the ClonTech SMARTer Stranded RNA-Seq Kit V2 for mammalian pico RNA input (Takara) and RNA sequencing was performed using HiSeq 2500 sequencer with 50bp read lengths (Illumina).

Differential expression analysis. DESeq2 (v_1.22.2)⁴⁵ Wald test was used to assess differential expression between groups. The input data consists of the raw count matrix where each row indicates the transcript, each column indicates the sample and each cell indicates the number

of reads mapped to the transcript in the sample. After independent DESeq2 filtering⁴⁵, P-values for selected genes were adjusted for multiple comparisons correction using Benjamini–Hochberg correction for FDR at threshold of $P < 0.05$ and no minimum LFC threshold was applied.

Gene set enrichment analyses. Gene set enrichment analysis (GSEA) (functional scoring method (FSC)⁴⁶) was conducted using the `runGSA()` function in `piano` R package⁴⁷ was performed on the ranked list of genes. For this, the result table from DESeq2 was ordered by the $-\log_{10}(\text{adjusted p-value})$ from the Wald statistic test. Mouse Gene Symbol and Entrez IDs were added using the `org.Mm.eg.db` (v_3.7.0) and `AnnotationDbi` (v_1.44.0) Bioconductor Packages, using the Ensembl genes as keys, and duplicated entries identifiers were then collapsed by keeping the one with the lowest adjusted p-value. Gene set collections from the mouse version of the Molecular Signatures Database MSigDB v6 were downloaded from <http://bioinf.wehi.edu.au/software/MSigDB/>

Visual representation. Heatmap were made using R package `heatmap` (v_1.0.12) and other visual representations (barplot and PCA biplot) were made using R package `ggplot2` (v_2_3.1.0)⁴⁸. **R Session info.** All the analyses have been performed using R⁴⁹ version 3.5.1 (2018-07-02), running under: OS X macOS Sierra 10.12.6 on x86_64-apple-darwin15.6.0 (64-bit) platform.

Bead phagocytosis assay

To assess Kupffer cell phagocytosis, Young (2-4 months) and Old (19 months) mice were injected intravenously twice with 2 μ m Red fluorescent polymer microspheres (100 μ l of 0.2% solid beads in NaCl 0.9%) (FluoroMax, Molecular Probes). Livers were then analysed 24 hours after the last injection.

Histology

Quantification of neutrophils and resident macrophages was performed in cryo-sections from Young (2-4mO) and Old (20-25mO) *Flt3^{Cre} Rosa^{YFP}* mice. Half of the organs were fixed overnight in 4% paraformaldehyde (Sigma) under agitation. After fixation, organs were washed in PBS 1X and incubated overnight in 30% sucrose, then embedded in OCT compound (Sakura Finetek). Cryoblocks were cut at a thickness of 12-14 μ m and then blocked with PBS 1X containing 5% normal goat or donkey serum (Invitrogen); 0.2% BSA(w/v), 0.3% Triton X 100 for 1hour at room temperature. Samples were incubated overnight at 4°C with primary antibodies (goat anti mouse MPO (1:100, Abcam); armenian-Hamster anti mouse CD31 (1:200, Abcam); rat anti Siglec-F (1:100, BD Pharmingen); rat anti mouse F4-80 (1:200, Bio-Rad); rabbit anti KI67 (1:200, Abcam); rabbit anti GFP (1:300, Fischer Scientific); chicken anti GFP (1:300, Abcam) and rat anti PU.1 (1:200, R&D), rabbit polyclonal anti Desmin (1:100, abcam), goat polyclonal anti MCSF(1:50, bio-techne), sheep polyclonal anti IL-34 (1:20, bio-techne)) in PBS 1X; 1% serum; 0.2% BSA and 0.3% Triton X 100. After washing with PBS 1X- 0.3% Triton X 100, slides were incubated for 2h at room temperature with DAPI (1:1000, Invitrogen) and appropriate secondary antibodies (goat anti Armenian Hamster AF647 and AF555(1:500,

Jackson Immuno); goat anti Rat AF555 and AF647, goat anti Rabbit AF488, goat anti Rabbit AF555, goat anti Chicken AF488 and goat anti Chicken AF555 (1:500, Invitrogen); chicken anti Goat AF647 (1:500, Thermo Fischer)) in PBS 1X; 0.2% BSA and 0.3% Triton X 100. Samples were then mounted with Prolong Gold antifade medium and imaged using an inverted Leica SP8 confocal microscope with 20 x/1.3 and 40x/1.3 (oil) objectives or a slide scanner (Olympus VS120) with 20x and 40x objectives.

Liver and lung neutrophils were identified as MPO positive cells and were quantified in nine fields of view (FoV, 0.3mm²) per animal (n=3 per age group), acquired with the 20x objective from a confocal microscope. The intra-tissular fraction was defined as MPO positive cells not colocalising within CD31+ sinusoids or large vessels. Liver and kidney macrophages were identified as F4-80+ cells and were quantified in six FoV (1mm²) per animal (n=3 per age group), acquired with the 20x objective from a slide scanner (Olympus VS120). Lung and brain macrophages were identified as F4-80+ and PU.1+ cells, respectively, and were quantified in nine FoV (0.3mm²) per animal (n=3 per age group), acquired with the 40x objective from a slide scanner (Olympus VS120). Proliferating macrophages were identified by nuclear Ki67+ staining and YFP positivity of macrophages was also scored. Quantifications were performed blindly to age by two independent observers.

SA β Gal staining on liver cryosections was performed as previously published⁵⁰ Briefly 12-20 μ m liver sections WT and TRIF Lps2 mice were stained as follows : sections were fixed 10 min in PBS 2% v/v PFA, 0,2% v/v Glutaraldehyde, washed three times in PBS and incubated 16 hours in a staining solution (40 mM Citric Acid at pH 6, 150 mM NaCl , 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ and 1mg/mL X-Gal in H₂O). Sections were then washed 3x5 min in PBS and fixed in 4% formaldehyde. After washing 3x5 min in PBS, they were counterstained with fast-red for 10-15 seconds, before being rinsed under tap water. Sections were then incubated with Ethanol 95% for 5 min, Ethanol 100% for 2x5 min, and mounted in Eukitt mounting medium. Brightfield Images were then acquired with an Olympus VS120 Slide Scanner at a 20X magnification. Quantifications were done using the Fiji open-source software. Images were thresholded using a global thresholding algorithm (MaxEntropy) and the fraction of stained area was quantified. Eight to ten 0,34mm² FOV were quantified per animal, in 5 animals per group.

For the evaluation of lipid deposition, 12 μ m liver sections from WT and TRIF Lps2 mice were stained with Oil Red O (Sigma) as per the manufacturer's instructions. Brightfield Images were acquired with an Olympus VS120 Slide Scanner at a 40X magnification, within 24 hours after the staining. Quantifications were done using the Fiji open-source software. Images were thresholded using a local thresholding algorithm (Phansalkar with a 50 pixel radius) and the fraction of stained area was quantified. Eight to ten 0,34mm² FOV were quantified per animal in 5 animals per group minimum.

For the niche component analysis, 12-14 μ m liver sections from WT, Flt3-Cre Rosa^{YFP} and Ticam^{Lps2} were stained with desmin (1:100), CSF1(1:50) and IL-34 (1:20). Stellate cells were identified as Desmin positive cells. Tunel staining was performed with the In Situ Cell Death detection kit (Sigma) as per the manufacturer's instructions.

Statistical Analyses:

Statistical analyses were performed using GraphPad Prism 7.0a (GraphPad software). Non-parametric t-test (Mann-Whitney) and non-parametric ANOVA (Kruskall Wallis) tests were

performed, followed by a post-hoc Dunn comparison tests when significant differences were found.

Data availability:

All data generated or analysed during this study are included in this article (and its supplementary information files). Sequencing data sets described in this work will be submitted to the Gene Expression Omnibus (GEO) repository before final publication.

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Author contributions

EGP proposed the concept, designed the experiments EGP and KA wrote the paper. JSC and KA performed and analysed most experiments. CK, PD, YL, LK performed experiments. SM performed RNA sequencing analysis. CC and HL performed SA-bgal experiments. CW provided Ticam^{Lps2} young and aged mice. All authors read and agreed on the manuscript.

Author information

The authors declare no competing interests. Correspondence and requests for materials should be addressed to elisa.gomez-perdiguero@pasteur.fr.

Figure 1

A, Number of liver resident macrophages (Kupffer cells) per gram (g) of tissue and **B**, Number of neutrophils per g of liver from Young 2-4 month-old (mO) (n=40), and Old 16-18mO (n=23), 20-25mO (n=24) and 29-30mO (n=9) animals. Mean±s.e.m; one-way ANOVA (Kruskal Wallis) and post-test Dunn multiple comparison test against Young group: #, p<0.0001; ¶, p<0.001; §, p<0.01. **C**, Frequency of neutrophils, Gr1⁻ and Gr1⁺ monocytes (Mo) and lymphocytes in blood from 2-4 mO (n=36), 16-18mO (n=17), 20-25mO (n=29) and 29-30mO (n=12) animals. **D**, YFP labelling efficiency in blood monocytes and liver resident macrophages (Kupffer cells, KC) in Young (2-4 mO, n=10) and Old (20-25 mO, n=8) *Flt3^{Cre} Rosa^{YFP}* mice. Mean ±s.e.m; Mann-Whitney test. **E**, Number of (*Flt3^{Cre}*)-YFP⁺ and (*Flt3^{Cre}*)-YFP^{neg} resident macrophages per g of liver. Dashed line represents the mean density of (*Flt3^{Cre}*)-YFP⁺ resident macrophages, mean±s.e.m; Young (2-4mO, n=10) and Old (20-25mO, n=8). Mean ±s.e.m; Dunn multiple comparison test between Young and Old (*Flt3^{Cre}*)-YFP^{neg} resident macrophages: ‡, p<0.05. **F**, Pulse labelling of *Csf1r*-expressing cells in Young (2-4 mO) and Old (24-31 mO) *Csf1r^{MeriCreMer} Rosa^{tdTomato}* mice by a single injection of tamoxifen (TAM) and analysis 3 days (D3) and 72 days (D72) after. Tomato labelling efficiency in liver resident macrophages, Mean ±s.e.m; two-way ANOVA analysis; n=3 per age group.

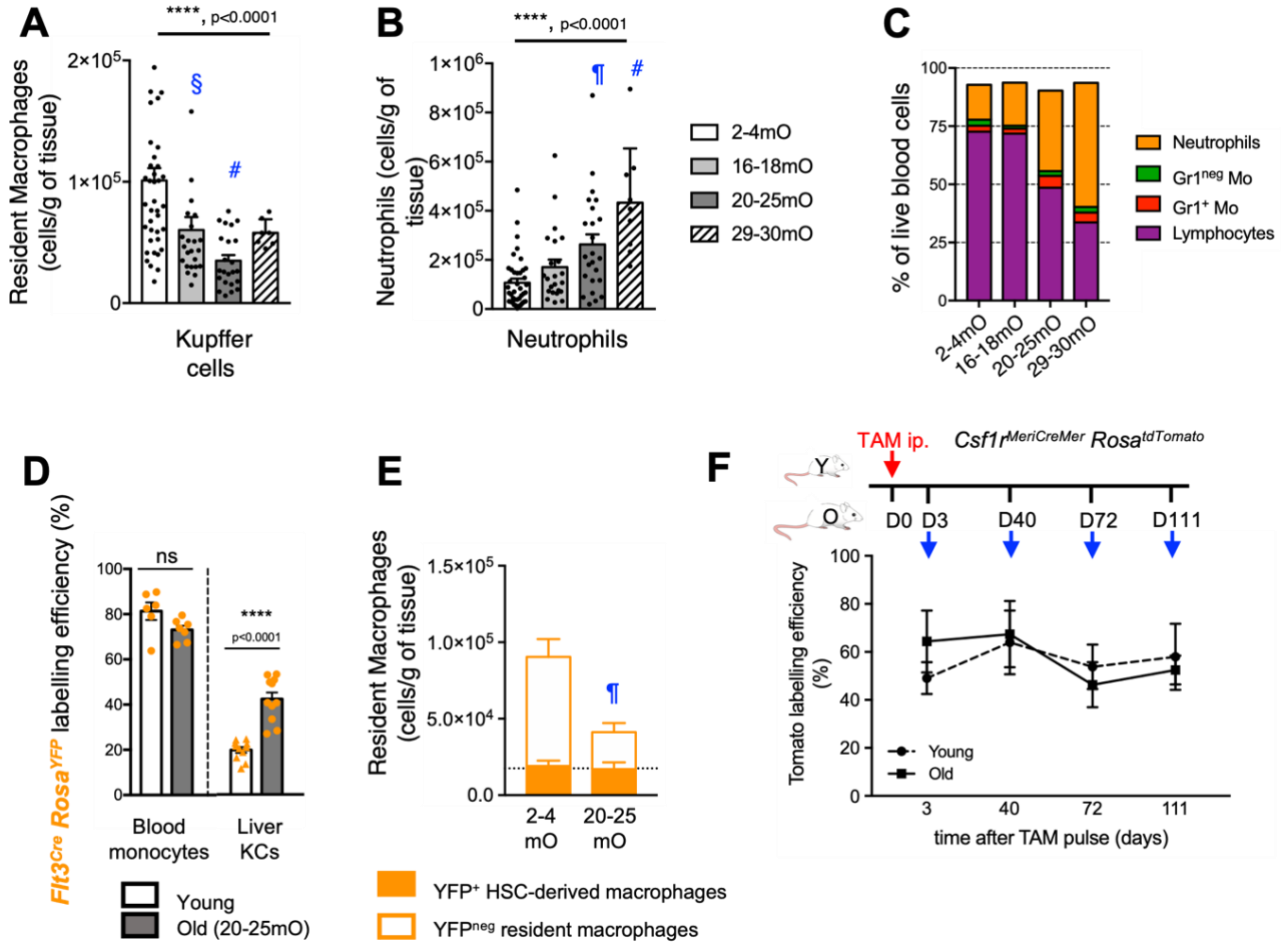
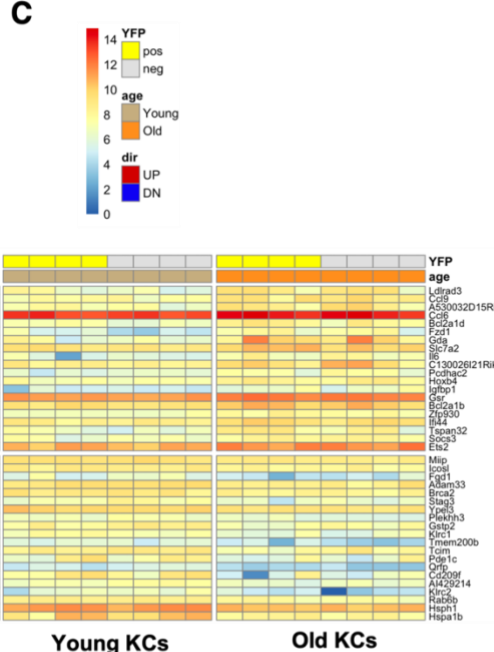
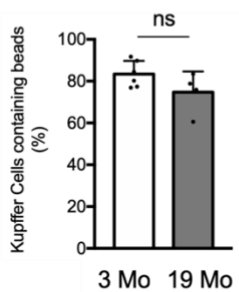
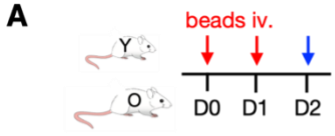


Figure 2

A, Analysis of in vivo phagocytosis of 2 μ m beads injected intravenously (iv., red arrows). Frequency of Kupffer cells containing beads 24h after the last injection from Young (3 mO; n=6) and Old (19 mO; n=4) animals **B**. RNA-sequencing analysis of Young (4mO) and Old (22-23mO) liver KCs. Right panel : Heat map of the up- or down-regulated genes between old and young KCs (log-transformed normalized values scaled by rows). Dendrogram shows the hierarchical clustering using the Euclidean distance between samples. Left panel : binary matrix showing the genes affiliation to the gene collections found enriched either in the young KCs (lower panel) or in the old KCs (upper panel). Top panel : Barplot displaying the adjusted p-value (-log₁₀-transformed) of the gene set enrichment analysis (GSEA). Blue, and orange bars for gene collections upregulated in young or old KCs, respectively.

C, RNA sequencing analysis of Young and Old liver KCs in a *Flt3^{Cre} Rosa^{YFP}* model, with a comparison between YFP⁺ and YFP^{neg} cells. Heat map shows up- (red) and down- (blue) regulated genes between young and old KCs.



B Pathway analysis

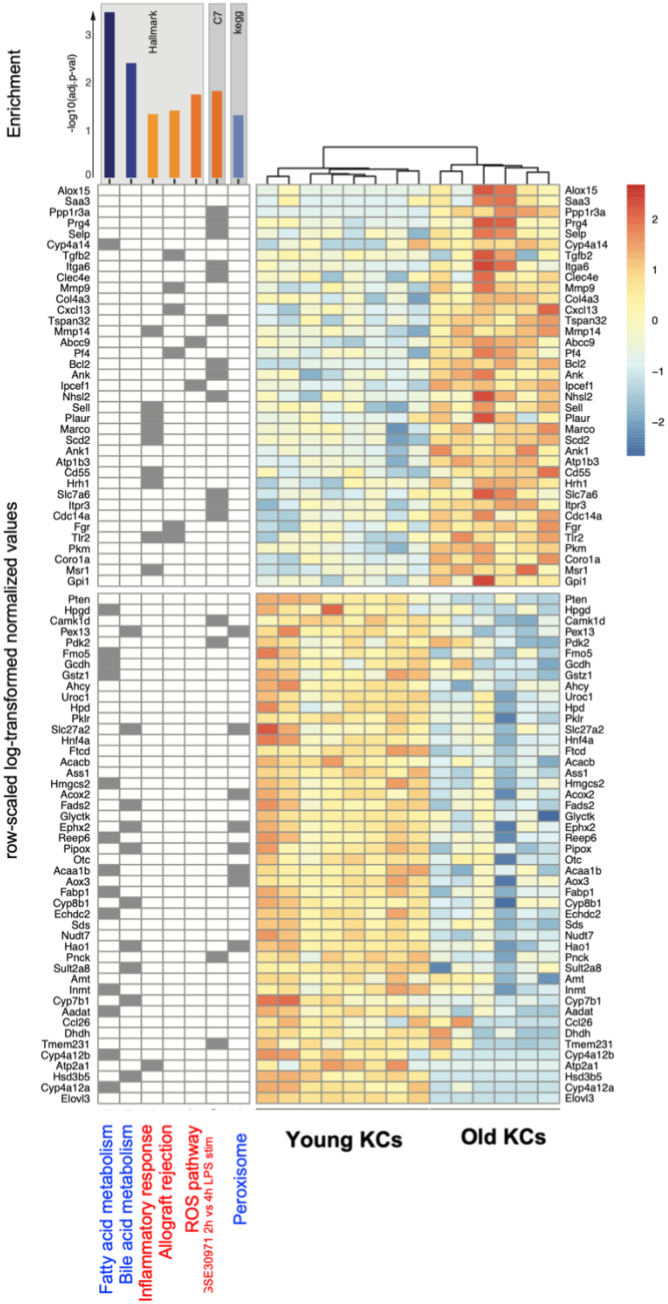


Figure 3

A, Frequencies of proliferating Ki67⁺ F4/80⁺ Kupffer cells (KCs), Ki67⁺ F4/80⁺ *Flt3*^{Cre} YFP^{neg} (HSC-independent), and Ki67⁺ F4/80⁺ *Flt3*^{Cre} YFP^{pos} (HSC derived) KCs in liver cryo-sections from Young (2-4 mO) and Old (20-25 mO) *Flt3*^{Cre} *Rosa*^{YFP} mice. Mean \pm s.e.m; Mann-Whitney test; n=3 per age group. **B**, Frequencies of proliferating EdU⁺ KCs in Young (2-4 mO) and Old (20-25 mO) *Flt3*^{Cre} *Rosa*^{YFP} mice . Mean \pm s.e.m; Mann-Whitney test; n >3 per age group. **C**, Number of liver resident macrophages (Kupffer cells) per gram of liver from Young and Old mice CTL, after PLX treatment and after PLX treatment + recovery. A Two-Way ANOVA showed an effect of the treatment factor ($p= 0,0037$) but no significant effect the age factor ($p=0,1633$). **D**, YFP labelling efficiency in Young (2-4 mO) and Old (20-25 mO) *Flt3*^{Cre} *Rosa*^{YFP} mice, in CTL, or after PLX treatment and recovery.

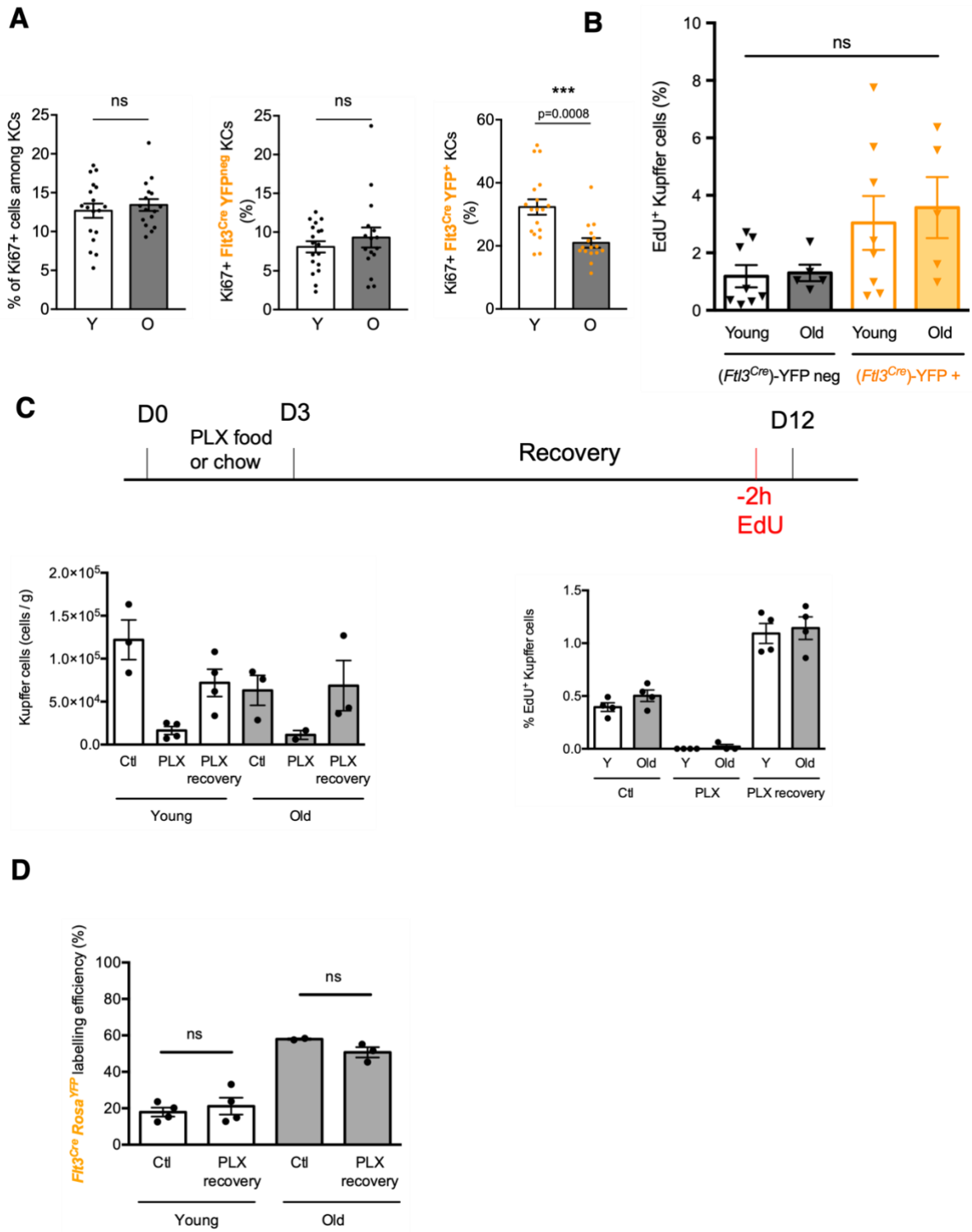


Figure 4

A, Frequencies of apoptotic TUNEL+ F4/80⁺ CLEC4F⁺ Kupffer cells (KCs), in liver cryo-sections from Young (2-4 mO) and Old (20-25 mO) Flt3^{Cre} Rosa^{YFP} mice. Mean \pm s.e.m; Mann-Whitney test; n=5 per age group. **B**, Experimental protocol for sustained poly I:C injections and **C**, Number of liver resident macrophages (Kupffer cells) per gram of liver from saline (CTL), 2w and 6w after sustained Poly(I:C) injections in Young adult mice. Mean \pm s.e.m; Dunn multiple comparison test against saline control group: ‡, p<0.05; saline controls, n=9-11; Poly(I:C) 2w, n=8 and Poly(I:C) 6w, n=4. **D**, Number of liver resident macrophages (Kupffer cells) per gram of liver from saline (CTL), 2w and 6w after sustained Poly(I:C) injections in Young adult Flt3Cre Rosa^{YFP} mice. Mean \pm s.e.m; Dunn multiple comparison test against saline control group. **E**, Representative liver cryosections of saline (CTL) or 2w after sustained Poly (I:C) injection in young adult mice, stained with CLEC4F(green), Col IV (grey), DAPI (blue) and TUNEL (red) and quantification of the frequencies of apoptotic TUNEL+ F4/80+ CLEC4F+ Kupffer. **F**, Frequency of neutrophils (orange), Gr1⁻ and Gr1⁺ monocytes (green and red, respectively) and lymphocytes (purple) in blood from Young (5 mO, n=5) and Old (21mO, n=8) Ticam^{Lps2} mutant animals. **G**, Number of neutrophils and **H**, Number of KCs per g of liver from Young (5 mO, n=5) and Old (21mO, n=8) Ticam^{Lps2} mutant animals. Mean \pm s.e.m; Mann-Whitney test. **I**, Ratio of the number of Old TUNEL+ KCs compared to Young TUNEL+ KCs in WT and Ticam^{LPS2} mice. Mann Withney test; ****: p<0,0001.

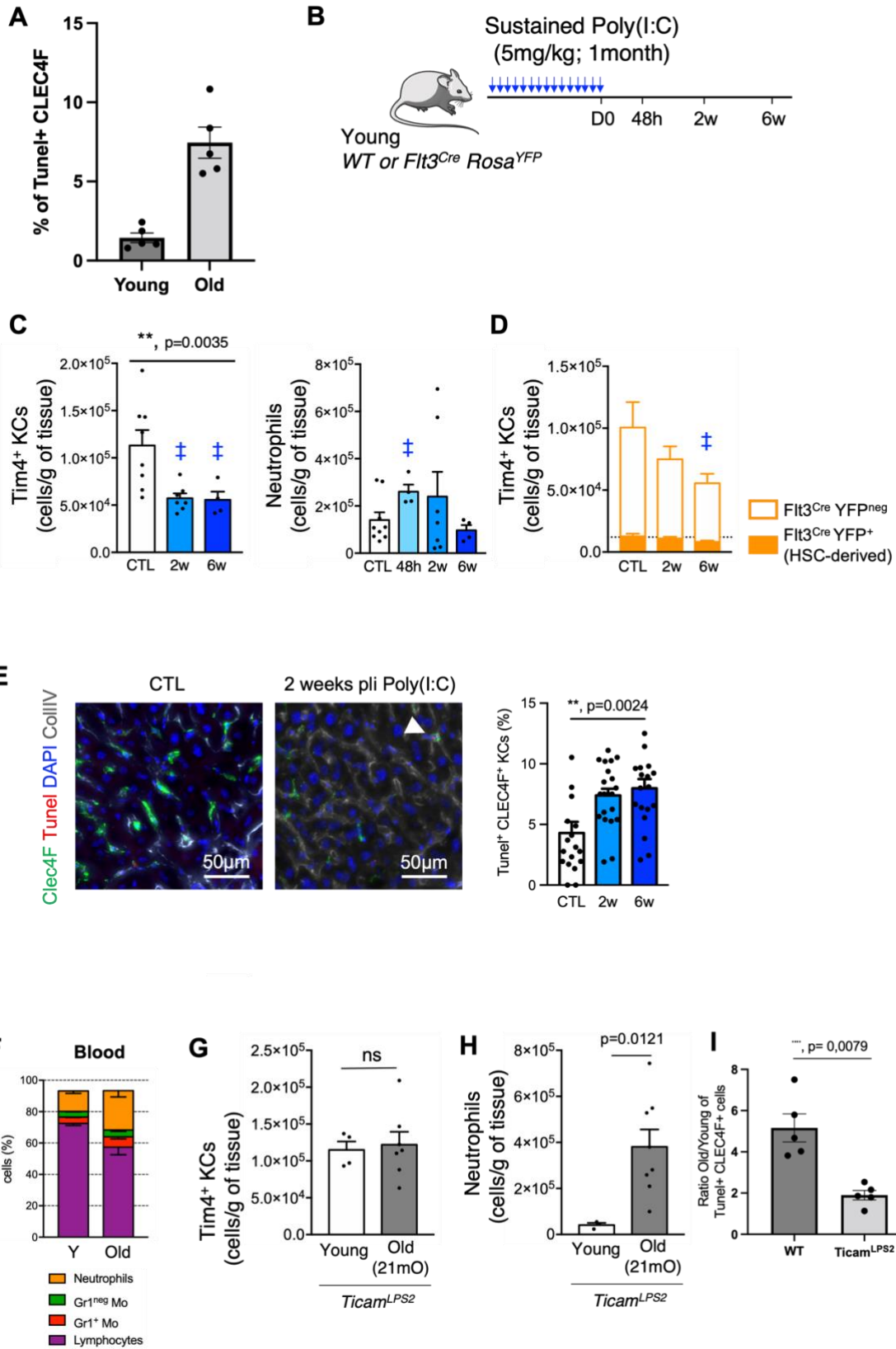
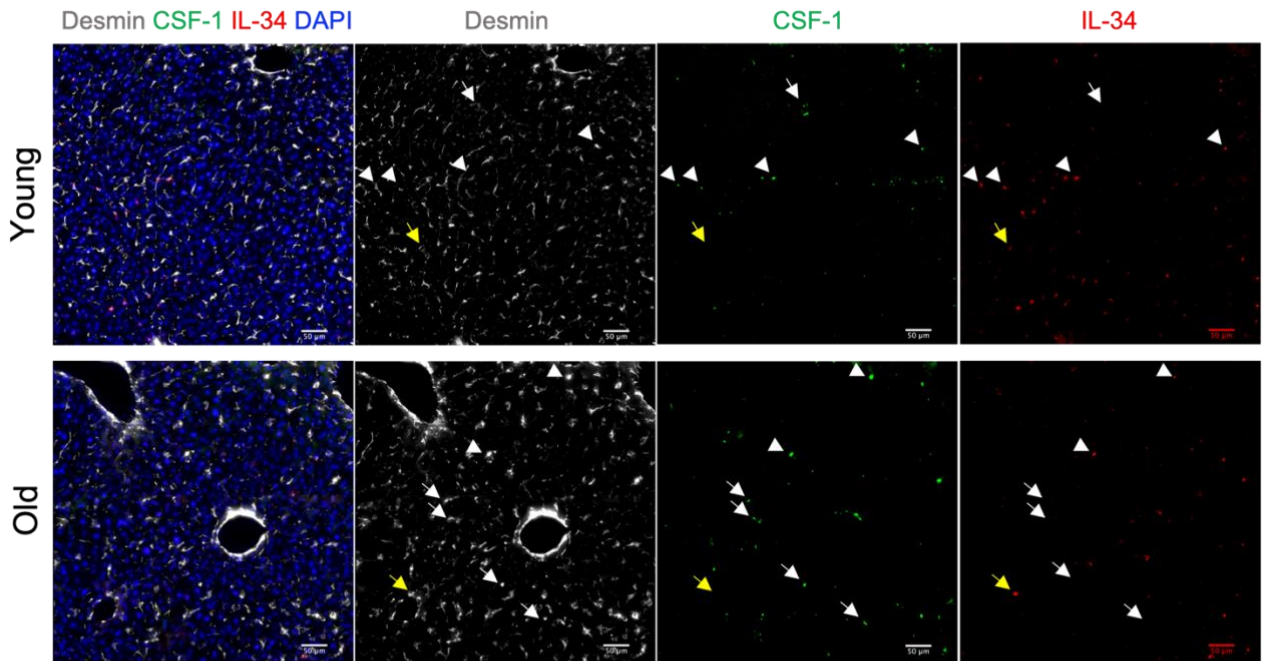


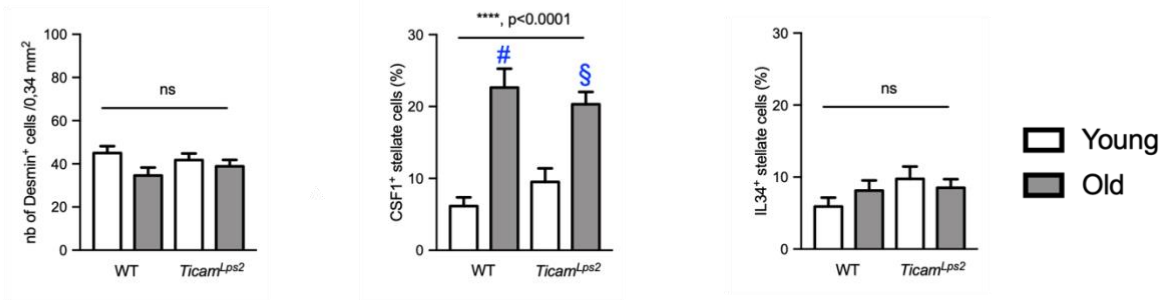
Figure 5

A, Representative liver cryosections from young (2-4 Mo) and old (21-24 Mo) mice stained with desmin (grey), CSF1 (green), IL34 (red) and dapi (blue). **B**, Proportion of Desmin positive (left panel), CSF1+ in Desmin+ (middle panel) and IL34+ in Desmin+ (right panel) in liver sections from young and old WT and Ticam^{Lps2} mice. n=5 per age. **C**, Proportion of Desmin positive (left panel), CSF1+ in Desmin+ (middle panel) and IL34+ in Desmin+ (right panel) in liver sections from saline (PBS), 2w and 6w after sustained Poly(I:C) injections in Young adult mice. Mean± sem . One-way ANOVA (Kruskal Wallis) #, p<0.0001; ¶, p<0.001; §, p<0.01.

A



B



C

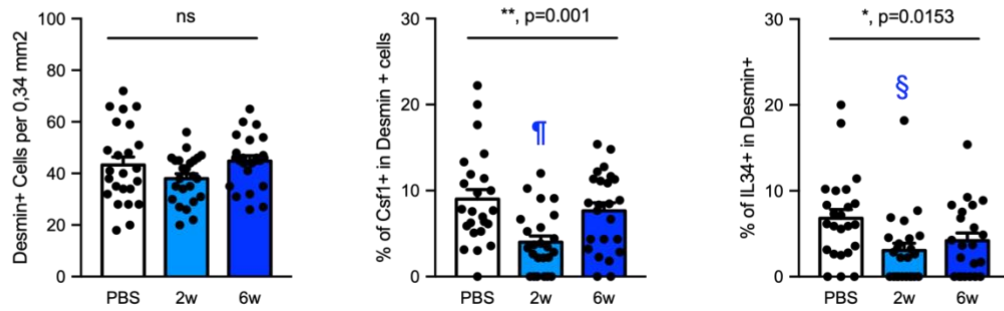
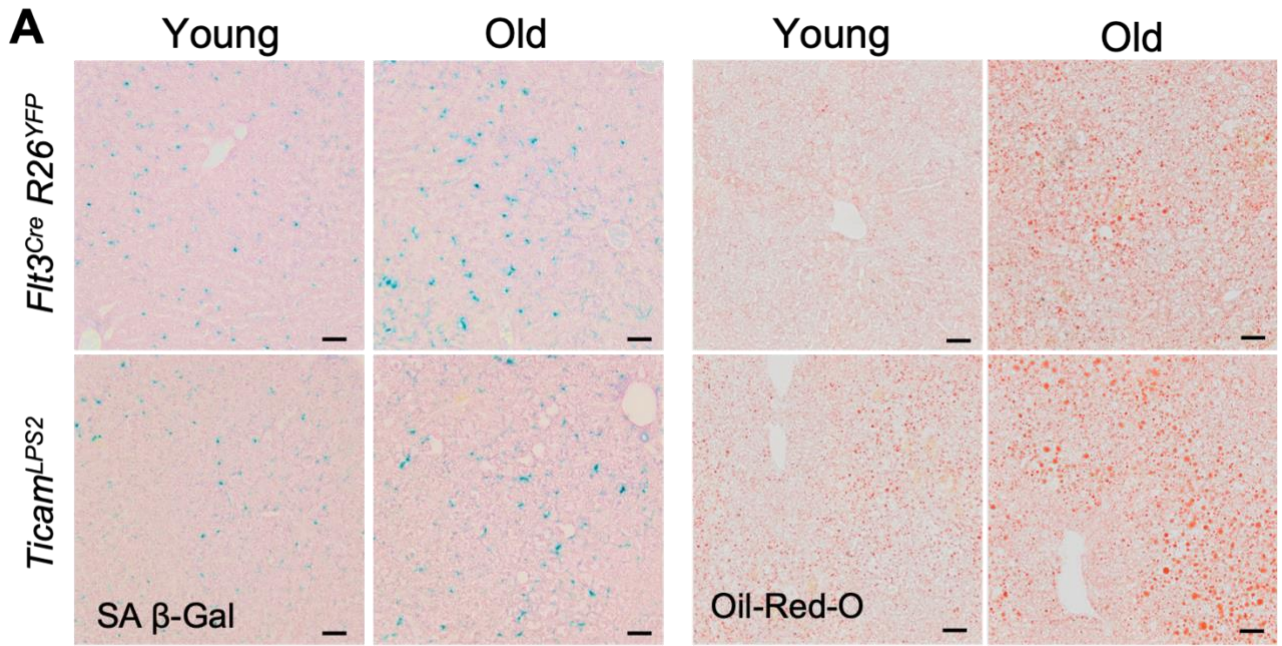
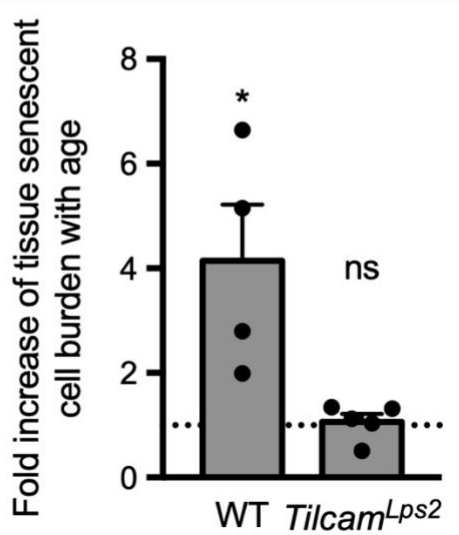


Figure 6

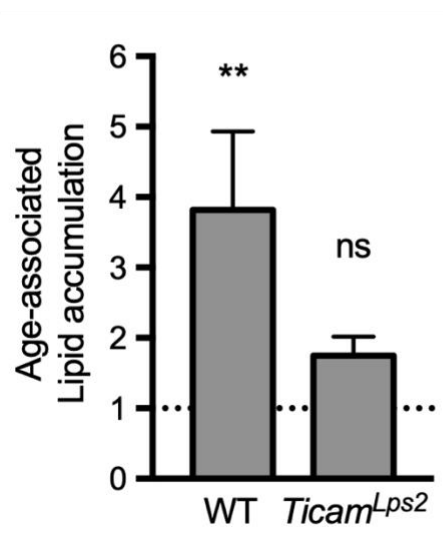
A, Representative images of SA B-Gal and Oil-Red-O stained liver sections from Young and Old Flt3^{Cre} Rosa^{YFP} mice and Ticam^{Lps2} mice. Bar: 50 μ m **B**, Fold change (old /young) in tissue senescent cell burden as measured by the SA B Gal positive area measured in liver sections from Young and Old Flt3^{Cre} Rosa^{YFP} mice and Ticam^{Lps2} mice. Mean \pm s.e.m. **C**, Fold change (old/young) or lipid accumulation measured by the Oil-Red-O positive area in liver sections from Young and Old Flt3^{Cre} Rosa^{YFP} mice and Ticam^{Lps2} mice. Mean \pm s.e.m. *: t-test, *:p<0,05; **:p<0,01 compared to young of the same genetic background.



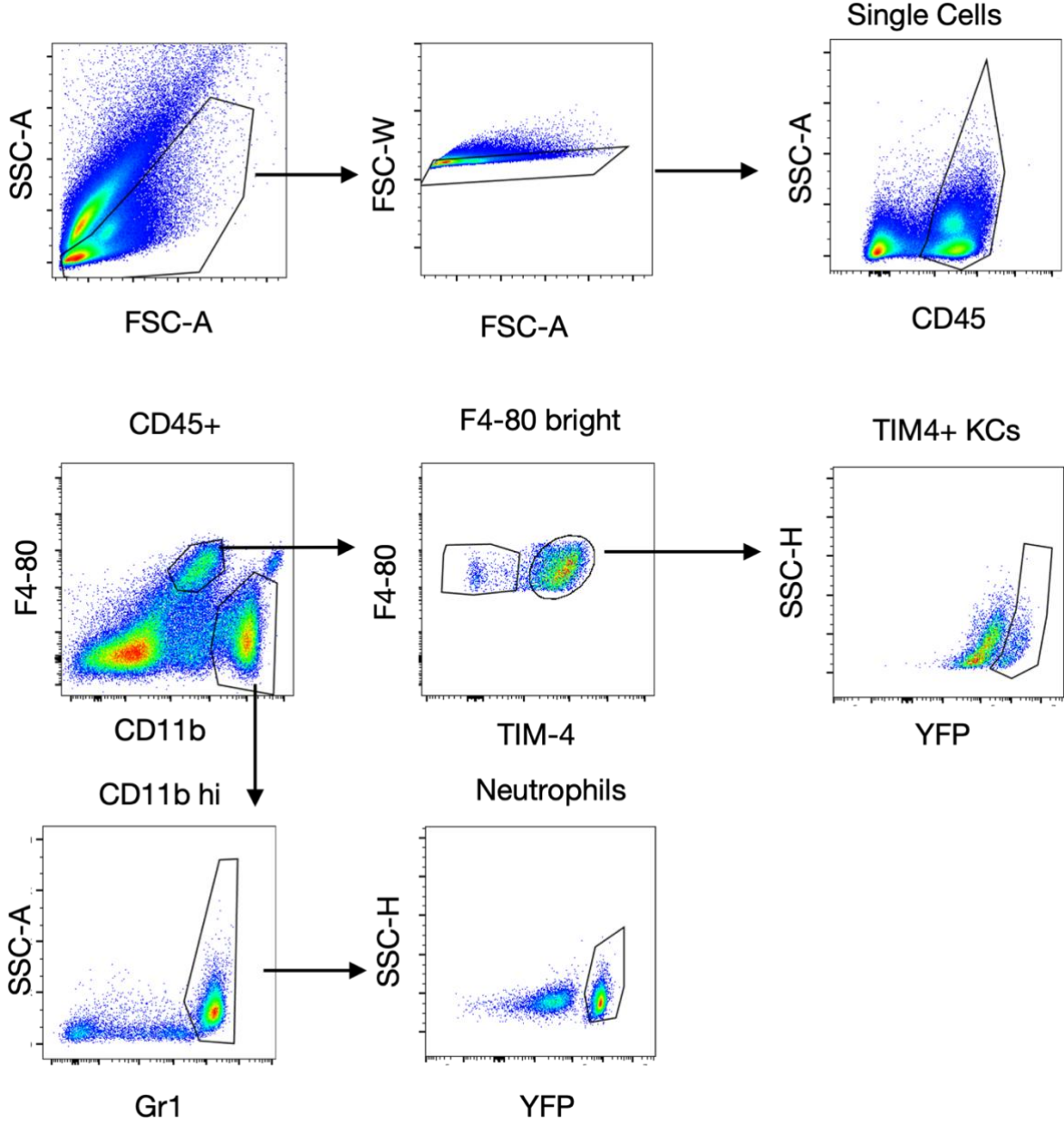
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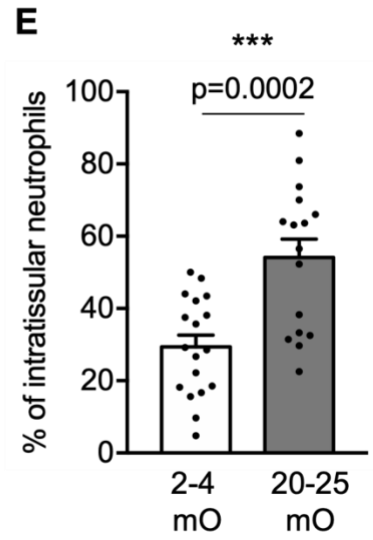
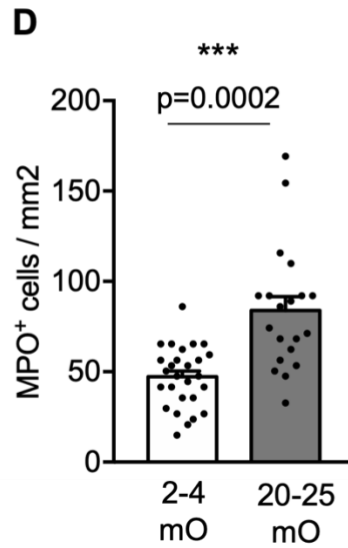
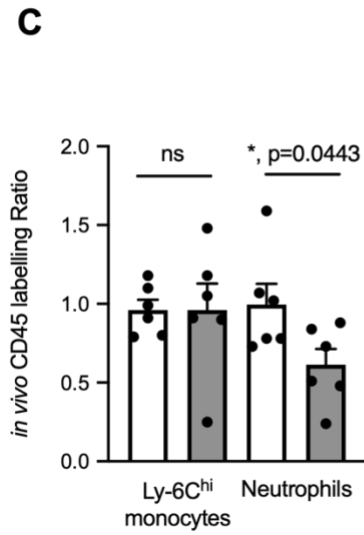
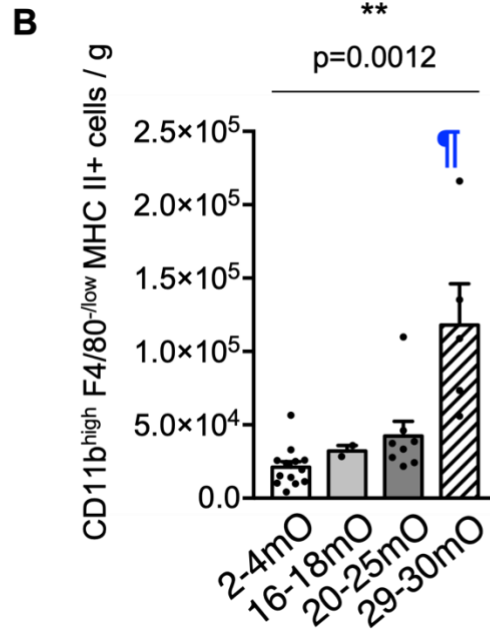
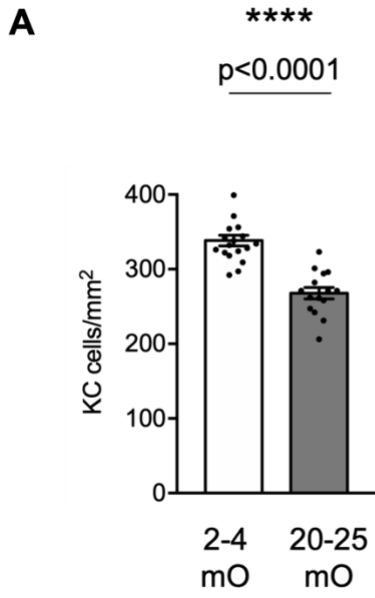


Extended data Figure 1 : Main gating strategy for the analysis of liver myeloid populations

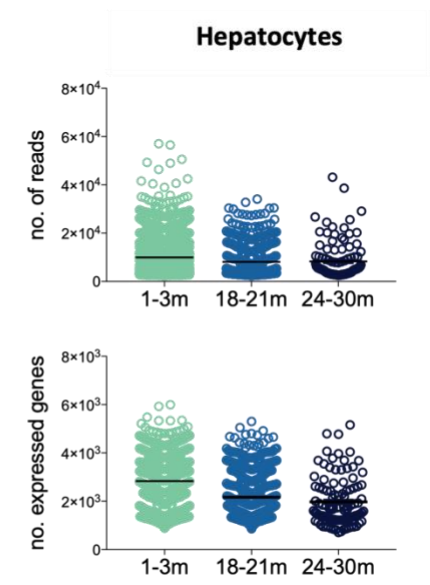
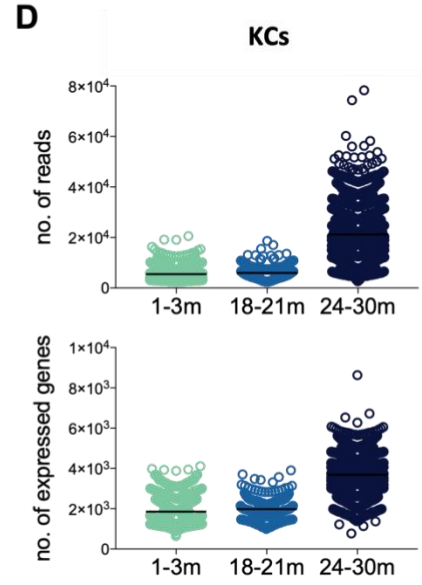
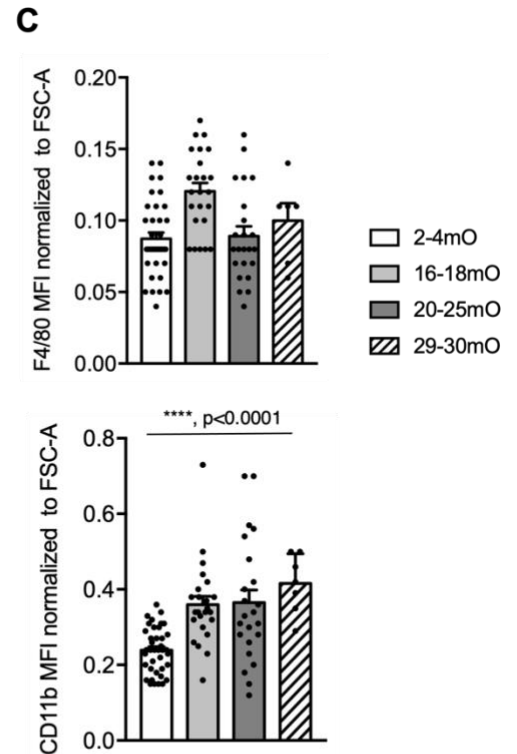
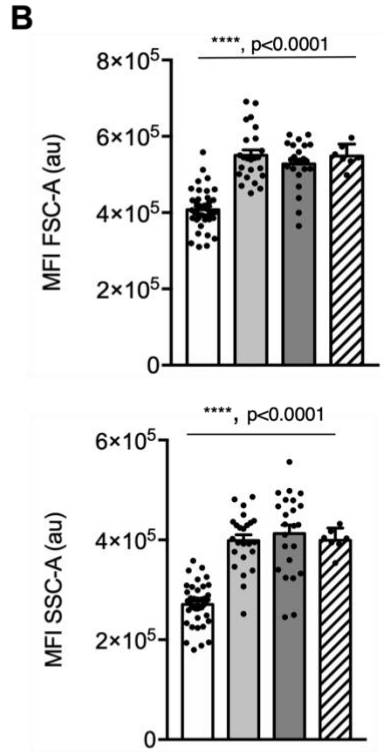
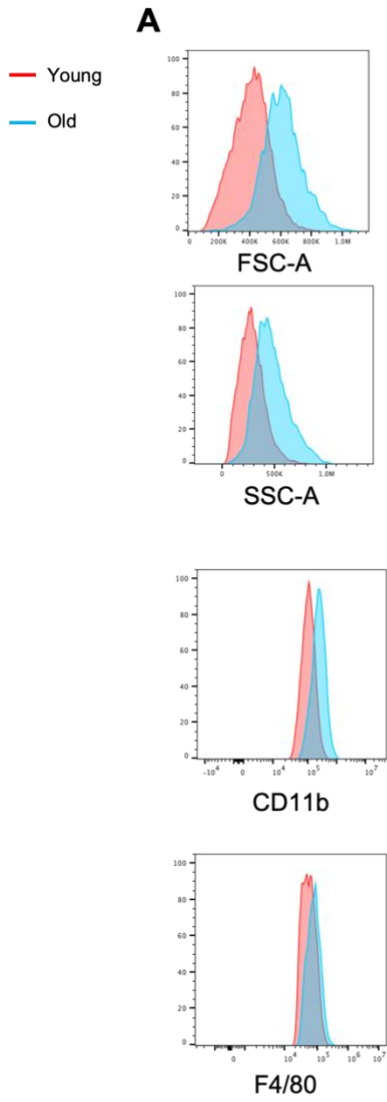


Extended data Fig. 2: Age-associated changes in liver innate immune cells.

A, Number of F4/80⁺ liver Kupffer cells (KC) per mm² in cryo-sections from Young (2-4 mO) and Old (20-25 mO) mice. Mean \pm s.e.m; Mann-Whitney test; n=3 per age group. **B**, Number of CD11b^{high} F4/80^{-/low} MHC-II⁺ cells per g of liver from Young (2-4mO; n=13) and Old 16-18mO (n=3), 20-25mO (n=8) and 29-30mO (n=5) animals. Mean \pm s.e.m; one-way ANOVA (Kruskal Wallis) and post-test Dunn multiple comparison test against Young group: ¶, p<0.001. **C**, ratio of CD45 labelling in the tissue compared to the labelling of the circulating counterparts, after in vivo injection of CD45 antibody 2 minutes before sacrifice **D**, Number of MPO⁺ neutrophils per mm² in liver and **E**, Frequency of intra-tissular liver neutrophils in cryo-sections from Young (2-4 mO) and Old (20-25 mO) mice. Mean \pm s.e.m; Mann-Whitney test; n=4 per age group.



Extended data Fig. 3: Age-associated morphological changes in liver resident macrophages. **A**, Representative histograms of SSCA (granularity), FSC-A (size), CCD1b and F4/80 expression in Young (red line) and Old (20-25 mO; blue line) liver Kupffer cells (KC). **B**, Median Fluorescent Intensity (MFI) of SSC-A and FSC-A and **C**, of F4-80 and CD11b expression normalized to size in Young (2-4 mO, n=40) and Old 16-18mO (n=24), 20-25mO (n=23) and 29-30mO (n=7) KCs. Mean±s.e.m; one-way ANOVA (Kruskal Wallis). **D**. Number of reads for the KC and Hepatocyte clusters, extracted from the Tabula Muris Senis consortium data set.



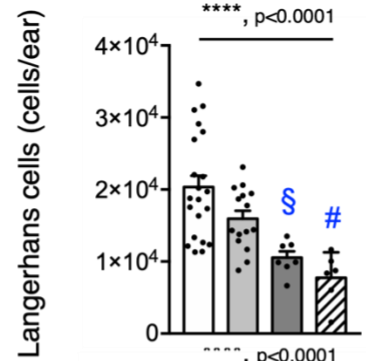
Extended data Figure 4: Loss of HSC-independent resident macrophages with ageing in most tissues except in the kidney.

A, Number of resident macrophages per ear for epidermis (Langerhans cells) and per g of tissue for lung (Siglec-F⁺ F4/80^{bright} CD64⁺), brain (F4/80⁺), spleen (F4/80^{bright} CD64⁺) and kidney (F4/80^{bright} CD64⁺) from Young (2-4mO; n=19-41) and Old 16-18mO (n=4-24), 20-25mO (n=7-24) and 29-30mO (n=3-11) animals. Mean \pm s.e.m; one-way ANOVA (Kruskal Wallis) per organ and Dunn multiple comparison test against Young group: #, p<0.0001; ¶, p<0.001; §, p<0.01; ‡, p<0.05. **B**, Number of (Flt3^{Cre})-YFP⁺ and (Flt3^{Cre})-YFP^{neg} resident macrophages per ear for epidermis and per g of tissue for lung, brain spleen and kidney. Dashed lines represent the mean density of (Flt3^{Cre})-YFP⁺ resident macrophages; mean \pm s.e.m; Young, n=9 and Old, n=8; Mann Whitney test of Young versus Old YFP^{neg} cells: ¶, p<0.001; §, p<0.01; ‡, p<0.05.

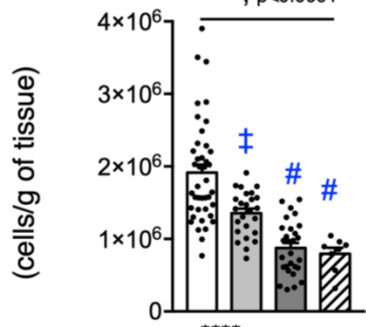
A

- 2-4mO
- ▒ 16-18mO
- 20-25mO
- ▨ 29-30mO

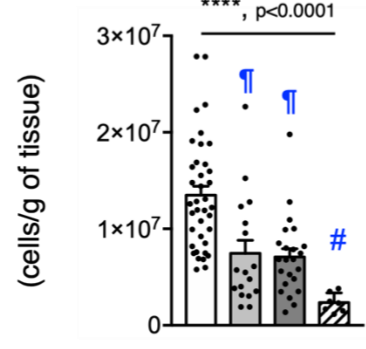
Epidermis



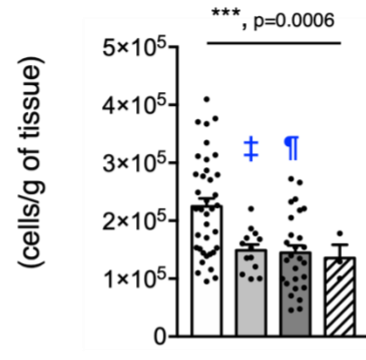
Lung
Alveolar
macrophages



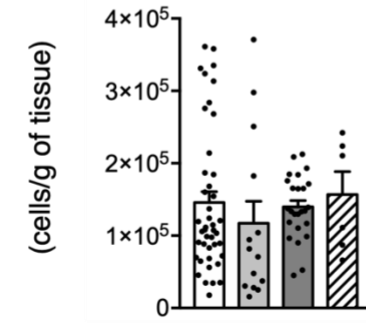
Spleen
Red Pulp
macrophages



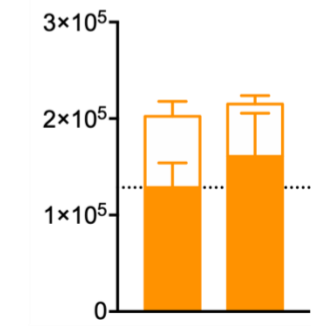
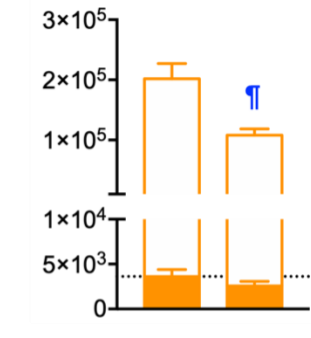
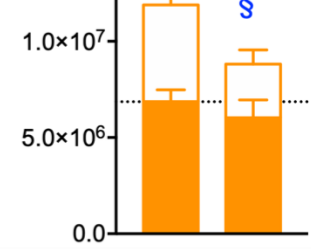
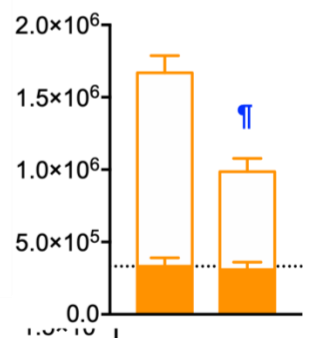
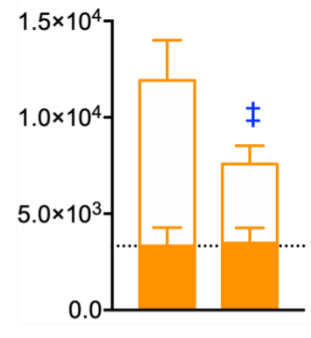
Brain
Microglia



Kidney
macrophages



B

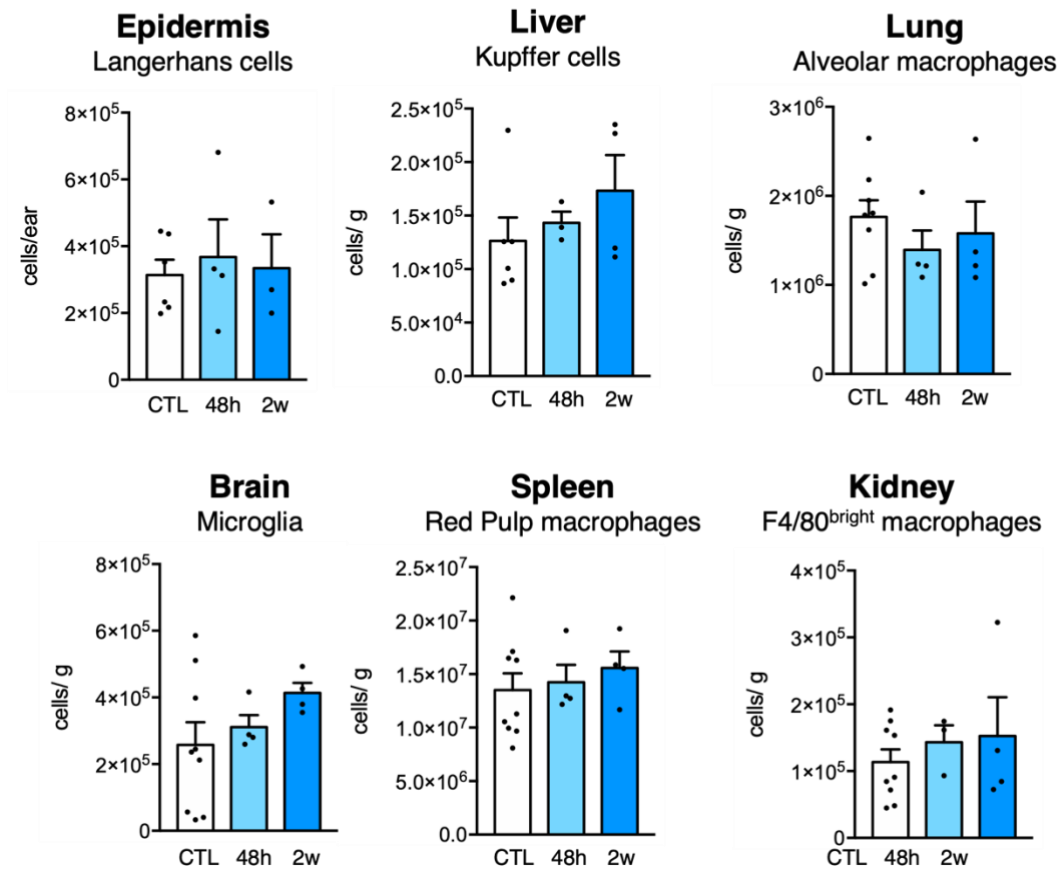
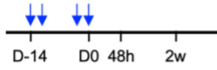


2-4 mO 20-25 mO

Extended data Fig. 5: Macrophage and Neutrophil density after acute Poly(I:C)-induced inflammation, respectively.

Analysis 48h and 2 weeks (2w) after acute (4 injections within ten days) saline (control, CTL) or Poly(I:C) injections. Number of resident macrophages per ear (Langherans cells) and per g of liver (Kupffer cells), lung (alveolar macrophages), brain (microglia), spleen (red pulp macrophages) and kidney. Mean \pm s.e.m; saline control, n=6; 48h, n=4 and 2w, n=4.

Acute Poly(I:C)
4x(5mg/kg)

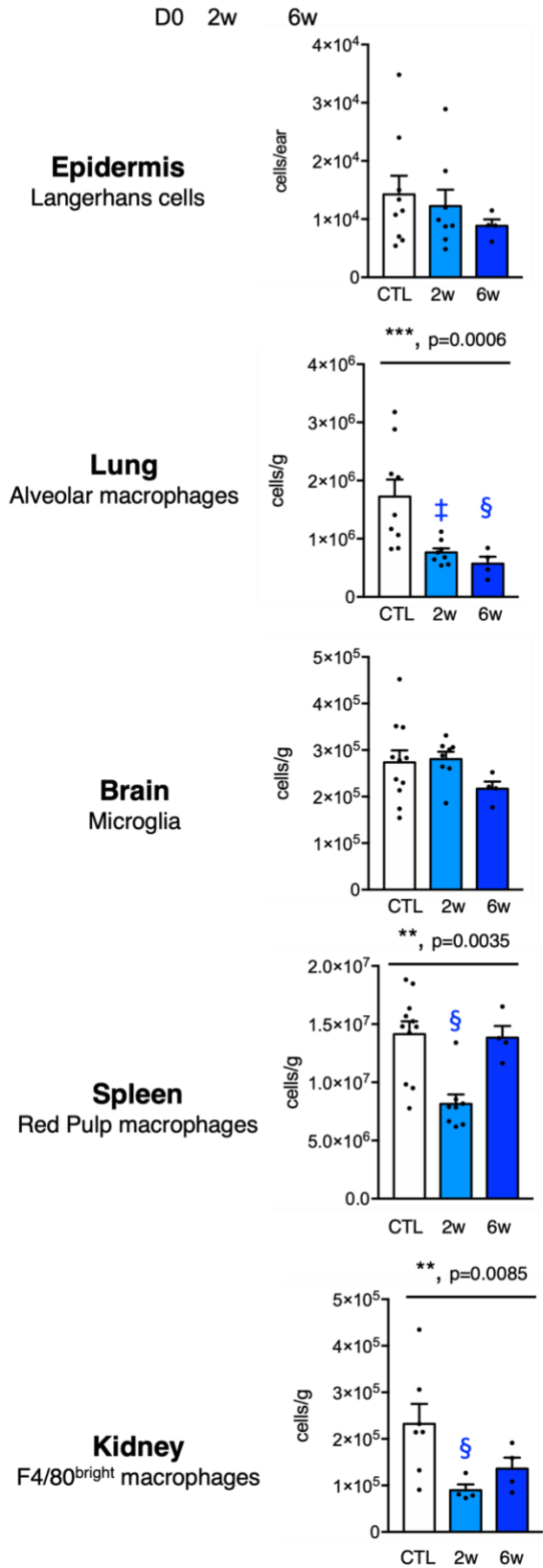


Extended data Figure 6 : resident macrophage density after repeated poly(I:C) inflammation in epidermis, lung, brain, spleen and kidney

A, Analysis 2 weeks (2w) and 6 weeks (6w) after sustained challenge with every other day injections for a month of saline (control, CTL) or Poly(I:C) in Young adult mice. Number of resident macrophages per ear for epidermis and per g of tissue for lung, brain spleen and kidney. Mean \pm s.e.m; one-way ANOVA (Kruskal Wallis) per organ and post-test Dunn multiple comparison test against saline group: §, $p < 0.01$; ‡, $p < 0.05$; saline control, $n = 9-11$; sustained Poly(I:C) 2w, $n = 4-8$ and 6w, $n = 4$. **B**, Analysis 2w and 6w after sustained saline (control, CTL) or Poly(I:C) injections in Young Flt3^{Cre} Rosa^{YFP} mice. Number of (Flt3^{Cre})-YFP⁺ and (Flt3^{Cre})-YFP^{neg} resident macrophages per ear for epidermis and per g of tissue for lung, brain spleen and kidney. Dashed lines represent the mean density of (Flt3^{Cre})-YFP⁺ resident macrophages in saline controls, mean \pm s.e.m; one-way ANOVA (Kruskal Wallis) per organ and Dunn multiple comparison test against YFP^{neg} cells in saline group: ‡, $p < 0.05$; saline control, $n = 3$; chronic Poly(I:C) 2w, $n = 4$ and 6w, $n = 4$.

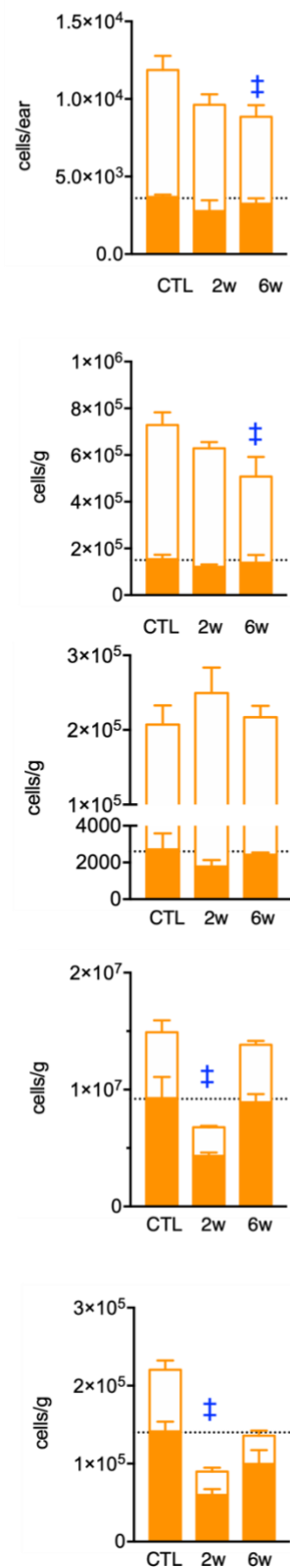
A

Sustained Poly(I:C)
(5mg/kg; 1month)

**B**

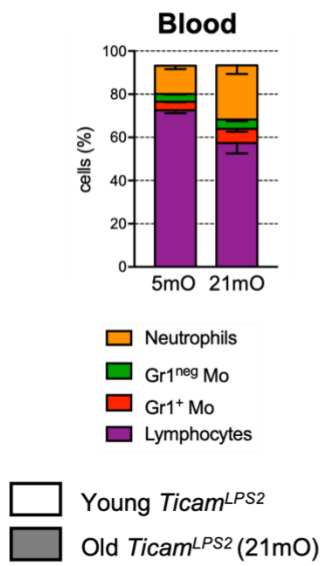
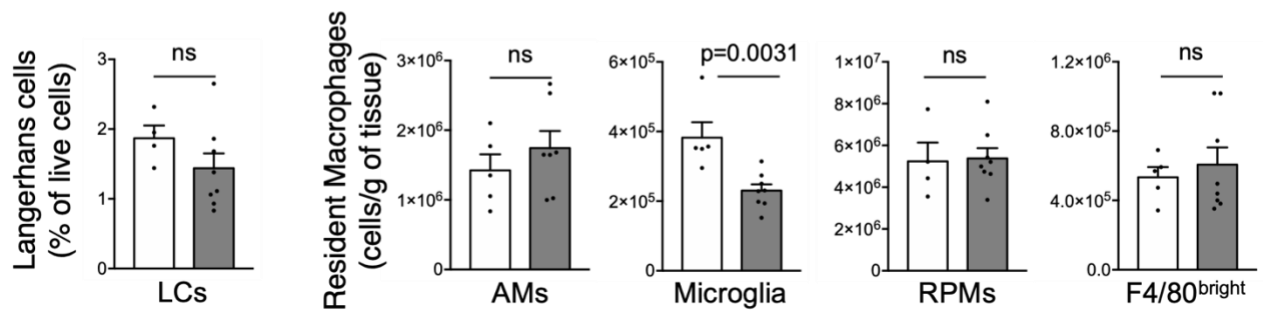
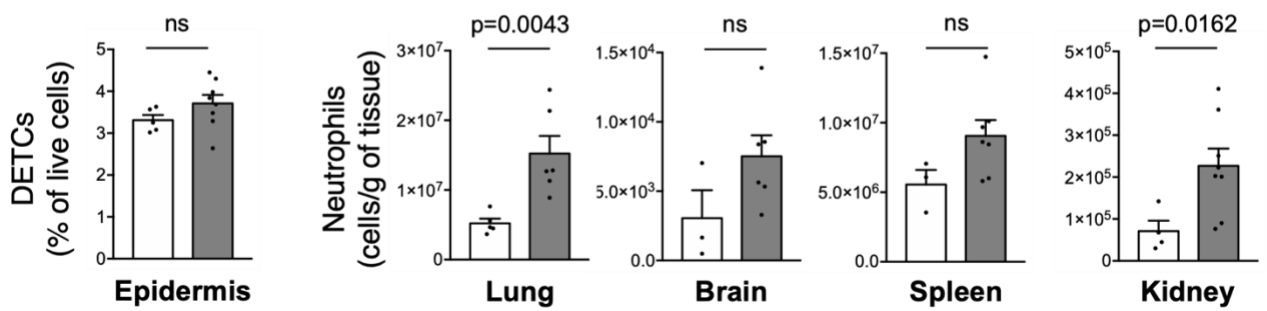
Flt3^{Cre} Rosa^{YFP}

YFP⁺ HSC-derived macrophages
YFP^{neg} resident macrophages



Extended data figure 7: neutrophil and macrophage density in epidermis, lung, brain, spleen and kidney of Ticam^{Lps2} mutants

A, Frequency of neutrophils (orange), Gr1⁻ and Gr1⁺ monocytes (green and red, respectively) and lymphocytes (purple) in blood from Young (5 mO, n=5) and Old (21mO, n=8) Ticam^{Lps2} mutant animals. **B**, Frequency of epidermal Langerhans cells (LCs) and number of resident macrophages per g of lung (alveolar macrophages, AMs), brain microglia, spleen (red pulp macrophages, RPMs) and kidney (F4/80^{bright} CD64⁺ macrophages) and **C**, Frequency of dendritic epidermal T cells (DETCs) and number of neutrophils per g of lung, brain spleen and kidney from Young (5 mO, n=5) and Old (21mO, n=8) Ticam^{Lps2} mutant animals. Mean \pm s.e.m; Mann-Whitney test.

A**B****C**

2. Preliminary data: Kupffer cell maintenance after PLX depletion

The functions of KCs during injury and the ability of circulating monocytes to contribute to the KC pool has been studied in models of massive macrophage depletion using chemicals like gadolinium chloride, clodronate liposomes and Diphtheria Toxin Receptor (DTR)-based selective depletion.

In these models, Kupffer cells die by apoptosis and/or necrosis, and the production of molecules like CCL2 helps recruit monocytes to the liver. There, monocytes differentiate into macrophages, and in the absence of KCs to compete with them, they persist and upregulate KC specific markers such as CLEC4F and TIM4 and adopt a KC-specific transcriptional signature (Scott et al., 2016a).

I sought to determine if, after depletion induced by the impairment of the CSF1R signalling through PLX5622 administration, bone marrow-derived macrophages contributed to the restoration of the KC pool as observed in classical models of depletion.

To deplete macrophages, PLX food was provided *ad libitum* for three days to 8-week old mice, while control mice were fed with control chow. To quantify depletion, mice were analysed on the day following the withdrawal of the PLX food (PLX group). Macrophage numbers and ontogeny were then analysed 2 or 6 weeks after PLX withdrawal (PLX-recovery groups) (Figure 1 A).

As CSF1R is also expressed on bone marrow progenitors and monocytes, I first checked the numbers of circulating leucocytes. While there was no difference in the number of circulating monocytes in control and PLX-recovery, I did observe a non-statistically significant reduction of monocytes in the PLX group (Figure 1B). Since there is such variability in the number of monocytes per μl of blood in control animals, I did not sample sufficient mice in the PLX group to formally conclude here. Understanding whether monocyte numbers are affected by the PLX treatment is crucial for a correct interpretation of the results, and there are discrepancies in the literature. I will thus repeat this experiment to clarify if monocyte numbers are affected, and if so, to what extent.

In the liver, more than 80% of Kupffer cells were depleted. Two weeks after the end of treatment, KCs were back to 50 % of their initial numbers (Figure 1C). KC numbers were back to steady-state levels after six weeks of recovery. TIM4 negative infiltrating macrophages (IMs) followed the same trend but not in a statistically significant manner (Figure 1D). There was no significant increase in the number of neutrophils at any analysed timepoint, suggesting little to no inflammation (Figure 1E).

To better understand the relative contribution of circulating cells and local proliferation of KC, I assessed the labelling efficiency of KCs in our monocyte fate mapping reporter line ($CCR2^{CreERT2} Rosa26^{Tomato}$) and I quantified KC proliferation during recovery using EdU incorporation. I decided to focus on the contribution of monocytes to KC two weeks after recovery for two reasons: (1) analysis of KC ontogeny in $Flt3^{Cre} Rosa26^{YFP}$ mice (see previous section, Figure 4D) showed no contribution of bone marrow-derived cells to KC two weeks

after PLX withdrawal; and (2) monocyte numbers were normal at this timepoint (Figure 1A), which will avoid confounding factors.

To address the contribution of monocytes to KC recovery, I analysed *CCR2^{CreERT2} Rosa26^{Tomato}* mice, where a single pulse of 4-hydroxytamoxifen (OH-TAM) leads to efficient (80%) Tomato labelling in monocytes 24h after (Lacerda Mariano et al., 2020). Five days after pulse, monocyte labelling drops below 20%. If monocytes contribute to KC pools at the time of pulse labelling, it will be reflected in the labelling efficiency of KCs several days after pulse. I thus pulse-labelled monocytes 12 days after PLX withdrawal and analysed Tomato labelling in myeloid cells 5 days later. Blood monocyte labelling 5 days after pulse was comparable between control and PLX-recovery *CCR2^{CreERT2} Rosa26^{Tomato}* animals, around 20%. Similarly, for liver macrophages, no significant differences were observed between control and PLX-recovery animals. Five days after OH-TAM pulse, 20 % of IMs, but only about 1% of KCs are labelled (Figure 1F). These results suggest there is no contribution of circulating monocytes to KC pool during recovery. Since Tim4 is only expressed by monocyte-derived KC after 30 days in other depletion models, I will need to repeat these experiments 30 days after OH-TAM pulse to investigate whether Tomato- labelled IMs can persist and adopt a KC-like phenotype.

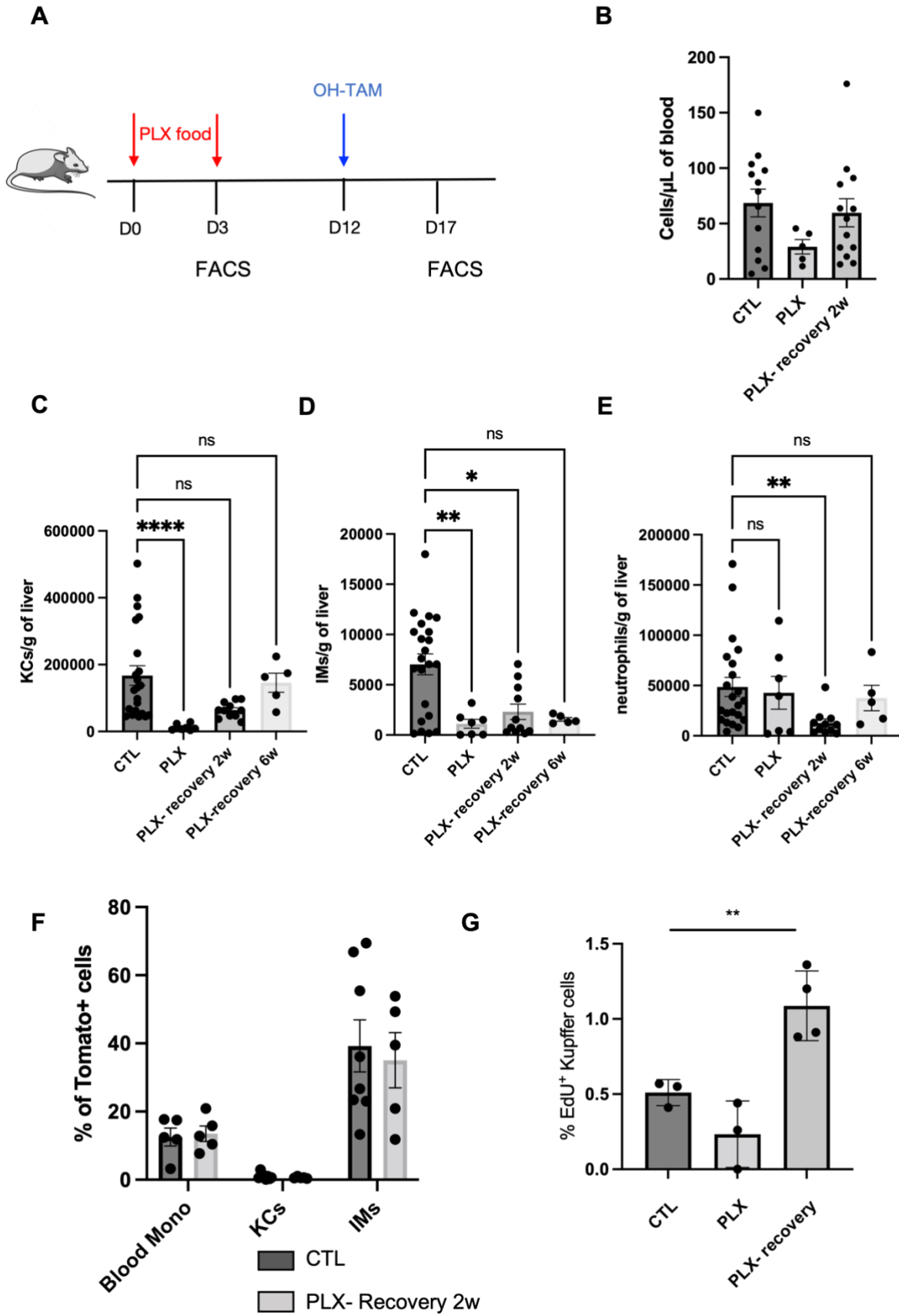
I then investigated KC local proliferation during recovery. In steady state, only 0,5% of KCs were EdU positive after a single pulse of EdU incorporation administered 2h before sacrifice. This proportion was doubled in recovery, supporting the hypothesis that KCs locally proliferated to restore their steady state number when the CSF1R signalling was restored (Figure 1G).

In this model of KC depletion, the restoration of KC pools occurs through local proliferation of the remaining cells. As CSF1R is also expressed on monocytes, this could also reflect the impairment of both recruitment and differentiation of monocytes by CSF1R blockade. It is possible that both monocytes recruitment and KC proliferation were triggered to repopulate the liver KC niche, but that the competition between the two mechanisms was skewed toward Kupffer cells.

In conclusion, although PLX represents a very efficient tool for resident macrophage depletion with limited inflammation, we must consider and better characterize its effects on monocytes.

Figure 1: PLX depletion is not inflammatory and does not induce KC replacement from the circulation by IMs

A, Schematic of the experimental procedure. OH TAM was injected 5 days before analysis in *CCR2^{CreERT2} Rosa26^{Tomato}* mice. Number of : **B**, monocytes per μL of blood; **C**, KCs per gram of liver; **D**, IMs per gram of liver and **E**, neutrophils per gram of liver, in control and after PLX, PLX+ 2-week recovery, PLX+6-week recovery. Kruskal Wallis and multiple comparisons. **F**, Frequency of (*CCR2^{CreERT2}*)-Tomato⁺ blood monocytes, liver KCs and IMs in *CCR2^{CreERT2} Rosa26^{Tomato}* mice five days after OH TAM pulse from control and PLX+ 2-week recovery groups. Mean +/- SEM. **G**,: Frequency of EdU⁺ KCs in CTL, PLX and PLX+ 1 week recovery. Kruskal Wallis. Mean +/- SEM. ****: $p < 0,0001$, **: $p < 0,001$, *: $p < 0,05$



3. Preliminary data: Maintenance of Kupffer Cells in Acetaminophen Induced Liver Injury

3.1. Acute Acetaminophen Induced Liver Injury

The contribution of macrophages has been studied in different inflammatory settings and they have been deemed extremely plastic. But in the light of our new understanding of their heterogeneity, we must reassess whether the diversity of conflicting functions observed in macrophages is not at least in part due to the differences between recruited and resident populations.

In bacterial infections, such as *Listeria monocytogenes*, a massive death of Kupffer cells has been reported. It has been suggested that KC death in this context could be an “altruistic” sacrifice to trigger the recruitment of effectors specialized in microbial clearance (Blériot et al., 2015; Ginhoux et al., 2017). In non-infectious acute liver injury, like acetaminophen-induced liver failure, some studies have also reported a substantial death of KCs in the first days of injury. But as many reports focused on KC frequency (%), it is difficult to conclude as there is a massive recruitment of other cell types. It is thus important to re-examine the maintenance of KCs in this context. It has further been suggested that both Kupffer cells (Campion et al., 2008; Ju et al., 2002) and recruited monocytes are beneficial to the repair process, while other reports emphasized their changing roles over the course of the injury and repair (Triantafyllou et al., 2018; Zigmund et al., 2014)

Before unravelling macrophage functions during repair, I first characterised a model of acetaminophen-induced acute liver injury, in order to determine the dynamics of myeloid cells throughout the repair process and investigate the maintenance of KCs. The gating strategy is presented in [Figure 2A](#).

I identified 3 phases in myeloid cell dynamics over the 12 days following injury ([Figure 2B](#)):

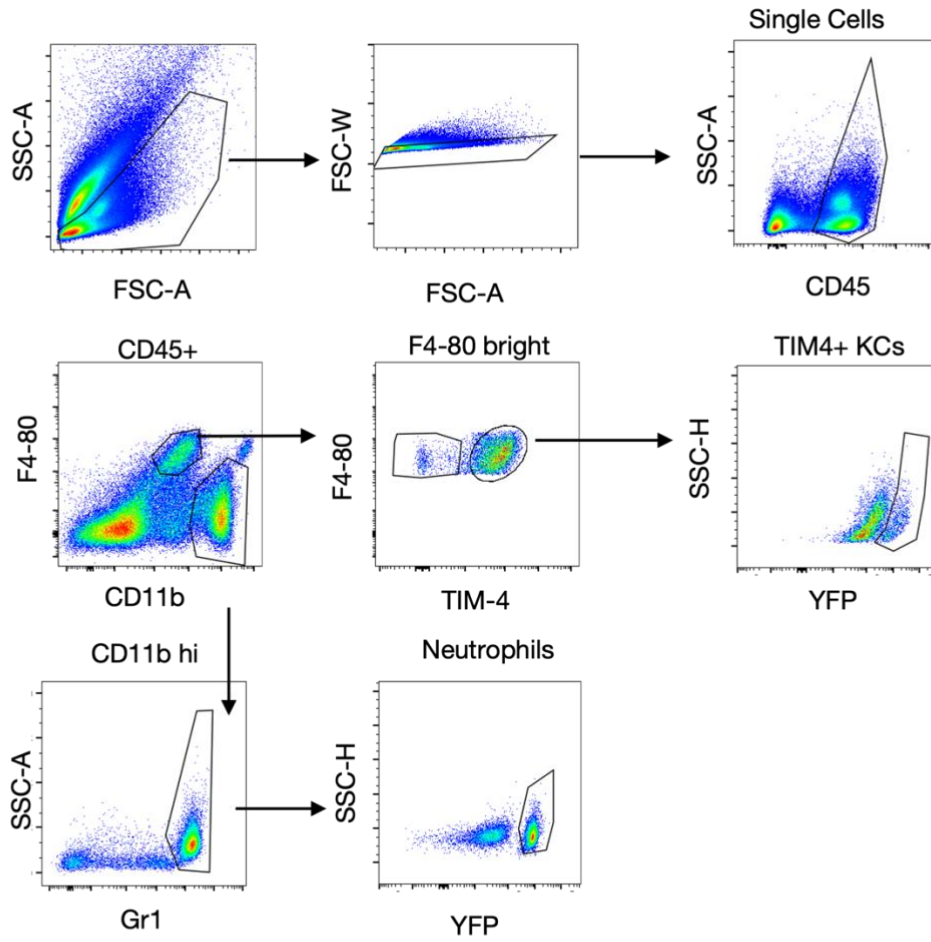
- a first phase (D1-D2) was characterised by a large influx of neutrophils and monocytes from the circulation.
- A second phase (D2-D5) showed an expansion of the macrophage compartment, with an increase in the number of F4-80+ TIM4+ Kupffer cells and a massive infiltration of TIM4 negative macrophages
- A third phase (D5 onwards) saw the resolution of the inflammation with the clearance of the infiltrating cells and the return of Kupffer cells to their steady state levels

To quantify cell abundance, I have relied on absolute numbers of cells per gram of tissue and not their relative contribution to the leukocyte compartment. This latter method may not properly capture the real dynamics of each population in a context where many leukocytes populations are affected. This is particularly striking for KCs. If one compares the percentage of KCS among CD45+ cells between control and the early to mid-phase of injury, one may

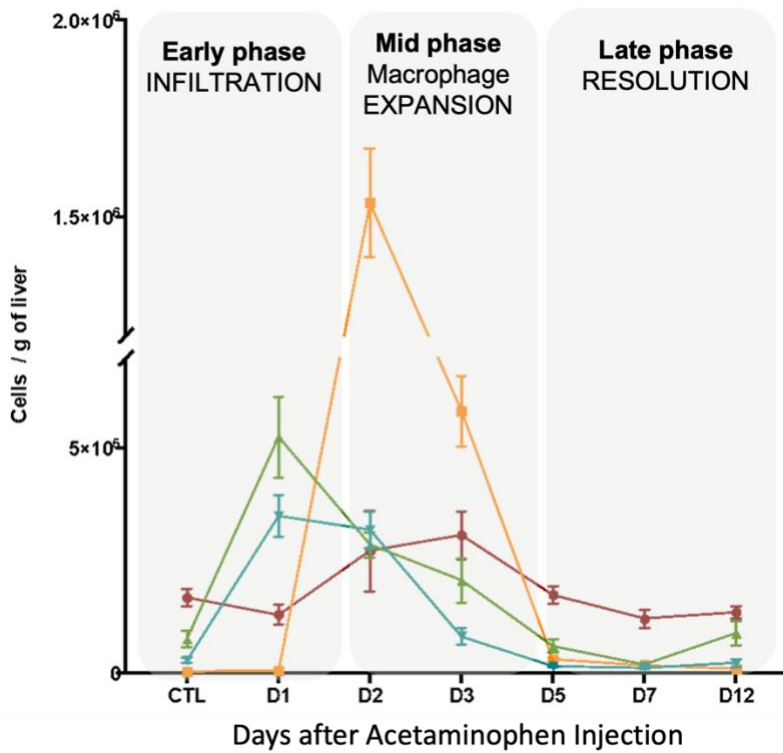
assume that KC numbers are significantly diminished while cell counts per gram of tissue rather suggest that the population remains fairly stable (Figure 2C).

Figure 2: Characterisation of myeloid cell dynamics in Acetaminophen Induced Liver Injury
A: Gating Strategy **B:** Number of cells per g of Kupffer cells, Infiltrating Macrophages, Neutrophils, and other myeloid (CD11b+) cells in CTL (PBS) and up to D12 after injection of acetaminophen. **C:** Evolution of the proportion of liver myeloid subsets among CD45+ cells upon injury and over time, macrophages were gated based on F4-80 and Tim-4 expression. Other myeloid cells were gated based on CD11b and F4-80 (negative). Bar graphs represent mean (\pm sem) percentage of cells among CD45+ cells

A

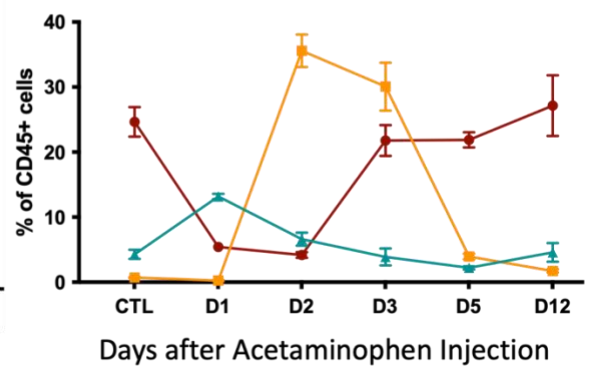


B



- Tim4+ Kupffer cells
- Infiltrating Macrophages
- ▲ Neutrophils
- ▼ Other CD11b+

C



In a first approach to study KC maintenance, we injected twice 2µm PE beads two weeks before injection of acetaminophen. These beads were efficiently phagocytosed by KCs and two weeks after, about 60% of KCs were still labelled and contained PE beads (data not shown). We measured the labelling efficiency over time after injury and calculated the ratio of labelling normalised to control. The stability of the labelling over time suggested that after injury, there was no significant contribution of newly arrived, not labelled cells to the pool of KCs. (Figure 3A)

I then used *Flt3^{Cre} Rosa^{YFP}* mice to investigate the contribution of bone marrow progenitors and circulating cells to the KC compartment up to three weeks after injury. In the blood of *Flt3^{Cre} Rosa^{YFP}* mice, about 80 % of monocytes are labelled. The same proportion was observed in IMs. In steady state, only 20% of KCs are labelled. Any increase in the YFP labelling among KCs after injury could indicate the contribution of labelled cells from the circulation to this compartment. Throughout the course of the injury and repair process, there was no significant change of this labelling, suggesting no input from the circulation (Figure 3B).

To evaluate KC proliferation, BrDU was injected two hours before sacrifice. We observed a peak of KCs proliferation at D2 post injury, and a second peak after the resolution (Figure 3C). But these results need to be confirmed with another approach.

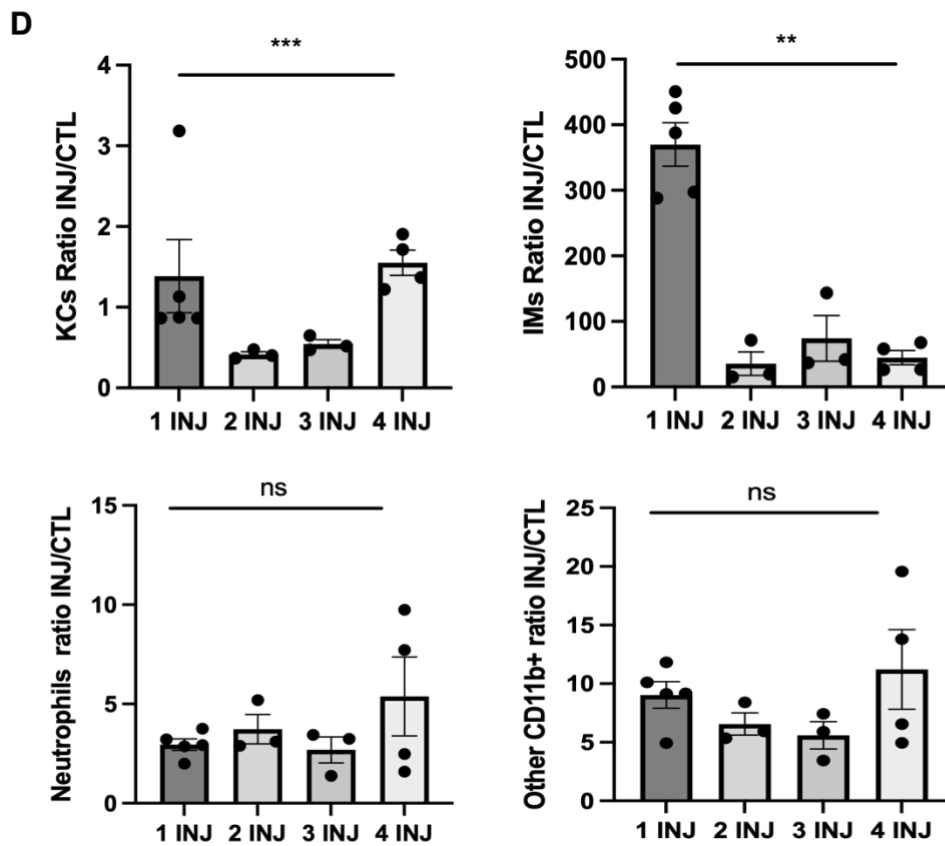
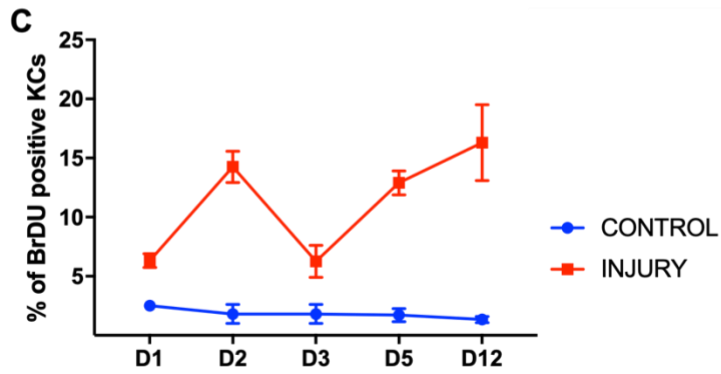
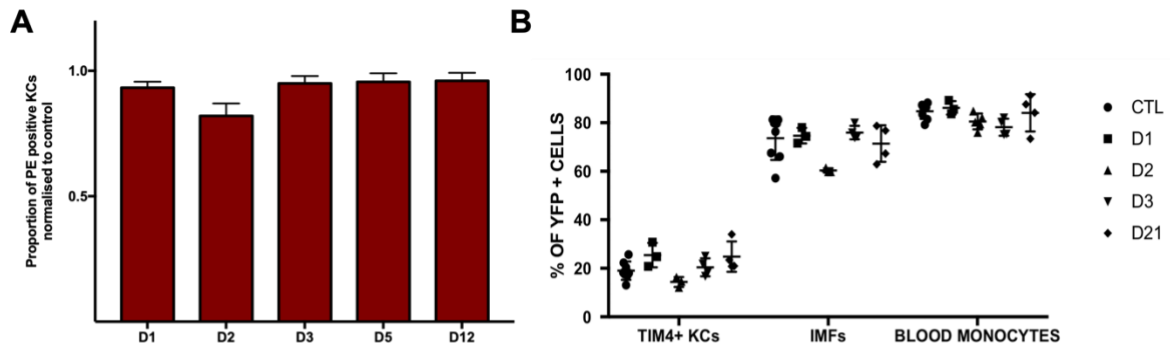
3.2. Repeated Acetaminophen Induced Liver Injury

In acute liver injury, KCs were able to self-maintain throughout the injury and repair process, through local proliferation. But that does not preclude an exhaustion of this maintenance potential after repetitive challenges. As a first approach, I performed a pilot experiment where animals underwent 1 to 4 rounds of acetaminophen injections, with a month of recovery in between. I then calculated the ratio of the number of KCS and IMs on day 2 (D2) after the last injury compared to the controls, in each experimental setting. I observed a reduction in the number of KCs after 2 and 3 rounds of injury. But surprisingly, KC numbers after 4 rounds of injury were not different from baseline (a single challenge (Figure 3D, upper left). This could indicate a transient proliferative exhaustion of KCs. But although the statistical significance of these trends appeared in the overall one-way ANOVA test, multiple comparisons did not show any significance, probably due to the small number of animals. Regarding the number of IMs, their recruitment seemed surprisingly impaired at 2, 3 and 4 rounds of injury. (Figure 3D, upper right). This may reflect a prominent role for KC in the recruitment of infiltrating macrophages or an exhaustion mechanism intrinsic to this population. Neutrophils and other myeloid cells in contrast do not seem affected. But here again, more data points would be needed to yield more statistically robust data. The “Other CD11b+” populations that notably comprises monocytes, seem to show a decrease in numbers after 2 and 3 rounds of injury, which would indicate that like IMs (for which they are a proposed precursor) their recruitment

is impaired. But statistical analyses show now significant difference with 1 round of injury, maybe due to the limited number of data points. If pursued, I will also couple this phenotypical KC analysis with proliferation studies and enzymatic tests to measure liver enzymes serum levels (ALT/AST) and assess the efficiency of the repair process. I will also evaluate whether KC proliferation is impaired after two or three rounds of ALI.

Figure 3 : Kupffer Cell maintenance in Acetaminophen Induced Liver Injury

A, Ratio of PE bead labelling efficiency compared to Control (PBS) from D1 to D12 after injection of acetaminophen. **B**, Labelling efficiency in Kupffer Cells (KCs), Infiltrating Macrophages (IMs) and blood monocytes of Flt3 Cre RYFP mice, in CTL (PBS) and up to 21 days after injection of acetaminophen. Bars : mean +/- SEM. **C**, BrDU labelling in KCs in CTL (blue) and Injury (red). Bar +/- SEM. **D**, Ratio of KC (left) and IM (right) numbers per g of liver, compared to Control (PBS) after 1 to 4 rounds of acetaminophen injection, at D2 after the last injection, Bar +/- SEM. Kruskal Wallis **: $p < 0,01$; ***: $p < 0,001$.



4. Methods (Maintenance after PLX5622 and Injury)

Mice models: Animal procedures were performed in accordance with the Care and Use Committee of the Institut Pasteur (CETEA) guidelines and with their approval.

Acetaminophen Induced Liver Injury: Six-week-old male C57NL/6JRj mice were purchased from Janvier Labs and experiments were performed at 7-8 weeks of age, after acclimation to our facility. Males were used as they have been proven more sensitive to AAP induced liver failure compared to females (Dai et al., 2006; Mohar et al., 2014), and having animals from the same gender allowed to exclude the gender factor of variability. They were handled humanely, monitored closely after AAP administration, and sacrificed by cervical dislocation.

Maintenance: Flt3-Cre Rosa^{YFP}, Csf1r^{MeriCreMer} Rosa^{Tom} and CCR2^{Cre-ERT2} Rosa^{Tom} mice received PLX or chow food for three days ad libitum. They were analysed after 3 days of this regimen, or after 3 days and a period of 2 to 6 week-recovery.

Pulse labelling of CCR2^{Cre-ERT2} Rosa^{Tomato} mice : OH-Tamoxifen (OH-T) (10 mg/mL OHT in 80% NaCl 0,9%,10% Ethanol, 10% Castor oil) was mixed with progesterone (10 mg/mL in 90% castor oil, 10% ethanol) to obtain a 2:3 OHT /1:3 progesterone solution. Mice were weighted and intraperitoneally injected with 75 µg/g of this solution.

Acetaminophen Induced Liver Injury: Two weeks before the onset of liver failure, animals received two iv injections of 2µm PE- beads. Animals were fasted overnight (16h) and weighted before AAP administration and re- introduction of food. Fresh solutions of AAP (Sigma Aldrich) at 20 mg/mL were prepared immediately before use by dissolving the compound in warmed sterile PBS 1X. Acute liver injury was induced by injecting intraperitoneally (ip) a sub-lethal but toxic dose (300 mg/kg) of acetaminophen (AAP) (Mohar et al., 2014; Mossanen and Tacke, 2015; Zigmond et al., 2014). Animals were monitored twice a day for signs of distress or pain and weighted daily.

Proliferation: **Acetaminophen Induced Liver Injury:** Proliferation rates were assessed with BrDU incorporation analysis using the BD BrDU flow Kit (BD). 1 mg of BrDU was injected intraperitoneally 2 hours before sacrifice. Liver single- cell suspensions were prepared as described page 53 and stained with a live/dead dye (Yellow fixable Live/dead, Molecular probes) and with antibodies directed against CD45, CD11b, F4/80 and Tim4. Cells were then fixed, permeabilised and treated with DNase according to manufacturer's instructions and stained with the APC-BrDU antibody. **Maintenance:** Proliferation rates were assessed with EdU incorporation analysis using the BD EdUU flow Kit (BD). 1 mg of EdU was injected intraperitoneally 2 hours before sacrifice. Liver single- cell suspensions were prepared as described page 53 and stained with antibodies directed against CD45, CD11b, F4/80 and Tim4. Cells were then fixed, permeabilised and treated with DNase according to manufacturer's instructions and stained with the APC-EdU antibody and an antiGFP antibody (Fischer Life).

CONCLUSION

During my thesis, I analysed the mechanisms of maintenance of Kupffer cells (KC) in different physiopathological and experimental contexts. My results challenged the idea that experimental or physiological depletion of Kupffer cells systematically led to the repopulation of the KC niche by bone marrow derived cells. We showed that age was associated with a decrease in KCs and proposed inflammation as a driver for this loss. Experimental Poly (I:C) induced repetitive inflammation and the analysis of Trif^{Lps2} mutants further supported this hypothesis. The loss of KCs was not compensated by the recruitment and differentiation of circulating cells. This suggests that KC death and the resulting (partial) availability of the KC niche are not in themselves sufficient for the engraftment of monocyte derived macrophages in the liver. These findings raise the question of the determinants of monocyte participation into the KC pool. What are the conditions permissive of their recruitment, differentiation, and eventual persistence in the tissue? These questions will be discussed below.

DISCUSSION

I will first discuss the pathways through which inflammation can alter Kupffer Cell viability. Second, I will discuss the potential determinants of the contribution of circulating cells to the Kupffer cell pool. I will then explore some functional implications of the age induced Kupffer cell loss. Finally, I will discuss the challenges faced in the investigation of KC-specific function in liver injury and discuss future perspectives.

1. Effects of inflammation on Kupffer Cell viability

Kupffer cells are well described as drivers of inflammation, mostly through cytokine production. Conversely, inflammation responses induce profound remodelling in KC themselves and sometimes can lead to KC death. Cell death can be distinguished between necrosis and programmed cell death, of which apoptosis is the most studied. Apoptosis is generally regarded as a protective response, whereas necrosis is thought to favour inflammation and disease progression. However, new pathways of programmed cell death have been identified that render this dichotomy in part obsolete.

1.1. Apoptosis

Macrophage apoptosis has been mainly studied in atherosclerosis and infections, and not much in other inflammatory contexts.

Atherosclerosis is the accumulation of lipid particles within vessels walls, an in particular in large arteries. This results in the activation of endothelial cells, triggering inflammation and the recruitment of monocytes and other leukocytes. There, monocytes can then differentiate into macrophages, and uptake low density lipoproteins (LDL) to form “foam cells”. The atherosclerotic plaque is composed of a build-up of lipids, matrix, foamy macrophages and other trapped cells (Moore & Tabas, 2011). In this pathological context, macrophages are a heterogenous population where some subsets are deemed both beneficial as they can scavenge lipids and promote vascular growth, but also detrimental as some activated, inflammatory macrophage produces metalloproteases that digest the extracellular matrix and can trigger the rupture of the plaque and thrombosis as a result. In consequence, macrophages have emerged as a potential target for the treatment of atherosclerosis. As high levels of macrophage death have been observed in late-stage atherosclerosis, the mechanism of this death has been extensively studied and pharmacologically targeted (Martinet et al., 2019). Studies have shown that at different stages of the atherosclerosis plaque, macrophage death play different roles. Several inducers of macrophage death have been identified: oxidative stress, the presence of inflammatory cytokines such as TNF α , ER stress, scarcity of growth factors and activation of the FAS ligand pathway. Endoplasmic Reticulum (ER) stress occurs when there is an accumulation of misfolded proteins that exceed the processing

capabilities of the ER. This triggers apoptosis pathways through the induction of C/EBP Homologous Protein (CHOP). Ligands (LDL, endotoxins, phospholipids) of macrophage Pattern Recognition Receptors (PRRs), notably scavenger receptors, have been shown to trigger macrophage apoptosis through the ER stress/CHOP pathway.

Interestingly, foamy macrophages and macrophage apoptosis are also involved in *Mycobacterium tuberculosis* (*Mtb*) infection, with opposing roles. Macrophage apoptosis during *Mycobacterium* infection through ER stress/CHOP pathway has been proposed to limit *Mtb* infection by depriving the bacterium of its reservoir (Gan et al., 2008; A. Li et al., 2020). However, *Mtb* infection leads to the formation of granulomas, where foamy macrophages are very abundant. And foamy macrophages have been linked to *Mtb* latency, as they could sustain intracellular *Mtb* in a physiological state similar to the non-replicating quiescent state, invoked to explain persistence (Peyron et al., 2008).

As ageing is accompanied by the accumulation of cellular debris, we can hypothesize that an increased scavenging burden with age could negatively impact general macrophage survival, for both IM and KC. Recently differentiated macrophages (IM) could have limited survival in such environment, contributing to their inability to persist in the aged liver. In regard to KC, we could hypothesise that this progressive scavenging burden could contribute to the gradual loss of KC by cell death observed during ageing. Supporting this hypothesis, we did observe an increase in cell size and granularity of KCs with age, which suggests an increased vacuolar burden in old macrophages. This warrants a more detailed analysis at the ultrastructural levels of KCs with age, to characterize the accumulation of scavenging vesicles with age and their contents.

1.2. Pyroptosis

Recently, an inflammation-induced programmed cell death was described in macrophages and other immune cells: pyroptosis. It involves a caspase mediated cell death triggered by the activation of the inflammasome: activation of the NLRP3 classical pathway of the inflammasome formation can lead both to the production of cytokines and the cleavage of Gasdermin to liberate a pore forming domain. The formation of the pores leads to extensive membrane damage and the subsequent death of the cell. (Frank & Vince, 2019). Pyroptosis is thought to be a mechanism of defence against bacterial endotoxin, and it is not clear whether the mechanism exists in “sterile” inflammation.

1.3. Necroptosis

Necroptosis is another form of inflammation related cell death, mediated by the fixation of Tumor Necrosis Factors (TNF) to their death receptor in the absence of active caspase 8. RIPK1 recruits RIPK3 and their autophosphorylation creates the necrosome complex. This complex

recruits and phosphorylates the Mixed Lineage Kinase-domain Like (MLKL) protein, which then translocates to the membrane and destabilizes it (Weinlich et al., 2017). Necroptosis is observed in KC during *Listeria monocytogenes* infection (Blériot et al., 2015) and notably, release of KC cellular contents into the environment leads to the recruitment of monocytes into the liver and their differentiation into KC-like macrophages.

Apoptosis leads to the presentation of PS at the cell surface, which can trigger their swift removal by professional phagocytes. In their absence, apoptosis leads to the formation of smaller apoptotic bodies that can then be uptaken by non-professional phagocytes, such as fibroblasts (Martin et al., 2003). In contrast, both pyroptosis and necroptosis are particularly pro-inflammatory through the release of the cell content. These three mechanisms could play a role in the inflammation-induced age-associated cell death of Kupffer cells, depending on the succession of insults sustained by the liver throughout age. Currently, the identification of the different programmed cell deaths pathways relies on the usage of genetic mutants that are selectively impaired in one specific pathway. This is thus not suited to study the ageing-induced KC cell death, since knocking down only one specific pathway may not be sufficient to reveal its overall contribution as other pathways are still active. Further, the relative contribution of each pathway may be highly variable between animals, depending on the challenges they responded to throughout their lifetime. Since KC loss is gradual and cumulative over time, pharmacological interventions to inhibit apoptosis would be very difficult to interpret. Further, this approach needs to be macrophage specific as changing the cell death rate of tissue cells could also have indirect effects on KC survival by reducing the scavenging burden described above.

2. Determinants of IMs persistence and differentiation into KCs

2.1. Absence of competition

A first determinant that can be proposed for monocyte contribution to KC pool is the number (or density) of remaining KCs. It can be hypothesized that there could be an initial threshold of KC depletion that would create a “window of opportunity” for monocyte contribution (Guilliams et al., 2020; Scott et al., 2016a). This hypothesis requires two conditions. On one hand, loss of KC would create an available niche (in terms of space and growth factor availability) that would trigger the recruitment and differentiation of monocytes to replace them. On the other hand, the remaining pool of KCs would not have sufficient proliferation capacity to replenish their stock, either by lack of a proliferative signal, presence of a proliferation inhibitory signal or by lack of time. In the latter, KC re-entering the cell cycle would be slower than monocyte recruitment. In this scenario, monocyte differentiation into KC can be explained by their microenvironment (discussed below). But how the partial or local absence of KC would trigger monocyte recruitment is still unknown, especially in a non-inflammatory context. Moreover, it is still unknown for how long monocyte-derived KCs can persist in the liver and whether they can self-maintain by local proliferation over extended periods of time.

Thus far, we have not been able to experimentally test this hypothesis, as we must develop and characterize tools to manipulate KC numbers in a manner that would allow us to evidence such threshold. Most available tools totally (or almost) deplete macrophages and would need further tuning to finely modulate KC numbers. For example, using Diphtheria toxin receptor (DTR)-based depletion, such as CLEC4F DTR (Scott et al., 2016a) 100% of KCs are eliminated within 24 hours, precluding any reconstitution from a remaining, actively proliferating pool. In this experimental paradigm, KC depletion induces the production of chemoattractants and leukocyte adhesion molecules (CCL2 secretion and expression of adhesion molecules on LSECs, such as VCAM-1 and SEL-E) leading to monocyte recruitment (Bonnardel et al., 2019). In this context, monocyte-derived KC were proposed to persist for four months and were Ki67+ seven days after recruitment. However, this timepoint is too early to infer on their proliferation capacities, as monocyte-derived KC were not yet fully differentiated (as evidenced by the lack of Tim4 expression). By injecting lower amounts of DT, 80% depletion was achieved using the same CLEC4F-DTR strain (Scott et al., 2016a) In this case, both monocyte recruitment and proliferation of the remaining KCs were identified as contributing to KC replenishment. Monocyte contribution, estimated by donor chimerism in liver-shielded chimeras, was highly variable between animals. Since irradiation is known to trigger systemic inflammation and partial chimeras can be difficult to interpret as they involve subtle variations of already low numbers, it would be most interesting to perform these experiments in mouse strains where monocyte contribution could be evaluated directly, using for example the *CCR2^{CreERT2} Rosa26^{Tomato}* pulse labelling.

To contribute to this debate, I analysed a depletion model based on CSF1R inhibition using PLX, where 15% of KCs survive (85% depletion). In contrast to results from low DT (80% depletion), no monocyte recruitment and differentiation into KC was observed here, using two distinct fate mapping strategies (*CCR2^{CreERT2} Rosa26^{Tomato}* and *Flt3^{Cre} Rosa^{YFP}*).

I privileged PLX inhibition as it has been reported that PLX inhibitors induce macrophage cell death by apoptosis, and is thus presumed to not induce inflammation. While most studies with PLX relies on a long window on inhibition (up to 3 weeks) to reach full depletion, we favoured a short inhibition, to reduce potential alterations in other CSF1R-dependant populations. In the liver, IM numbers did not increase in response to the depletion of KCs and neutrophils did not accumulate either. This confirms that PLX-induced macrophage death provoked little to no inflammation. The apparent transient decrease of circulating monocytes numbers after PLX treatment was not significant. A larger sample will be needed to conclude on the effect of PLX on monocytes. However, normal circulating monocyte numbers in PLX treated animals does not preclude an impairment of their recruitment, their viability in tissues, and their differentiation into macrophages. To fully conclude on the mechanisms of KC recovery after CSF1R inhibition, I will need to further investigate these issues.

Regarding ageing, it could be hypothesized that although monocytes are recruited, the aged liver environment does not support their differentiation into KC. In my analysis of old animals, where only 50% of KCs remain, I observed a gradual increase in infiltrating macrophage numbers (defined as F4/80+ Tim4 neg). This increase did not make up for the loss of phenotypic Tim4+ KC, as the total density of F4-80+ macrophages still decreased with age until 24 months. Moreover, these macrophages did not acquire TIM4, the characteristic KC marker. One reason for that could be their short time of residency in the liver. It has been reported that infiltrating macrophages started expressing CLEC4F between D4-D14 after depletion and TIM4 even later (Scott et al., 2016a). If the recruited macrophages in aged individuals in a context of systemic low-grade inflammation only transit through the liver before being eliminated, they may not stay long enough in the liver to be imprinted with the KC identity. This is further supported by our pulse labelling of adult macrophages in *Csf1r^{MeriCreMer} Rosa26^{Tomato}* animals: while Tomato labelling in KCs remained stable up to 3 months after Tamoxifen injection in old animals, the labelling among TIM4 negative macrophages (IM) dropped from 70% at day 3 after pulse to less than 10% six weeks later (Figure 10). The permanent inflammatory state in the liver, sustained in part by the perpetual death of old KCs may induce a constant recruitment of IMs that are only transiently present in the liver, in a process similar to what is observed in Acetaminophen Induced Liver Injury (AILI) (our results and (Zigmond et al., 2014).

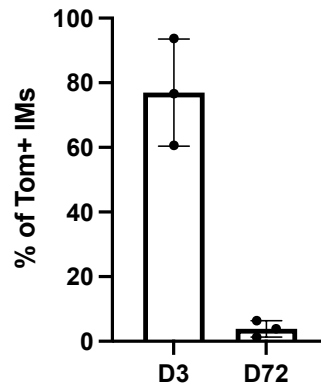


Figure 11 : Tomato labelling of Infiltrating Macrophages (IMs) in 21-24 Mo *Csf1^{MeriCreMer}* *Rosa26^{Tomato}* mice at D3 and D72 after injection of Tamoxifen (n=3 per condition); Mean +/- SD).

2.2. An adequate niche

Competition does not only apply to space, but also to the availability of the factors needed for IM survival and their differentiation into KCs. These factors are part of the KC niche.

The KC niche is composed of all the stromal and parenchymal cells that are in contact with KCs and provide them with trophic factors, notably CSF1, which is one of the key regulators of the survival, proliferation, and differentiation of macrophages.

In inflammatory contexts, when monocytes are recruited in high numbers to the liver parenchyma, there is a competition between these newly arrived cells and the proliferating KCs for the binding of CSF1. In DT-induced KC depletion, monocyte differentiation into KC can be explained by CSF1 availability for their differentiation, survival and potential proliferation. Then, tissue-specific cues from hepatocytes (BMP) and hepatic stellate cells (Vitamin A, CSF1, Notch and BMP) would instruct KC transcriptomic signature and tissue-specialisation (upregulation of *Id3*, *LxRa*, ..) (Bonnardel et al., 2019). This tissue specification would be then further reinforced once they become functional, for example with the expression of the transcription factor *SpiC* after efferocytosis. In aged mice, a deficiency in these factors could explain not only the failure of monocyte derived macrophages to persist in the liver, but also the inability of the ageing liver to sustain its pool of KCs.

In ageing and acute injury, where KCs remain, the competition could be skewed in favour of KCs by a different affinity for CSF1 or an uneven access to it, due to their precise localisation. Kupffer cells are located within the liver sinusoids, with an increased density at the periportal regions of the liver. They extend processes across vessels and into the space of Disse where they interact with endothelial cells, hepatic stellate cells and hepatocytes. These cells are the main purveyors of CSF1 for the maintenance of KCs. A less favourable location of IMs could impact their ability to receive these signals.

2.3. Inhibition of infiltrating macrophage clearance

If IMs are only transiently present in the liver, there could be a well-controlled mechanism for their clearance. In the case of thioglycolate-induced peritonitis, it has been shown that monocyte-derived macrophages can be eliminated by two routes: either by local apoptosis (the major route) or by emigration of these cells to lymph nodes (Gautier et al., 2013).

2.3.1. The role of CSF1/CSF1R

Among the pathways activated by CSF1R signalling, there are several anti-apoptotic genes like *Bcl-xl* and *Bcl2*. Any impairment of this signalling pathway may thus affect the maintenance of infiltrated macrophages. We showed that with age there was an increase in the levels of stellate cell membrane bound CSF1 in mouse liver, and it was reported circulating CSF1 was also elevated in aged individuals (Larsson et al., 2015; Suehiro et al., 1999). This could be a response to the scarcity of KCs. But in 24 month-old mice, when this decrease has been ongoing for a year, the levels are not mitigated by the recruitment of IMs and their consumption of CSF1. This could reflect an impaired ability of recruited monocytes to integrate CSF1 signalling. Analysis of MacBlue (Δ Csf1r^{ECFP}) reporter mice (Ovchinnikov et al., 2008) that lack a conserved distal element from the Csf1r promoter, suggests at least differential *Csf1r* gene regulation between KCs and other liver macrophages. While all liver macrophages express *Csf1r*, only monocytes and a subset of F4/80^{+/low} macrophages are labelled in the MacBlue strain (Sauter et al., 2014). It would thus be important to investigate CSF1-CSF1R signalling pathways in IMs and KCs, not only at the level of *Csf1r* gene regulation but also at the downstream signalling pathways.

2.3.2. The role of CX3CL1/CX3CR1

CX3CR1 (fractalkine receptor) is expressed in several mature macrophage populations and in monocytes, with differential expression between monocyte subsets: Ly-6C^{low} monocytes are GFP^{hi} in CX3CR1^{GFP} reporter mice, while Ly6C^{hi} monocytes express lower levels of GFP (GFP^{int}). Ly6C^{hi} monocytes that infiltrate tissues gradually upregulate this receptor. Many tissue resident macrophages, such as KCs, do not express CX3CR1 in adult tissues (Yona et al., 2013). CX3CL1 (fractalkine) is the only known ligand of this receptor. It is produced in a membrane-bound form, but it can be released through the action of ADAM 10 and ADAM 17 metalloproteinases. CX3CL1 serves as a chemoattractant when in soluble form and promotes adhesion when expressed at the surface of endothelial cells. In the steady state liver, CX3CL1 is only found in the bile duct, but in case of inflammation it is upregulated in liver endothelial cells (Aspinall et al., 2010).

It has been demonstrated that CX3CL1 – CX3CR1 signalling also promotes monocyte and macrophage survival. CX3CR1 signalling has been shown to activate the BCL2 anti-apoptosis pathway (Karlmark et al., 2010). In cancer contexts, CX3CR1 promotes the survival of

angiogenic tumour associated macrophages (Zheng et al., 2013). In humans, the CX3CR1-M280 mutation has been linked to decreased monocyte survival (Collar et al., 2018).

In aged mice, a decrease of *Cx3cr1* gene expression has been observed in cardiac resident macrophages (Pinto et al., 2014). A decrease of CX3CR1 expression in human patrolling monocytes has also been described (Seidler et al., 2010). This decreased expression of CX3CR1 may be linked to a greater susceptibility to apoptosis in recruited macrophages.

3. Age-associated ultrastructural changes in liver

Ageing is accompanied by structural changes in the liver that may affect KC biology.

3.1. LSEC fenestrations

Although macrophage-intrinsic changes described above could underlie the age-related scarcity of KCs in the liver, other factors may come into play. We proposed inflammation as a driver for the loss of KCs observed in ageing, and it is unlikely it would only affect them and/or their monocyte-derived counterparts. Other members of the KC niche may be affected.

LSECs for instance express a broad range of TLR receptors and scavenger receptors (Mannose Receptor, Lyve 1, pro-LDL receptor related protein) and they are efficient phagocytes. They are antigen presenting cells, play a role in the initiation and response to inflammation and regulate immune reactions. As such, they are susceptible to repeated inflammatory cues and share the burden of the scavenging effort. The increase in these solicitations with age could damage them.

Their architecture itself is subject to age-related alterations. I mentioned earlier that capillarisation of the sinusoid endothelium is a hallmark of the aged liver that could impact the diffusion of molecules between blood and the parenchyma. But cells from the parenchyma can also extend microvilli through these fenestrae into the space of Disse to communicate with the sinusoid lumen (Wise, 1970). Conversely, KCs microvilli can be found in LSEC fenestrae, where they enter the space of Disse to mingle with hepatocyte microvilli. KCs can further insert part of their cellular body through rare gaps in the endothelium cell wall (Billiar & Curran, 2017). The loss of fenestrae together with the establishment of a basement membrane could thus form a physical barrier to communication with hepatocytes and stellate cells, and impair the engraftment of newly arrived, monocyte-derived macrophages. Moreover, LSEC fenestrations allow the transit of lipids from blood to be uptaken by hepatocytes. Their closure can lead to lipid accumulation in the sinusoids, increasing the burden for macrophages.

To study the role of pseudocapillarisation in the aged liver phenotype, it would be interesting to chemically reverse the process and analyse its effect on KC density. Although the mechanisms underlying this loss of fenestrae are not completely understood, some molecules

like serotonin have shown potential in reverting this phenotype (Furrer et al., 2011). Other molecules that modulate the regulate actin cytoskeleton and lipid rafts related pathways have also been used to reverse age-related pseudocapillarisation : 7-ketocholesterol, TNF-related-apoptosis-inducing ligand (TRAIL). Nutrient sensing pathways have also been targeted by metformin and nicotinamide. These molecules delay the onset of some ageing related traits and increase fenestrations (Hunt et al., 2019).

Another approach would be to induce pseudocapillarisation in young mice. Several substances and notably hepatotoxins (nicotine, carbon tetrachloride, alcohol) have been shown to decrease the size and/or number of LSEC fenestrae(Braet & Wisse, 2002). But as their effects are pleiotropic, they may have direct effects on KCs, hepatocytes and hepatic stellate cells and thus could be difficult to control for.

3.2. Liver fibrosis

It has been shown that ageing is associated with an increased susceptibility to fibrosis, and the incidence of fibrosis-related diseases is high among the elderly. Unfortunately, it is delicate to quantify and visualise physiological fibrosis in mice, even aged, without having to experimentally exacerbate it through the injection of hepatotoxins such as Carbon Tetrachloride or with a High Fat regimen.

Deposition of extracellular matrix in the liver replaces active cells, alters the liver structure, and perturbs blood flow. By crowding the space of Disse, it may impair the interactions between Kupffer cells and the parenchyma, thus dysregulating the KC niche.

Fibrotic areas themselves represent a specific microenvironment, where different subsets of Scar Associated Macrophages (SAM) have been identified (Beljaars et al., 2014). The cues from the fibrotic area could prevent the switch of IMs towards a KC phenotype.

3.3. Ageing is a dynamic and multi-factorial process

When investigating myeloid cell dynamics with age, we observed a shift between the 21–24-month-old and 29-30-month-old age groups. While we observed a gradual decrease of KC numbers until two years of age, the numbers of KC and overall macrophages had stopped declining in geriatric 30-month-old mice, and there was a burst in CD11b^{high} F4/80⁻/low MHC II⁺ myeloid cell infiltration (see Results, Supplementary figure 2B). This could reflect a switch in inflammageing: either by the sudden increase of systemic inflammation, or the reaching of an inflammation threshold that induces an increased recruitment of monocytes and/or overcomes the resistance of the aged liver to the installation of new myeloid populations. In line with this hypothesis, we observed a limited increase in the number of Tim4^{neg} F4/80⁺ macrophages (Infiltrating Macrophages, IMs) over time, until two years old, and they did not seem to persist in the liver: their numbers remained too low to compensate KC loss, and Csf1rMeriCreMerRosa26^{Tomato} results showed their short lifespan. This increased infiltration is observed in Ticam^{LPS2} mice as well, suggesting that it is independent of the loss of KCs and is

rather a result of the global increase of liver and/or systemic inflammation (Figure 12). In mice older than two years old, IM number did not increase further, KC numbers had stabilised, and the burst in non-macrophage myeloid cells mentioned above was observed. It would be interesting to better characterise what happens in the liver environment from mice older than two years, that allows increased myeloid cell recruitment to the liver and stabilisation of KC loss. I would also need to characterise the expression of other KC specific markers like Clec4F on Tim4neg F4-80+ “infiltrating” macrophages and to analyse their transcriptomic profiles, to understand whether these macrophages, although only transiently present, start a differentiation process towards a KC-like profile.

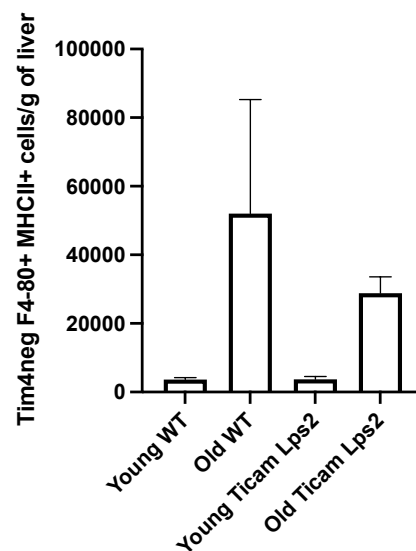


Figure 12 : Number of Tim4neg F4/80+ MHCII+ macrophages in young (2-6 Mo) and old (18-24 Mo) WT and Ticam^{Lps2} mice

Altogether, these data suggest a model where repeated inflammation throughout life leads to gradual KC loss by different cell death mechanisms. In parallel there is an increased trafficking of monocytes and neutrophils in the aged liver. As inflammation remains low grade and the liver architecture undergoes changes that are not favourable to KC maintenance, IM turnover in the liver remains high. But a switch in systemic inflammation, or the reaching of an unsustainably low level of KCs could provide the cues needed to open KC niches to the long-term establishment of monocyte derived IMs in geriatric animals, as KC-like cells or as “inflammatory” macrophages similar to those observed in inflamed livers (Figure 13).

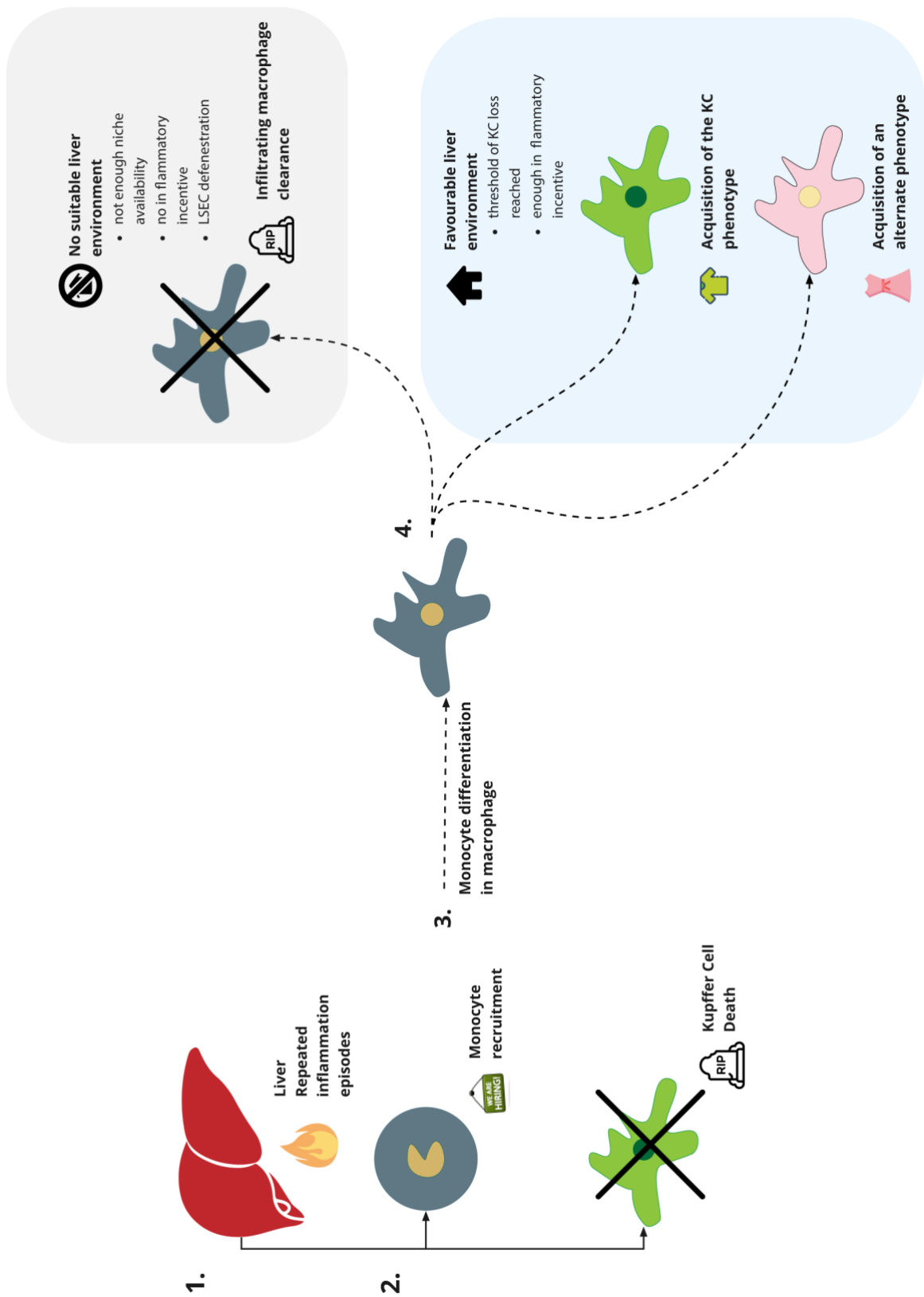


Figure 13 : A proposed model for the age-related alterations of liver macrophages

4. KC loss in ageing: impact in age related diseases

We have observed that the decrease in KC density with age correlated with increased accumulation of senescent cells and lipid droplets in aged livers. Conversely, a “youthful” KC density in old livers correlated with improved liver ageing hallmarks. It is thus possible that KC loss could be involved in the progression of age-related liver diseases.

4.1. Lipid accumulation

A role of KCs in lipid metabolism has been shown in a few recent studies (Bieghs et al., 2012; Blériot et al., 2021; Remmerie et al., 2020), their decline could thus contribute to the lipid accumulation we and others observed in the aged liver (Chung, 2021).

4.2. Senescent cells and fibrosis

We found that the age-related decrease in KC density correlated with an accumulation of senescent cells (defective cells that are in permanent cell cycle arrest). This phenotype was attenuated when KC numbers remained stable in aged *TrifLps2* mutants, where TLR sensing was impaired. As KCs have been suggested to participate to the removal of senescent cells, it is plausible that their gradual decrease would play a role in this accumulation. We also know that aged livers are prone to fibrosis, unfortunately we could not quantify liver fibrosis in histological sections.

A link between senescent cells and fibrosis has been proposed. It has been shown in the lung that the senescence-associated secretory phenotype (SASPs) drove at least in part the onset of fibrosis and that clearance of these cells improved lung function (M. Hashimoto et al., 2016; Schafer et al., 2017). Senolytic drugs and transgenic mice were used to clear senescent cells in this model.

The causal relationship between KCs and senescent cells must be investigated: on one hand, the accumulation of senescent cells with age could induce the activation of KCs and an inflammatory response to recruit immune effectors. Further, hepatic stellate cells are drivers of liver fibrosis, and they could be activated either directly by the pro-inflammatory cytokines contained in the SASPs, or indirectly by the activated KCs. On the other hand, the decrease of liver macrophages with age, could impair senescent cells removal, leading to their increased accumulation.

4.3. Relevance in age related human diseases

In humans, fate mapping and parabiosis experiments can of course not be used to track the contribution of circulating cells to resident macrophage pools over time. Organ transplant can be used as a proxy, as mismatched HLA or sex chromosomes allow the distinction between macrophages from donor and host origins. Evidence has been conflicting so

far(Patel et al., 2021). Notably, some skin graft studies have shown potential for maintenance of Langerhans cells from host origin(Kanitakis et al., 2011). Other studies looking at Langerhans cells origin after bone marrow transplants have shown the replacement of donor LCs by host derived cells over time (Collin et al., 2006; Mielcarek et al., 2014). In the liver, macrophages from donor origin were observed up to 11 years after transplant (Pallett et al., 2020). However, they constituted only a small fraction of the liver macrophages. Importantly, host and donor macrophages showed phenotypic differences in their surface expression of CCR2, CX3CR1 and CD163 notably. But these analyses were performed on a very limited cohort, and individual histories may have influenced the result.

It has been shown that in humans like in mice, macrophages play a role in the onset of various liver diseases and that they may represent promising targets in the treatment of NASH, cirrhosis or infection (reviewed by (Tacke, 2017)). It would thus be relevant to understand how changes in liver macrophage ontogeny after transplants affects liver function. Comparisons of transplant recipients of different ages would also shed a light on their susceptibility to infiltrating macrophage engraftment. However, for functional studies in human, the skin might provide a more amenable model for now as it is more accessible for biopsies and can be cultured ex vivo (Jenner et al., 2014).

5. Challenges in studying KC specific functions in injury

5.1. Inflammation and depletion

Depletion is the ideal way to study the function of a specific cell population. But it is important to ensure that the extensive cell death created does not in itself create an inflammatory response that could be a confounding effect when interpreting results.

In DTR-based models for example, it has been reported that depletion occurs at least in part through necrosis (measured with propidium iodide incorporation in a iDTR;Gfi1-Cre model (Hu et al., 2016) which is a known trigger for inflammation (Sims et al., 2010), especially if the targeted cell population is abundant. Others have contended, using CD11b-DTR lines, that inflammation was induced in a tissue dependent manner: inflammatory gene expression was elevated in the brain and kidney after depletion but not in the liver (Frieler et al., 2015). In CD64-DTR (Baranska et al., 2018) or CLEC4F-DTR (Scott et al., 2016b) strains, no significant inflammation has been reported, based on neutrophil infiltration. However, to assess this infiltration, neutrophil numbers should be provided, as the evaluation of cell abundance based on frequency of CD45+ or live cells may be misleading, as discussed with our ALLI results. All in all, the effects of DT-based depletion may be dependent on the line used and the tissue targeted, notably in relation to the abundance of the targeted cell population, and it is important to systematically investigate it. And to strengthen the conclusions drawn, it may be beneficial to compare different depletion methods.

During my thesis I evaluated PLX5622-induced depletion of Kupffer cells. Although it allowed the depletion of more than 80% of KCs without overt signs of inflammation, it may have affected circulating monocyte populations, which would be a confounding effect. This will be further assessed with a larger mouse cohort. A further analysis of the effects of PLX treatment on the other cell types that constitute the KC niche is also needed. Moreover, although a direct measure of monocyte infiltration may be biased by the direct effect PLX has on these cells, a measure of the mechanisms triggered to attract them could provide a useful insight: the elevated production of CCL2 and adhesion molecules by the endothelium for instance would indicate that KC loss has indeed triggered replacement mechanisms.

Finally, the definition of inflammation is broad, as it involves leukocyte migration and recruitment but also production of inflammatory mediators and activation of adaptive immune cells. We chose neutrophils as a readout for macrophage induced inflammation, but this may mask other mechanisms at play.

It would thus be far more interesting to target specific functions of KCs without depleting them. We could look at phagocytosis or specific cytokine production for instance. Given the vast diversity and redundancy of KC receptors and pathways involved in these processes,

genetic targeting might prove challenging in mice. Other model organisms like the fly may be more amenable in a first approach.

5.2. Heterogeneity beyond ontogeny

There is mounting evidence that there are not just two types of liver macrophages: infiltrating and resident, with two ontogenies, that specifically delineate two sets of functions. Liver macrophages instead present heterogeneity in their location (along the liver zonation axis, along biliary ducts, and capsular macrophages), in different pathological contexts, and plasticity over the course of injury (Ritz et al., 2018; Zwicker et al., 2021).

In injury and disease contexts, IMs represent the most abundant macrophage population, and they have shown great plasticity, adopting a range of phenotypes and transcriptomes adapted to the context (Krenkel et al., 2020; Remmerie et al., 2020). The extent of KC plasticity remains under question, as most bulk and single cell RNA sequencing approaches have not yet revealed significant transcriptomic changes in KCs over time and in pathological settings. Although they can indeed respond to a variety of stimuli to orchestrate the immune reaction, this may represent a transient activation rather than a long-term plasticity leading to transcriptionally different KC subsets. If KC plasticity appears limited, the mechanisms of their phenotypic “rigidity” should be explored, at all levels (metabolic, proteome, transcriptome and epigenetic). It may reflect their long time of residency in the liver as it allows a strong imprinting from the microenvironment.

The heterogeneity of KCs in steady state has also been difficult to assess, at least transcriptionally; besides the recent characterization of two subsets of KC characterised by their expression of CD36 and CD206 (Blériot et al., 2021). It may be useful to look at other levels of regulation like the epigenome or metabolome.

6. Perspectives

Having characterised macrophage maintenance in ageing and injury, I would like to further study i) the mechanisms that determine the age-related loss of KCs, ii) the process that underlies the inability of IMs to persist in aged livers and iii) the extent of KC plasticity over time and in injury.

6.1. In situ observation of KCs

I have hypothesized that age-related morphological changes in LSEC could impact the ability of macrophages to persist in the liver. Using electron microscopy, I would like to visualise if and how ageing alters the interactions between KCs and LSECs. I will also seek to better assess the localisation of KCs and IMs in ageing.

6.2. Macrophage transplant

These observations could be accompanied by transplantation of sorted “young” KCs into old individuals, to directly evaluate the ability of the liver to sustain their engraftment. If engraftment is possible, it would allow us to compare different macrophage sources (monocytes vs IM vs KC) but also to investigate whether KC transplantation can alleviate hallmarks of aged liver (senescent cell and lipid accumulation).

6.3. Phagocytosis and cellular longevity

I am currently breeding and characterising a line of Albumin-Cre x Rosa^{mTmG} mice to study the fate of Kupffer cells after phagocytosis of hepatocytes, in injury contexts. As shown in the trachea, horizontal transfer of the Cre protein between a cargo cell and a phagocytic macrophage can lead to Cre-recombination in macrophages (Lim et al., 2020) which would permanently label them with GFP expression. Loss of Tomato expression associated to GFP expression will allow me to differentiate between transient transfer of the GFP protein and genetic recombination. This would allow me to characterise the contribution of IM and KC to the clearance of apoptotic hepatocytes after injury and to monitor the longevity of Tomato+ phagocytic macrophages. This would create an experimental paradigm where I could follow the fate of phagocytic macrophages (apoptosis, proliferation, life span) but also to investigate the transcriptomic and epigenetic changes induced by phagocytosis over time (are they transient?). This approach would thus help us understand how intense phagocytosis can influence macrophage fate. It may also provide insight into the respective functions of KCs and IMs.

6.4. Repeated Injury

I have started establishing a protocol for Acetaminophen Induced Injury. After refining, it will provide a framework to better understand if and how repeated “sterile” inflammation could lead to the loss of KCs observed in aged mice.

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RESUME DÉTAILLÉ EN FRANCAIS

Les macrophages, phagocytes professionnels, sont au centre du système immunitaire inné. Ils participent à la première ligne de défense contre les infections et les lésions des tissus. Ils assurent la surveillance permanente de l'homéostasie tissulaire, nettoient les débris, éliminent des agents pathogènes et orchestrent la réponse immunitaire en produisant des cytokines inflammatoires.

Selon le modèle du système phagocytaire mononucléé établi par Cohn et van Furth¹ dans les années 60, les macrophages sont issus de la différenciation de cellules souches hématopoïétiques (CSH). Ceux-ci par l'intermédiaire d'une cascade de précurseurs, donnent naissance à des monocytes circulants qui peuvent être recrutés dans les tissus en cas d'inflammation. Là, les monocytes peuvent se différencier en macrophages. Cependant, de travaux récents ont mis en évidence une deuxième voie de production des macrophages, indépendante des CSH. En effet, la plupart des macrophages qui résident de façon permanente dans les tissus, sont produits dès la vie embryonnaire à partir de progéniteurs erythro-myéloïdes (EMPs en anglais)^{2,3} (Ces macrophages indépendants des CSH s'établissent dans leur tissu de résidence durant le développement, et y persistent à l'âge adulte. En cas d'inflammation, deux lignées de macrophages coexistent donc dans les tissus. Il nous faut maintenant réexaminer la dynamique des macrophages et leur rôle à la lumière de cette diversité d'ontogénie.

Durant ma thèse, je me suis particulièrement intéressée aux macrophages résidents du foie, les cellules de Kupffer (KCs). Macrophages résidents les plus abondants du corps, ils résident dans les sinusoides, les capillaires fenêtrés spécifiques du foie. Ils ont été étudiés dans de nombreux types de lésion : infection⁴ surdose de paracétamol^{5,6} (et inflammations chroniques du foie⁷ Mais les rôles qui leur ont été attribués sont parfois contradictoires.

Par ailleurs, il est établi que le vieillissement s'accompagne de changements importants dans le système immunitaire, ces changements sont connus sous le nom d'immunosénescence, et l'une de leurs caractéristiques principales est l'« inflammaging » : une inflammation systémique de faible intensité qui s'accompagne d'une moins bonne résistance aux infections, et une réponse diminuée aux vaccins. L'« inflammaging » est à replacer dans le contexte des maladies chroniques liées à l'âge, qui sont elles aussi attribuées à une dérégulation de la réponse inflammatoire⁸. Dans le foie, le vieillissement se traduit par une perturbation du métabolisme lipidique, et d'une plus grande susceptibilité à la fibrose^{9,10}.

Afin de comprendre le devenir des KCs dans différents contextes inflammatoires, j'ai utilisé une combinaison de cytométrie en flux, modèles génétiques de traçage de lignées (fate mapping), séquençage d'ARN et histologie pour i) caractériser les mécanismes de maintien des KCs dans un modèle de lésion aiguë induite par une overdose de paracétamol ainsi que dans le cadre du vieillissement et ii) développer des outils pour élucider les fonctions des différentes lignées de macrophages dans ces contextes.

Persistence des KCs dans le vieillissement

J'ai dans un premier temps cherché à déterminer si les KCs, dont on sait qu'ils se maintiennent principalement par prolifération à partir du pool établi à la naissance, gardent leur potentiel de persistance au cours de la vie, jusqu'à chez des souris âgées de plus de deux ans. Avec mes collègues, nous avons établi que les KCs, comme d'autres macrophages tissulaires, diminuaient avec l'âge en densité (nombre de cellules par g de tissu) et cette perte n'était pas compensée par le recrutement et la différenciation de monocytes circulants. En effet, même si la densité de macrophages infiltrants (MI) augmentait dans le foie avec l'âge, ceux-ci n'acquerraient pas le phénotype des KCs, défini notamment par l'expression du marqueur TIM4. De plus, malgré cela, la densité totale de macrophages (définis par le marqueur F4-80 chez la souris) diminuait avec l'âge. Cette perte de macrophages s'accompagnait d'une accumulation de neutrophiles dans les tissus, ce qui est un marqueur d'inflammation.

Les KCs de souris âgées présentaient aussi des changements morphologiques : une augmentation de la taille et de la granularité. Cependant, un séquençage ARN de KCs issus de souris jeunes et vieilles n'a mis en évidence que peu de changements transcriptomiques avec l'âge. Seulement une centaine de gènes étaient différentiellement exprimés. Une analyse des voies de signalisation régulées par ces gènes suggère une baisse de métabolisme et une augmentation de l'inflammation dans les KCs de souris âgées. Ce sont des altérations qu'on peut observer dans de nombreux types cellulaires avec l'âge, ce séquençage n'a donc pas mis en évidence des altérations fonctionnelles spécifiques des KCS liées à l'âge. Cependant l'une des voies métaboliques les plus affectées semblait être celle des lipides, et il a été rapporté que les c KCs jouaient un rôle dans l'homéostasie lipidique du foie. Il serait donc intéressant de regarder de plus près les voies de signalisation impactées. Un test de phagocytose de billes fluorescentes a n'a pas mis en évidence une altération de cette fonction dans les KCs de souris âgées. Cependant ces fonctions doivent être examinées en contexte pathologique. Enfin, l'hypothèse la plus simple pour expliquer cette perte de KCs chez les souris âgées était un défaut de prolifération, soit de leur capacité basale, soit en réponse à un stimulus.

Cependant, en utilisant des marquages EdU en cytométrie de flux et Ki67 sur lames, je n'ai pu mettre en évidence aucun défaut de prolifération chez les KCs âgées (21-24 mois), à l'homéostasie et en réponse à une levée d'inhibition de CSF1R. En revanche, la mortalité des macrophages (mesurée par marquage Tunel sur lames) était multipliée par 5 chez les souris âgées. Collectivement, mes données montrent que la perte de KCs avec l'âge n'est pas la conséquence d'un défaut de prolifération mais plutôt due à une augmentation de leur mortalité. De plus, les données transcriptomiques et la littérature nous suggèrent un potentiel rôle de l'inflammation dans ce processus.

Je me suis donc ensuite intéressée au rôle de l'inflammation et nous avons montré que des épisodes d'inflammation répétés pourraient expliquer cette perte de KCs avec l'âge. Nous avons utilisé du poly(I :C), un analogue de l'ARN double brin viral. Il constitue un signal pour les récepteurs de type TLR-3 des macrophages et induit une réponse inflammatoire¹¹. Des injections répétées de poly(I :C) à des souris jeunes induisaient un phénotype similaire à celui observé dans le vieillissement : une perte de KCs et une augmentation de l'infiltration des neutrophiles. Inversement, les souris *Trif*^{Lps2}, chez lesquelles la transmission du signal inflammatoire via TLR était inhibée, ne présentaient pas de baisse significative du nombre de leurs KCs avec l'âge. La mortalité des KCs dans ce fond génétique augmentait peu avec l'âge.

La survie et le maintien de l'identité des KCs dépend de facteurs de leur microenvironnement qui définissent la niche. Les hépatocytes, les cellules stellaires et les cellules endothéliales des sinusoides hépatiques (CESH) sont les principaux acteurs cellulaires de cette niche. Les cellules stellaires en particulier produisent le CSF1 (Colony Stimulating Factor 1 aussi connu sous le nom de MCSF ; Macrophage Colony Stimulating Factor), cytokine clé pour le maintien des KCs¹². Une analyse de coupes de foies jeunes et vieux par immunofluorescence n'a pas mis en évidence d'altération du nombre de ces cellules qui pourraient expliquer la perte de KCs. En revanche l'augmentation de la proportion de ces cellules qui présentaient du CSF1 à leur surface pourrait indiquer soit une intensification de leur production pour pallier la perte de KCs, soit une baisse de consommation de ces cytokines du fait d'un pool de macrophages diminué.

Pour mieux appréhender l'état de la niche des KCs dans un foie vieillissant, il faudra regarder de plus près l'ultrastructure du foie, notamment les CESH. En effet, ces cellules sont impliquées dans la communication de la circulation hépatique avec les cellules du parenchyme¹³. Il a été montré qu'avec l'âge, elles perdent une partie de leur fenestrations, diminuant ainsi la perméabilité de cette interface¹⁴. Cela pourrait avoir des répercussions sur la capacité des KCs à recevoir les facteurs trophiques et sur le recrutement et la différenciation des monocytes circulants.

Enfin, nous avons cherché à déterminer si la perte de KCs avec l'âge était corrélée à des altérations fonctionnelles du foie mesurables. Il a été montré que les KCs pouvaient intervenir dans le métabolisme des lipides¹⁵ et le nettoyage des cellules sénescentes. Nous avons réalisé des marquages au Oil-Red-O et senescent-associated β Gal (SA- β Gal) pour quantifier leur accumulation. Nous avons ainsi mis en évidence leur augmentation chez des souris âgées « wild type ». Cette augmentation était moindre chez les souris Trif^{Lps2}. Ces données suggèrent que la perte de KCs avec l'âge pourrait jouer un rôle dans les dysfonctionnements de la fonction hépatique avec l'âge.

Outils pour une étude fonctionnelle des KCs

Pour étudier plus en avant les fonctions des macrophages, l'approche classique vise à les éliminer et identifier les conséquences de leur absence. Les KCs peuvent être éliminées par injection de chlorure Gadolinium ou des liposomes de clodronate. Il existe aussi des approches génétiques basées sur l'expression du récepteur à la toxine diphtérique (DTR) par ces cellules et l'injection de la toxine pour les éliminer spécifiquement. La lignée CLEC4F-DTR a montré son efficacité et sa spécificité¹⁶. Cependant ces différentes approches présentent certaines limites : i) en causant la mort des cellules par des mécanismes inflammatoires comme la nécroptose, elles peuvent créer une inflammation dont les effets seront difficiles à distinguer de ceux liés à l'absence de KCs. ii) les KCs sont des cellules abondantes dont la mort rapide et massive peut avoir des conséquences sur le tissu qui ne sont pas directement liées à leurs fonctions spécifiques.

Pour répondre au premier point, nous avons testé une autre approche pour la déplétion des macrophages. Le Plexikon (PLX) est un antagoniste des récepteurs CSF1R. Développé initialement pour cibler la microglie (macrophages résidents du cerveau)¹⁷, ils ont montré leur efficacité dans d'autres organes. J'ai dans un premier temps évalué l'effet d'un traitement court au PLX (fourni dans l'alimentation) sur la persistance des cellules de Kupffer. Un traitement de 3 jours induisait la perte de 80% des cellules de Kupffer, sans recrutement

significatif de macrophages dérivés des monocytes circulants. Deux semaines après la fin du traitement, les KCs avaient retrouvé 50 % de leur nombre initial. Surtout, la mort des KCs dans le traitement Plexikon semble peu ou pas inflammatoire : nous n'avons pas observé d'augmentation significative du nombre de neutrophiles recruté dans le foie après traitement.

Les récepteurs CSF1R sont aussi exprimés sur les monocytes (et certains de leurs progéniteurs dans la moelle osseuse), mais la baisse de leur nombre observée dans la circulation sanguine après trois jours de traitement avec l'inhibiteur PLX n'était pas significative. Au vu de la grande variabilité du nombre de ces cellules en circulation, il me faudra augmenter la taille de mon échantillon pour conclure. Cependant, deux semaines après la fin de l'inhibition, leur nombre était revenu à la normale. C'est à ce moment que j'ai mesuré leur contribution au pool de KCs. Pour cela, j'ai utilisé la lignée de souris inductibles *CCR2^{CreERT2} Rosa26^{Tomato}*. Chez ces souris, l'injection d'hydroxytamoxifen induit l'expression de Tomato dans les monocytes (80% marqués 24 heures après injection de 75 µg/g). Ce marquage est transitoire, puisqu'à 5 jours post-induction, seuls 10 % des monocytes sont encore marqués. Comme attendu, les KCs ne sont presque pas marqués en homéostasie (1% de KCs sont positives). Cette proportion n'augmente pas deux semaines après le traitement PLX. Ceci suggère que les monocytes de la circulation ne participent pas à la reconstitution du stock de KCs après déplétion. En revanche, des analyses de marquage EdU en cytométrie de flux ont montré que les KCs proliféraient localement deux semaines après la fin du traitement. Le traitement PLX chez des souris âgées produisait des effets similaires, confirmant un maintien de leur potentiel prolifératif et leur réponse aux variations de stimulation au CSF1.

Étudier les fonctions des KCs sans induire leur déplétion (ii) requerrait l'inhibition sélective de voies fonctionnelles : phagocytose, réponse inflammatoire, etc. La grande diversité de récepteurs et voies associées, et leurs fonctions parfois redondantes rend cette approche délicate à mettre en place et interpréter chez les souris. Un autre organisme modèle comme la mouche, où le ciblage génétique est plus aisé, pourrait être utilisé dans un premier temps.

Modèle de lésions aiguës du foie

La surdose d'acétaminophène (paracétamol) est la première cause d'insuffisance hépatique non virale dans les pays développés ¹⁸. Elle se caractérise par une accumulation d'intermédiaires de dégradation toxiques qui conduisent à la mort massive des hépatocytes. Cette mort cause l'activation des cellules alentour, dont les KCs. Ces cellules activées vont produire des cytokines pour recruter plus de cellules immunitaires et préparer la réparation du tissu : c'est l'inflammation. Des rôles parfois contradictoires ont été attribués aux macrophages hépatiques dans ce contexte ^{19,20}. Afin de comprendre plus en détail l'hétérogénéité de ces fonctions j'ai dans un premier temps caractérisé ce modèle de lésion. Chez la souris l'injection de 300 mg/kg d'acétaminophène induit des lésions transitoires, qu'on peut estimer dans les premières 24 heures par la mesure sérique des enzymes hépatiques. Trois jours après l'injection, le foie a retrouvé son intégrité mais la réparation tissulaire se poursuit pendant deux semaines. J'ai étudié la dynamique de recrutement et d'expansion du compartiment des cellules myéloïdes dans les deux semaines suivant l'injection de doses toxiques mais non létales d'acétaminophène. J'ai mis en évidence 3 phases :

- Jour 1-Jour 2 : recrutement de neutrophiles et monocytes
- Jour 2-Jour 4 : expansion du pool de macrophages par augmentation de la densité de KCs et recrutement de macrophages issus de la circulation

- A partir de jour 4 : retour à l'homéostasie (disparition des population myeloides recrutées).

J'ai montré que durant ce processus, les KCs persistaient et même augmentaient leur nombre par prolifération locale. Les macrophages recrutés n'étaient présents que de manière transitoire. Il reste à évaluer si cette capacité de persistance est préservée après des lésions répétées. Enfin, il sera intéressant d'étudier la réparation du foie chez des souris âgées. En effet, le vieillissement a été associé à une réparation moins efficace des tissus ²¹. Nous avons établi que les KCs de souris âgées pouvaient proliférer aussi bien que les KCs dans les souris jeunes, à l'état basal. Mais leur nombre plus faible pourrait impacter leur capacité de réponse et modifier la dynamique de recrutement des cellules immunitaires circulantes. Par ailleurs, le fait que nous n'ayons pas mis en évidence beaucoup de différences transcriptomiques à l'état basal, entre les souris jeunes et âgées, n'exclut pas une plasticité amoindrie avec l'âge.

Conclusions et perspectives

J'ai étudié le maintien des macrophages résidents du foie dans deux contextes inflammatoires : le vieillissement et un modèle de lésion aiguë.

Dans le vieillissement, j'ai observé une baisse de leur nombre, non compensée par le recrutement de cellules issues de la circulation. Il faut maintenant établir les mécanismes de cette perte. Nous avons identifié l'inflammation comme déclencheur de cette baisse, mais les processus mis en jeu restent à définir. On sait notamment que le vieillissement s'accompagne de modifications structurelles des cellules endothéliales du sinusoiide hépatique (CESH), elles perdent notamment de leur perméabilité. Au vu des interactions intimes entre les KCs et ces cellules, et du rôle important de la perméabilité des CESH dans la communication entre le parenchyme et la circulation, il paraît plausible que la biologie des KCs soit impactée.

Il serait également intéressant de se pencher sur les voies de mort cellulaire des macrophages pour comprendre lesquelles sont impliquées dans leur perte.

Dans mon modèle de lésion aiguë, les KCs se maintenaient également sans contribution de la circulation. Cependant les MI recrutés transitoirement dans le foie participent à la réponse à la lésion. Il reste encore à élucider l'hétérogénéité des fonctions des macrophages dans ce contexte et à évaluer les mécanismes de réparation chez des souris âgées dont le pool de KCs est diminué. Par ailleurs, la capacité de prolifération locale des KCs pourrait être progressivement érodée après plusieurs épisodes de lésion aiguë.

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ABSTRACT

Kupffer cells (KCs) are resident macrophages of the liver. Professional phagocytes of the innate immune system, they take part in the first line of defence against infections and injury. They also actively regulate liver homeostasis. Recent works have elucidated their origin. We now know that, like most other tissue resident macrophages, KCs develop during embryonic life from Erythro-Myeloid Progenitors (EMPs), seed the liver during development and persist there in adulthood. During inflammation, however, they can be joined by recently differentiated macrophages that arise from circulating monocytes belonging to the Haematopoietic Stem Cell (HSC) descendance. Here I studied the ability of mouse KCs to maintain themselves throughout life, and into old age. Using flow cytometry and fate mapping strategies, we showed that KC density decreased over time and was not compensated by recruitment of circulating cells. RNA sequencing, analysis of *Ticam*^{LPS2} mutants and Poly (I:C)-induced repeated inflammation experiments highlighted the contribution of inflammation to the ageing phenotype. This phenotype correlated with lipid and senescent cell accumulation. We further studied KC maintenance in acetaminophen induced liver injury and after depletion induced by a CSF1R antagonist. In both contexts, KCs were able to maintain themselves through local proliferation without significant input from circulating cells. These experiments will provide a framework for the better characterisation of KC functions in injury and disease.

RESUME

Les cellules de Kupffer (KCs) sont les macrophages résidents du foie. Phagocytes professionnels, ils sont au cœur du système immunitaire inné, première ligne de défense de l'organisme contre les agressions extérieures. Ils participent également au maintien de l'homéostasie tissulaire. Des travaux récents ont permis de mieux comprendre leur origine. On sait maintenant que, à l'instar de la majorité des macrophages résidant dans les autres tissus, les KCs émergent durant la vie embryonnaire à partir des précurseurs érythro-myeloïdes (EMP), colonisent le foie durant le développement et y persistent à l'âge adulte. Cependant, en cas d'inflammation, ils sont rejoints par des macrophages nouvellement différenciés à partir des monocytes circulants descendants des cellules souches hématopoïétiques. Durant ma thèse, j'ai étudié la capacité des KCs à persister tout au long de la vie, y compris chez des souris gériatriques. En utilisant la cytométrie en flux et des techniques de traçage de lignées, j'ai montré que la densité des KCs diminuait avec l'âge sans être compensées par les monocytes circulants. Un séquençage ARN, l'analyse de mutants *Ticam*^{LPS2} et des expériences d'inflammation répétées induites par un analogue viral ont mis en lumière le possible rôle de l'inflammation dans ce phénotype. Ce dernier corrélait avec des altérations fonctionnelles liées à l'âge. J'ai ensuite étudié la persistance des KCs dans un modèle de lésion aiguë du foie induite par le paracétamol et après déplétion des KCs par un antagoniste du récepteur CSF1R. Dans ces deux contextes, les KCs étaient capables de persister ou se reconstituer sans contribution de la circulation. Ces expériences établissent un cadre et des outils pour une meilleure caractérisation des fonctions des KCs en contexte pathologique.