Gilded scars: decoding how metabolism and cancer cell-intrinsic features shape immunity in hepatocellular carcinoma

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Chapter 6

Discussion

Although significant progress has elucidated a myriad of mechanisms underlying liver cancer initiation and progression—facilitating the development of tumour microenvironment (TME)-targeting therapies that have notably extended patient survival—most individuals with advanced hepatocellular carcinoma (HCC) continue to face a poor prognosis due to the absence of universally effective treatments[1]. Nonetheless, enhancing anti-tumour immunity through immune reprogramming remains a highly promising—albeit more complex—therapeutic avenue. Fine-tuning the pro-tumorigenic and anti-tumorigenic roles of immune cells in the context of heterogeneous genetic mutations, metabolic dysfunction, and immune microenvironment dysregulation is particularly challenging, yet critical to developing effective personalized therapies.

In **Chapter 2**, we gathered evidence suggesting that the immune system plays a pivotal role in driving carcinogenesis in patients with metabolic disorders, highlighting a critical node as these cases become increasingly prevalent in an industrialized and aging population. In **Chapter 3**, we provide a comprehensive overview of technical procedures designed to maximize the use of ex vivo material from murine liver cancer models, thereby facilitating follow-up studies on HCC research. In **Chapter 4**, we demonstrate that implementing myeloid-centred treatments in a personalized manner holds promise for HCC, considering how the heterogeneous HCC genetic makeup remodels its surrounding microenvironment to foster tumour progression. **Chapter 5** provides further evidence of the efficacy of combinatory approaches aimed at rewiring the myeloid compartment to ultimately unleash an anti-tumour response, in this case in a genetically-defined model of MASH-HCC.

In this final section, the main conclusions and outstanding questions arising from this work are discussed, focusing on key aspects of hepatocarcinogenesis from the perspectives of cancer-intrinsic and immune cell-specific mechanisms driving disease progression. First, I will examine how cancer cells manipulate their own phenotype and the surrounding immune-metabolic landscape to their advantage. Next, I will explore the dynamics, influence, and therapeutic potential of targeting macrophages in liver cancer and then provide a perspective on lymphocytes in HCC. Finally, I evaluate how cancer metabolism can be leveraged as a therapeutic target. Collectively, these topics aim to highlight potential personalized approaches to advance treatment in this field.

Degree of oncogene activation dictates cancer phenotype

It is well established that cancer cells require the accumulation of mutations to sustain uncontrolled growth and the development of full-blown malignancy. These mutations drive changes in downstream signalling pathways, ultimately promoting cancer cell survival, proliferation, and dissemination. However, how cancer cell phenotype, metabolic alterations, and the impact on neighbouring and systemic cells are shaped by these mutations remains to be fully determined. To complicate matters further, distinct mutations can promote varying degrees of oncogenic pathway activation, which may result in different consequences for tumour fitness and phenotype. Exactly how this plays out remains the focus of ongoing research.

Chapters 4 and **5** shed light on how the gradient of mitogen-activated protein kinase (MAPK) activation—sustained by distinct oncogenic forms of *Nras*—can lead to vastly distinct cancers, both in cancer-intrinsic and immune-associated features, including their metabolic profile. Namely, *Nras*^{G12D}-induced MAPK hyperactivation led to the development of more aggressive, fibrotic, myeloid-enriched tumours, whereas *Nras*^{G12V}-driven tumours showed lower MAPK activation, were more indolent, lipid-rich, and lymphoid-abundant, resembling metabolic dysfunction-associated steatohepatitis (MASH)-

HCC. These findings suggested that the degree of oncogenic activation directly influences cancer cell viability and fitness. Corroborating this observation, overt MAPK activity can trigger anti-tumorigenic mechanisms, such as senescence, rendering cancer cells less fit to overgrow and eliciting immune clearance. If established, high-RAS liver tumours are aggressive with short latency and an undifferentiated phenotype, whereas mild RAS activation licenses immune escape and overgrowth of well-differentiated tumours[2], overall mirroring our findings with *Nras*^{G12D} and *Nras*^{G12V}-induced HCC.

The accumulation of additional genetic mutations may favour tumour escape, even in a scenario of overactive oncogenic pathways, as has likely been observed in the *Nras*^{G12D}-induced HCC model described in **Chapter 4**. On the other hand, it may well be that an intermediate activation of oncogenic pathways can also modulate to which degree ancillary signalling pathways are engaged, ultimately dictating cancer cell phenotype. For instance, despite the introduction of *Pten* invalidation in *Nras*^{G12V}-induced tumours—which has been shown to induce lipid accumulation in hepatocytes[3]—no evidence of phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathway activations, well-known to be upregulated upon MAPK enforcement, were seen in *Nras*^{G12V}/*Pten*^{KO}-induced tumours. These findings suggest that low levels of MAPK activity may elicit phosphorylated extracellular signal-regulated kinases (p-ERK) signalling without branching to neighbouring pathways. This showcases how elusive the regulators of lipid accumulation and consequent carcinogenesis in *Nras*^{G12V}/*Pten*^{KO}-induced tumours are, where the latter seemingly depends on the establishment of chronic inflammation and possibly associated metabolic catastrophe.

Inhibition of oncogene overactivation and downstream signalling has been a crucial pillar in cancer treatment. For instance, RAS proteins have long been considered "undruggable". However, recent advances have demonstrated that targeting these proteins is indeed feasible. Initial breakthroughs involved the development of KRAS^{G12C}-specific inhibitors, which promote regression of *KRAS*^{G12C}-mutated tumours and unleashes immune-mediated anti-tumour response[4]. Subsequent progress led to the creation of pan-RAS inhibitors capable of effectively inhibiting all RAS isoforms[5]. This is exemplified by the significant survival extension observed in *Kras*$^{LSL.G12D/+}$; *Trp53*$^{R172H/+}$; *Pdx*-1-Cre (KPC), pancreatic adenocarcinoma (PDAC)-bearing mice, a model known for its resistance to virtually all cancer-targeting therapies[6]. Interestingly, however, hyperactivation of several oncogenic pathways in colon cancer cells has recently been shown to suppress cancer fitness and induce cell death, in line with the concept of tumour-suppressing effects of oncogene overactivation, highlighting a novel strategy to be exploited in other cancer types[7]. The consequences of such actions in terms of immunomodulation must also be considered, given the extent by which cancer cells can modulate their surrounding environment, depending on its genetic makeup.

Cancer cells co-op tissue regeneration to fuel tumorigenesis

Cancer has long been touted as a "wound that does not heal". Indeed, the wound-healing process exhibits a plethora of parallels with oncogenesis[8], albeit cancers seemingly "halt" in the resolution phase, favouring their outgrowth. This can encompass an ongoing wound-resolving program encompassing the presence of immune-tolerant, extracellular matrix (ECM)-remodelling cells that persist—thus permitting tumour escape and providing the adequate niche to their growth—and the engagement of proto-oncogenic, proliferative pathways[8]—particularly Myc[9]. Importantly, cancer cells can co-op tissue regeneration and embryonic—or oncofetal[10]—pathways to foster their

plasticity[11], and these can be used to escape and reinitiate their malignant program once critical nodes, like RAS, are inhibited[6].

This concept is especially noteworthy for the liver. Indeed, the homeostatic liver is immune tolerant, a necessity owing to the constant, unrelenting bathing of gut-derived, pathogen-associated molecules from the hepatic portal vein[12]. Furthermore, the liver regenerative capacity is remarkable[13], as portrayed by the rapid regrowth upon liver transplant from healthy individuals. It may well be that the liver is naturally programmed to be tuned to this "injury-resolution" cycle. Unsurprisingly, liver insults can lead to a feedback loop of hepatocyte death and regenerative bursts, which may increase the risk of accumulating mutations and expansion of premalignant hepatocytes, serving as a source for HCC outgrowth[14-17]. Cancers in other organs hijack similar regenerative programs; one notable example being the colon, whose constant replenishment by Wnt-dependent leucine-rich repeat-containing G-protein coupled receptor 5 $(LGR5)^{+}$ stem cells support homeostasis, yet can also be co-opted by cancer cells for malignant outgrowth through corruption of the Wnt signalling[10]. The exploitation of regenerative processes by cancer cells can explain how they evolve from preneoplastic lesions. It can also explain how cancer cells escape overactive oncogenic signalling pressure and therapeutic strategies to block these pathways. *Myc* signature has been shown to allow the escape of RAS-overexpressing cells in HCC[2], thereby bypassing cancer-limiting mechanisms, such as senescence. Similarly, resistance in pan-RAS-treated PDAC cells arise from a Hippo-Yes-associated protein (YAP)-mediated Myc upregulation[6], pathways collectively well-known to dictate organ development and regeneration processes.

As described in **Chapter 4**, $Nras^{G12D}/Pten^{KO}$-driven HCC exhibits overactive MAPK/ERK signalling, suggesting that escape mechanisms are needed for cancer outgrowth. In fact, *Myc*/MYC is increased in $Nras^{G12D}/Pten^{KO}$ cancer cells, as observed in immunohistochemistry and bulk RNAseq analyses (unpublished). I hypothesize that the relative low penetrance of these tumours following hydrodynamic tail vein injection (HDTVi, about 30% penetrance) is partially explained by the possible requirement to acquire additional somatic mutations that allow *Myc* overexpression. One such candidate is *Trp53*, the famous "guardian of the genome" and most frequently mutated gene in cancer[18]. Indeed, p53 controls *Myc* transcription[19,20], and *Trp53* loss can lead to Myc activation to foster tumorigenesis[21]. Consistent with this, whole exome sequencing of $Nras^{G12D}/Pten^{KO}$ HCC bulk samples revealed *Trp53* loss in all tested samples. Notably, mice injected a combination of $Nras^{G12D}/Trp53^{KO}$ via HDTVi exhibit extremely aggressive, multinodular, MYC-activated tumours with massive myeloid infiltration, succumbing to disease in less than two weeks, highlighting a critical role for p53 control of Myc to avert overexpressing oncogene escape. The remarkable aggressiveness of this model was the main reason we originally decided to opt out of testing therapeutic strategies in $Nras^{G12D}/Trp53^{KO}$-driven HCC, considering its short therapeutic window. Provocatively, the unique fibrotic environment of $Nras^{G12D}/Pten^{KO}$ tumours may stem from this ongoing regenerative process, as fibrosis is a classical hallmark of non-resolving liver injury[22].

Apart from explaining tumour escape in $Nras^{G12D}/Pten^{KO}$ HCC, *Myc* upregulation can also underlie the massive myeloid infiltration observed in this model. This is expected, given the critical role myeloid cells play in resolving tissue injury. A clear example of this interplay stems from a report from Gerard Evan's lab using a Myc^{OE}-inducible model. In this report, KRAS-overexpressing tumours showed a less aggressive, adenoma-like phenotype, characterized by influx of lymphocytes. Upon Myc activation, a massive influx of myeloid cells and exclusion of tumour-fighting lymphocytes take place, correlating closely with increased cancer aggressiveness[23]. This suggests that a similar mechanism

might explain why *Nras*^{G12D}/*Pten*^{KO} HCC is so myeloid-enriched, even more than its constitutive *Myc*^{OE}-driven counterparts.

The mechanism driving wound resolution—and thus escape from the "stuck" regeneration phase—remains largely elusive and may potentially be a fruitful therapeutic venue. KRAS inhibition, for instance, can revert pancreatic cancer cells to a normal epithelial state[24]. p53, required to drive wound healing, could potentially assist in the wound resolution phase. In bulk RNAseq analyses not presented in this thesis, the p53 pathway is overactive in *Nras*^{G12V}/*Pten*^{KO} HCC tumours compared to *Nras*^{G12D}/*Pten*^{KO} counterpart, which may partially explain why the latter is more aggressive, suggesting that it may be involved in a better resolution phase and in sustaining a more differentiated phenotype. Interestingly, however, p53 has recently been shown to be required to maintain MASH-afflicted hepatocytes into a senescent state, preventing MASH-to-HCC transition[25]. Understanding the mechanisms underlying its possible activation in *Nras*^{G12V}/*Pten*^{KO} compared to other MASH-HCC samples may shed light on unique features of this model and for additional processes licensing MASH-HCC evolution.

Another intriguing observation is a possible influence of metabolic disorders in wound-resolving processes, thus potentially contributing to carcinogenesis. Indeed, metabolic disorders may deplete stem cell pools, thus impairing regenerative capacity and leading to defective repair processes contributing to carcinogenesis, as observed in obesity-associated colorectal cancer (CRC)[26]. Dysregulated secretion of adipokines (e.g., leptin, adiponectin) and pro-inflammatory cytokines (e.g., IL-6, TNF-α) may perpetuate damage and enhance pro-tumorigenic signalling pathways. Excess lipid accumulation in metabolic disorders induces endoplasmic reticulum (ER) stress and unfolded protein responses (UPR), disrupting cellular homeostasis and repair[27], and promoting carcinogenesis through sustained stress signalling pathways[28,29]. Ultimately, it remains to be established how metabolic disorder-targeting therapies, like glucose-like peptide-1 receptor (GLP-1R) agonists, play a role in shaping wound-healing and wound-resolving processes and carcinogenesis in these patients.

Heterogeneity in MASH-HCC and non-MASH-HCC

The heterogeneity underlying HCC development is notorious. This includes several possible underlying aetiologies, ranging from alcohol intake to metabolic dysfunction and hepatitis infection[1]; as well as multifaceted processes promoting tumorigenesis, including inflammation, oxidative stress, and fibrosis. Therefore, mimicking HCC heterogeneity in preclinical studies is crucial for investigating unique disease-promoting mechanisms and therapeutic combinations that can benefit distinct HCC subtypes. Despite the effort to develop molecular subclasses distinguishing human HCC subtypes[1], the decision for treatment in advanced HCC is still not tailored to specific patients.

To tackle this, we developed the genetically distinct preclinical HCC models described in **Chapter 4**. These specific combinations of oncogene overexpression and tumour suppressor genes led to unique pathophysiological and immunological presentations, successfully mimicking the breadth of human HCC subtypes. In future studies, exploiting the generation of additional models driven by common HCC mutations, such as *TERT* and *CNTBB1*, and the exploration of their unique features, may ultimately enrich the field and reveal novel therapeutic targets not discussed in this work. One important consideration for the findings reported in this work is the use of exclusively female mice for the preclinical studies. However, HCC shows a gender bias, being 5 times more common in men for HBV-related cancers, yet only 1.1 times more common in men for MASH-HCC[30]. Furthermore, sex

hormones, including androgens and oestrogens, have been implicated in modulating hepatocarcinogenesis, which may influence tumour kinetics and immune cell landscape. For instance, oestrogen has been shown to block pro-tumorigenic macrophage polarization and suppress growth of orthotopic HCC in mice[31], whereas androgens may support hepatocarcinogenesis by fuelling DNA damage and oxidative stress[32]. Therefore, considering factors associated with gender disparity in HCC development in an aetiology-dependent manner is also paramount. Novel research venues in the lab will now consider both genders.

Another challenge in HCC treatment is the dynamic evolution of underlying aetiologies. Although hepatitis infection is still the major cause of HCC worldwide, the ongoing shift to MASH-HCC—which is becoming increasingly more common worldwide[33]—must be watched closely. Interestingly, several preclinical findings in MASH-HCC show heterogeneous clinical presentations, which may depend on the stressor leading to MASH development (such as diet). This suggests that, even if efforts to tackle MASH-HCC advance successfully, patient outcomes may still vary significantly depending on potentially yet non-characterized MASH-HCC subtypes. Supporting this hypothesis, although patients typically develop HCC from MASH associated with fibrosis or cirrhosis, a sizeable proportion can develop HCC directly from MASLD, even in the absence of fibrosis, cirrhosis, or steatohepatitis[14,34]. Understanding what sets these patients apart will shed light on distinct mechanistic processes governing hepatocarcinogenesis in varied disease settings. **Chapter 5** aimed to partially resolve this conundrum by exploiting the unique features of a non-fibrotic MASH-HCC model, highlighting that future work to compare this with other models, such as diet-induced, fibrotic MASH-HCC ones, will be crucial to enrich the data reported in this thesis.

The role of fibrosis in driving HCC

Fibrosis—and its end-stage manifestation, cirrhosis—often accompany and predict liver cancer development and progression. In a simplified view, fibrosis refers to the deposition of collagen and other extracellular matrix components secreted by activated fibroblasts—hepatic stellate cells in the liver[22,35]. Fibrosis is directly linked to patient prognosis; for instance, mortality in MASLD/MASH increases with fibrosis and cirrhosis severity[36]. Additionally, it is likely that fibrosis may impair the resolution of liver damage through physical obstruction or limiting the action of injury-resolving cells. This could potentially contribute to disease worsening in *Myc*-enriched, *Nras*^{G12D}/*Pten*^{KO}-driven HCC, where fibrosis is rampant. However, whether fibrosis actively drives oncogenic transformation and cancer progression or is merely a byproduct of chronic liver injury and regenerative responses remains a topic of debate. Emerging evidence suggests that, in some contexts, fibrosis itself may be a direct driver of oncogenesis. Indeed, fibrosis can disrupt normal blood flow[37], creating hypoxic conditions that could promote the emergence of pro-tumorigenic niches. Mechanosensitive pathways are activated in response to tissue stiffness, leading to direct enhancement of oncogenic signalling in cancer cells, promoting proliferation[38]. Fibrosis can also act as a physical barrier to medication[39,40] and immune cell infiltration and may directly impair T cells, limiting the efficacy of anti-tumour responses[41]. Tissue stiffness can also mediate YAP activation, leading to immune cell reprogramming[40]. This could be one immunomodulatory mechanism governing YAP-activated, fibrosis-enriched *Nras*^{G12D}/*Pten*^{KO}-driven HCC (data not shown). Moreover, activated fibroblasts secrete chemokine ligand 2 (CCL2)[42], a monocyte-attracting chemokine that can further shape a permissive TME, which may figure as a mechanism driving myeloid infiltration in *Nras*^{G12D}/*Pten*^{KO}-driven HCC.

It is likely that the extent to which fibrosis is necessary for HCC development varies depending on the underlying aetiology. For instance, hepatitis C virus (HCV)-related HCC is almost universally linked to cirrhosis, whereas hepatitis B virus (HBV)-related HCC develops with a lower dependence on fibrosis[1]. Similarly, while cirrhosis is considered a major risk factor for MASLD-associated HCC, a subset of patients can develop HCC in the absence of cirrhosis[14]. Indeed, patients with MASLD without cirrhosis have a fivefold increased risk of HCC compared to those with HCV[43], highlighting the existence of alternative oncogenic mechanisms—such as oxidative stress—that may bypass the need for extensive fibrosis.

Lipid overload, oxidative stress, and inflammation in HCC

Lipid metabolism is a fundamental process in most cell types[44]. However, under conditions of metabolic overload, excessive lipid accumulation disrupts mitochondrial function, leading to electron leakage and the generation of reactive oxygen species (ROS), which can, in turn, drive lipid peroxidation[44]—topics described briefly in **Chapter 2**. Whether cells ultimately undergo ferroptosis—an iron-dependent form of cell death triggered by overwhelming oxidative stress—depends on their ability to mount an effective antioxidant response[45]. Nevertheless, oxidative stress also promotes inflammation, and inflammatory cells themselves can generate ROS as a defence mechanism against pathogens and premalignant cells[46]. In this context, epithelial cells exposed to excessive oxidative stress may undergo oncogenic transformation, driven not only by oxidative genomic damage but also by the chronic inflammation that accompanies it[46]. Given the strong link between lipid overload and hepatocarcinogenesis, oxidative stress likely plays a central role in liver cancer development and may even contribute to tumorigenesis independently of fibrosis, suggesting that this mechanism may play a role in *Nras*^{G12V}/*Pten*^{KO}-induced HCC described in **Chapters 4** and **5**.

Chronic inflammation—whether linked to fibrosis or not—is a central driver of hepatocarcinogenesis[17], potentially influencing all HCC subtypes. As discussed in **Chapter 2**, multiple studies highlight its importance. For instance, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in hepatocytes can induce a sublethal necroptotic phenotype, leading to the release of inflammatory mediators that sustain a tumour-permissive microenvironment[47]. The IL-6/STAT3 pathway promotes hepatocyte survival, proliferation, and immune evasion, further facilitating tumour development[48]. In MASLD/MASH, gut dysbiosis and increased intestinal permeability exacerbate hepatic inflammation[49], and NOD-like receptor protein 3 (NLRP3) activation drives fibrosis and steatohepatitis[50], which may collectively contribute to inflammation-driven carcinogenesis in fatty liver disease. The next section explores the role of inflammation and immune cells in greater depth, addressing both established mechanisms and remaining uncertainties in HCC development.

Ontogeny of liver macrophages in HCC initiation and progression

As previously discussed in **Chapters 2**, **4**, and **5**, macrophage ontogeny is highly context- and organ-dependent. Under homeostatic conditions, most tissue-resident macrophages originate from embryonic yolk sac progenitors or erythro-myeloid progenitors (EMPs). For instance, Kupffer cells (KCs) in the liver are derived from EMPs in the yolk sac, while microglia in the brain originate from earlier yolk sac progenitors that are independent of EMPs. However, in disease states, tissue-resident macrophages receive significant contribution from monocyte-derived macrophages (MDMs)[51] from bone marrow origin. In the brain, the distinction between embryonic and monocyte-derived

macrophages is relatively straightforward, as microglia—the brain's resident macrophages—maintain a relatively stable transcriptional and epigenetic profile, thus remaining distinct from brain-infiltrating MDMs, which are absent in the homeostatic brain[52]. However, this distinction is more ambiguous in the liver, as hepatic MDMs gradually acquire a KC transcriptional and proteomic signature, ultimately differentiating into monocyte-derived KCs[53]. To definitively trace these subsets, MS4A3-based lineage-tracing models, which specifically label granulocytic and monocytic progenitors[54], as well as CLEC4F-tracing, which specifically labels KCs, are indispensable. Recent studies exploring hepatic macrophage heterogeneity have increasingly leveraged this tool, providing a more precise understanding of their distinct functions[55,56]. In line with this, MS4A3 tracing models are currently being implemented in the lab, enriching the findings herein reported.

Despite the inherent challenges in definitively distinguishing these macrophage populations, accumulating evidence indicates that MDMs and KCs differ significantly in plasticity, inherent phenotypic programming, and function, particularly relevant in the context of injury. Indeed, MDMs tend to adopt a more inflammatory phenotype, whereas KCs are more tolerogenic and proficient in lipid metabolism in the liver[51,57]. Tissue-resident KCs likely possess self-renewal capacity and play a key role in long-term tissue repair and immune tolerance[51,57]. In contrast, monocyte-derived macrophages are more dynamic, requiring continuous replenishment from the periphery, and can progressively dominate the macrophage pool in late-stage HCC, potentially overshadowing KC contributions[58]. Supporting these distinctions, the IL1B+C-C chemokine receptor type 2 (CCR2)+ inflammatory macrophages identified in *Nras*^{G12V}/*Pten*^{KO}-driven HCC in **Chapter 5** appear to originate exclusively from monocytes. Similarly, CD36^{+} macrophages detected in pre-tumour *Nras*^{G12V}/*Pten*^{KO}-injected mice exhibit a pro-inflammatory signature and are largely MDM-derived. However, at later disease stages, these CD36^{+} cells also seem to be derived from KCs—thus potentially leveraging the KC's superior lipid-handling capacity[57]. Nonetheless, the precise mechanisms by which each macrophage subset influences tumorigenesis in a definitive and functionally distinct manner remain only partially understood.

One important consideration for the studies described in this Thesis work is the impact of the HDTV procedure on hepatic macrophages. Despite the high clinical relevance and strong tumour induction penetrance in the HCC models described in **Chapter 4**, the HDTVi procedure itself induces substantial, albeit transient, liver damage[59], which may impact the local pool of KCs. Indeed, preliminary data not shown here suggest a decline in Tomato^{+} cells two days after the embryonic KCs (emKCs) lineage tracing *Clec4f*-dTomato mice underwent HDTVi, with recovery to baseline levels by day seven post injection, when the liver has fully restored its size and function. This suggests a transient elimination of emKCs following HDTVi. A key consequence of this macrophage disruption may be a shift towards a more inflammatory microenvironment, since most KCs post HDTVi may likely be monocyte-derived, potentially impairing this replenished macrophage pool's capacity to process lipids. Alternative methods of oncogene delivery, such as AAV-based approaches, may thus exhibit distinct tumour progression kinetics and phenotypes even if the genetic landscape is similar, at least in part due to differences in the macrophage landscape following oncogenic induction.

The macrophage ontogeny dynamics described in **Chapter 4** are undeniably heterogeneous across the HCC models. More aggressive models consistently exhibit a greater contribution from MDMs[58]. One plausible explanation for this is that liver damage such as fibrosis, and the associated dedifferentiation observed in these models may disrupt the formation of a KC-supporting niche, a concept originally coined elsewhere[60]. This effect may be further amplified by the heightened demand

for tissue injury resolution, driven by *Myc* overexpression, which skews the balance toward monocyte-derived cells in the more aggressive HCC models. Conversely, the well-differentiated *Nras*^{G12V}/*Pten*^{KO}-driven HCC may better preserve interactions between endothelial cells, stellate cells, and macrophages, fostering an environment that promotes KC differentiation[61]. My hypothesis is that monocyte KCs (moKCs) constitute the majority of macrophages in *Nras*^{G12V}/*Pten*^{KO}-driven HCC, a hypothesis supported by the progressive decline in Prostaglandin_KCs—the likely bona fide emKCs—observed in this model. Future studies using MS4A3 and other lineage tracing mice will provide deeper insight into the relative contributions of moKCs and emKCs in this model.

Unravelling the influence of KCs and MDMs on MASLD-to-HCC progression remains an ongoing area of intensive research. So far, the majority of lipid-associated macrophages (LAMs) in MASH appear to be monocyte-derived[62]. As discussed in **Chapter 2**, KCs are activated following efferocytosis of dying, lipid-laden hepatocytes—following death due to lipid overload and ferroptosis, for instance[63]—, which may disrupt KC metabolism and function, becoming more inflammatory, recruiting MDMs, and undergoing cell death at disease onset. Upon infiltration, monocytes differentiate into MDMs in a Notch-dependent manner[55]. MDMs replace KCs, likely assisting in lipid handling and clearing debris, becoming LAMs—either as moKC LAMs or MDM LAMs—, fuelling inflammation and disease worsening. A recent discovery suggests that cells termed as "LAM-like KCs" —emKCs showing features of LAMs— exist in liver homeostasis and are crucial for resolution of liver injury, and deletion of triggering receptor expressed on myeloid cells 2 (*Trem2*) in both LAM-like KCs and LAM MDMs (but not when depleted only in either KCs or MDMs) comparably aggravated the liver capacity to return to homeostasis, suggesting overlapping roles between the two ontogenetic-distinct subsets in specific diseased settings[56].

Overall, these evidence highlight differing phenotypes of ontogenetic-distinct liver macrophages, but several outstanding questions still remain. For instance:

- Do KCs and MDMs interact differently with T cells, TLSs, or cancer metabolism in HCC?
- How do the distinct niches from where these macrophages originate ultimately dictate their intrinsic metabolic, inflammatory and immunomodulatory roles in disease?
- Is there a distinction between the role of emKCs and moKCs in disease progression?
- Do emKCs and moKCs differ in their capacity to present antigens to T cells?
- Beyond being better at handling excess of lipids, are KCs more resistant to lipotoxicity?
- What are other differences between LAMs originating from KCs and MDMs and can they be therapeutically targeted?

LAM as an umbrella nomenclature for distinct macrophage populations

Immunometabolic rewiring represents a trademark feature shaping carcinogenesis, as extensively discussed in **Chapter 2**. Arguably at the apex of immune-metabolic reshaping are LAMs, given the increased scrutiny around these cells in several recent studies[64,65]. Originally observed in cardiovascular and metabolic disorders, these lipid-loaded macrophages have been detected in several other diseases, including MASLD/MASH, neurological disorders, and cancer, but also in the homeostatic liver[56,60,64]. In **Chapter 2**, I dissected their role in metabolic disorders and associated cancers, with particular focus on TREM2^{+} macrophages, a subset of LAMs that was originally identified in obesity and are characterized by limiting overt metabolic diseases, like MASH, but are tumour-promoting via immunosuppression of T cells, for instance[64]. However, the role of LAMs in other

disorders and homeostasis still warrants investigation. Building on the previous section, LAMs are reportedly largely derived from monocytes, but as mentioned, a recent study suggests that emKCs can adopt a LAM-like phenotype when faced with tissue injury, mediating resolution in a TREM2-dependent manner[56]. In **Chapter 5**, we observed a likely contribution from Prostaglandin_KCs to the CXCL16^{+}, LAM macrophage pool, suggesting that KCs can transition to this LAM phenotype.

Notably, several reports have highlighted the appearance of LAMs in different diseases, often with distinct nomenclatures depending on their function, organ of interest, and set of markers[64]. Yet, most studies to date have overlooked the inherent heterogeneity of LAMs. In other cases, macrophages exhibiting classical characteristics attributed to LAMs, such as *TREM2* and secreted phosphoprotein 1 (*SPP1*) expression, have not been classified as LAMs, even though they express markers typically associated with these cells and should therefore be classified as such. Indeed, studies may focus mostly on mechanistic and underlying functions of these macrophages, rather than classifying them as LAMs—a valid approach.

In parallel with their underappreciated heterogeneity, LAMs may be either protective or disease-promoting, in a context-dependent manner. Reports suggesting a beneficial role refer to TREM2^{+} macrophages found in liver fibrotic areas, as *Trem2*-deficient macrophages promote fibrosis and have impaired lipid handling[66]. In MASLD, TREM2^{+} macrophages clear dying, steatotic hepatocytes, preventing MASLD-to-MASH evolution[67]. MASLD fosters the expansion of TREM2^{+} macrophages with disrupted mitochondrial homeostasis, marked by impaired oxidative phosphorylation and accumulation of damaged mitochondria[68]. In the absence of TREM2, these dysfunctional macrophages exhibit enhanced release of mitochondria-containing exosomes, which are taken up by hepatocytes, further exacerbating hepatic lipid accumulation and accelerating MASLD progression[68]. In mice fed a high-fat, high-cholesterol, high-fructose diet, TREM2^{high} SPP1^{+} macrophages sharing the classical LAM markers were shown to accumulate in the MASH liver. Here, unlike other reports for SPP1^{+} macrophages shown below, *Spp1* deletion in myeloid cells worsened MASH in this case, as SPP1 induced oncostatin and upregulated *Arg2* on hepatocytes, leading to increased fatty acid oxidation and reduced steatosis[69], highlighting that even SPP1 may have beneficial effects depending on the disease context.

Conversely, the majority of reports seem to suggest a detrimental role of LAMs, particularly in the context of cancer. Scar-associated macrophages—defined by expression of TREM2, CD9, SPP1 and galectin-3 (LGALS3)—expand in liver fibrosis and are mostly monocyte-derived[70]. Despite not being called LAMs, these cells clearly share typical LAM signature markers and are pro-fibrotic in cirrhotic human livers[70]. In Western diet-fed, MASLD-bearing mice, SPP1^{+} monocyte-derived macrophages replace emKCs[71]. Sharing the usual LAM markers (TREM2, CD9, perilipin-2 (Plin2) and others), these cells are key drivers of fibrosis development in MASLD[71]. SPP1^{+} macrophages expressing CD9, TREM2 and LGALS3—thus sharing LAM characteristics—were also shown to drive immunosuppression in prostate cancer via adenosine signalling[72]. Programmed cell death 1 (PD-1)$^{+}$ macrophages with LAM signature—but distinct from TREM2^{+} macrophages—were shown to accumulate in obesity and drive therapy resistance in CRC[73]. Metastasis-associated macrophages, expressing CD36 (and other markers like CD206, IL10 and arginase 1 (ARG1)) and exhibiting increased lipid content, were shown to promote an immunosuppressive TME to allow liver metastasis[74]. This subset appears distinct from TREM2 and SPP1 macrophage subsets described previously. Finally, our lab showed that lipid-laden macrophages (termed LLMs), expressing *Gpnmb*, *Spp1*, *Trem2*, and *Lgals3*, can recycle myelin to fuel tumour growth

in glioblastoma[52]. In this case, the lipid content had a major impact in the paper's conclusion and the cell's capacity to foster tumorigenesis—which is not always the case for reports about LAMs.

The three-stage theory of LAMs: from homeostasis to cancer

I propose a three-stage model for the evolution of LAMs, from homeostasis to overt disease, to describe their progressive roles in cancer development (**Fig. 1**). In homeostasis (or Phase I), specific macrophage subpopulations are exposed to lipid metabolites and must assist in their handling. For instance, microglia are critical in supporting myelin turnover[75], and lung-resident alveolar macrophages routinely clear surfactant—composed of proteins and phospholipids[76]. KCs assist in the clearance of harmful lipid species (like oxidized lipid particles), are central in regulating cholesterol homeostasis, and constantly communicate with hepatocytes to ensure proper lipid balance[51,77]. Given that the hepatic lipid profile has been shown to be dependent on their liver zonation—both in homeostasis and MASH[78]—this variable is likely to play a role in the context of lipid handling exerted by hepatic macrophages.

During early disease—or Phase II, be it in premalignant inflammatory conditions or other inflammatory ailments—macrophages are central to resolving tissue injury and managing the resulting lipid burden, thereby working to restore normal lipid metabolism. Here, recognition of lipotoxic molecules via receptors like Mincle and CD36 can initiate an NF-κB-mediated inflammatory program[79,80], further amplified by inflammasome activation—which can be induced by cholesterol, for instance[81]. Excess lipid accumulation can impair mitochondrial function, generating ROS that further fuel inflammation. Oxidative stress may also lead to macrophage death[63], undermining efforts to reestablish homeostasis. I hypothesize that TREM2 is a hallmark for this phase, reflecting its critical role in efferocytosis and the restoration of metabolic balance. This view aligns with reports linking TREM2 to beneficial LAM functions in various diseases[67,82] and in our own unpublished results, suggesting that categorizing $TREM2^+$ macrophages solely as disease-promoting may be premature. The timing, context, and potential of TREM2-blocking strategies must therefore be carefully considered.

Finally, during overt disease—or Phase III, encompassing uncontrolled inflammatory disorders or established solid cancers—LAMs experience profound metabolic stress with significant functional consequences. Lipid overload in this phase can activate transcription factors such as Liver X receptor alpha (LXRα), peroxisome proliferator-activator receptor gamma (PPARγ), mTOR, and hypoxia-inducible factor alpha (HIFα)[44], which may collectively promote an immunosuppressive polarization[83-86]. Additionally, lipid-associated ER stress may trigger further immunosuppressive signals[87], while excess lipids reduce macrophage phagocytic capacity[88], collectively hindering their anti-tumour responses. The local diseased tissue environment—including interactions with other immune cells, stromal elements, and cytokine gradients—could further modulate this LAM behaviour. In this context, LAMs are more likely to be tumour-permissive and therefore represent a promising target for immunotherapeutic and metabolic reprogramming strategies.

If this three-phase model is valid, identifying specific markers and cellular programs unique to Phase III will be crucial for developing therapies aimed at repolarizing or inhibiting disease-promoting LAMs. Preliminary data suggest that *Spp1*, commonly upregulated in LAMs during overt disease[64], serves as an effective marker for these pro-tumorigenic cells[89]. However, it remains to be determined whether SPP1 directly drives the evolution from a TREM2 phenotype to a tumour-promoting state, or if it is an indirect consequence of pro-tumorigenic pathway engagement.

Considering all these aforementioned points about LAMs, a more granular analysis is possible for the LAMs identified in HDTV-injected control empty vector, tumour-free livers and *Nras*^{G12V}/*Pten*^{KO}-induced HCC. In these contexts, I propose that LAM prostaglandin KCs are potentially Phase I cells; that LAM IL1B+ CCR2+ inflammatory macrophages in pre-tumour samples are Phase II cells; and that CXCL16^{+} macrophages—as well as IL1B+CCR2+ macrophages, at least to a certain extent—likely shift to a Phase III phenotype at end-stage. While only speculatory, it is worth mentioning that the possibility of HDTVi-mediated depletion of embryonic, lipid-proficient KCs may already skew the entire liver to a more disease-like, primed state, even when considering control empty vector injected livers.

Regarding the identification of a LAM continuum from homeostasis to disease, we performed DEG analysis between each LAM subcluster in *Nras*^{G12V}/*Pten*^{KO}, and we estimated three markers that can identify these three populations by flow cytometry: macrophage receptor with collagenous structure (*Marco*) (for LAM Prostaglandin KCs); *Clec4e* (or Mincle; for LAM IL1B+ CCR2+ MDMs); and *Cd63* (for LAM CXCL16+ macrophages). Interestingly, a recent publication identified similar macrophage populations in the context of MASH, where *Spp1*^{high} cells were classified as expressing *Cd63*, *Il1b* inflammatory cells as high for *Clec4e*, and *Marco*^{high} cells as moKCs[55]. Thus, we are likely the first to report similar findings in MASH-HCC. Work is currently underway to pinpoint the phenotype of each of these populations.

Interestingly, *Spp1* expression is largely absent in all monocytic subsets in *Nras*^{G12V}/*Pten*^{KO}—including supposedly Phase III CXCL16^{+} macrophages—suggesting that, at least in this scenario, SPP1 is insufficient to identify Phase III LAMs. Although puzzling, it may actually be in line with the lack of fibrosis observed in *Nras*^{G12V}/*Pten*^{KO}-induced HCC, as SPP1 is needed for fibrosis in liver injury[90], and SPP1 increases in the context of MASH-bearing mice in parallel with fibrosis[69]. Indeed, *Spp1* encodes the protein osteopontin, which can activate hepatic stellate cells, promoting fibrotic deposits[91]. Furthermore, Notch signalling on hepatocytes drives osteopontin secretion, which is needed for fibrosis in MASH[92]. Exactly why SPP1 is not expressed in the context of MASH-HCC LAMs remains to be assessed and causes for this are likely multifactorial.

CD36 versus TREM2 macrophages – non-overlapping functions in liver homeostasis and disease?

In line with the hypotheses of a three-phase LAM dogma and a context-dependent, distinct role for TREM2^{+} macrophages, our findings in **Chapter 5** suggest that blocking TREM2 macrophages promoted survival extension at post-tumour stage, which was associated with an increase in proliferative CD36^{+} cells with lower CD206 levels. We postulate that the combination of macrophage pro-inflammatory polarization via vascular endothelial growth factor (VEGF) blockade and inhibition of TREM2 function collectively promoted the influx and expansion of monocyte- and KC-derived CD36^{+} cells with anti-tumorigenic capacity. This conjecture reinforces the idea of LAM heterogeneity and suggests a potentially distinct role for CD36^{+} versus CD36^{-} macrophage subpopulation (KCs or MDMs) and subsets.

We propose that CD36^{+} and TREM2^{+} macrophages may represent non-overlapping cell populations in the liver. From control liver to *Nras*^{G12V}/*Pten*^{KO}-induced HCC—going through the three phases described above, but in this case specifically in MASH to MASH-HCC—we observe a progressive increase in *Trem2*^{high} macrophages and a concomitant decrease in *Cd36*^{high} macrophages as disease advances. This balance partially shifts upon treatment with standard-of-care (SOC) and anti-TREM2

(Chapter 5). Based on these findings, we propose a tentative mechanistic timeline for these cells (**Fig. 1**):

- **Homeostasis** (Phase I): CD36$^+$ KCs reside in the liver, playing a critical role in supporting lipid metabolic balance. Despite the capacity of CD36 to induce a pro-inflammatory cascade, the maintenance of a tolerogenic niche—highlighted by their CD206 expression—prevents an overt inflammatory response. In homeostasis, excessive lipid exposure is unlikely, suggesting CD36$^+$ KCs are able to handle this satisfactorily. Conversely, TREM2$^+$ KCs are unlikely to be present in the homeostatic liver, as all reports so far identified these cells only in the context of liver injury or metabolic overload[56,67,93].
- **Pre-tumour stage** (Phase II) **in MASLD/MASH:** as metabolic dysfunction progresses, the demand for lipid processing increases dramatically. In this scenario, CD36$^+$ macrophages may be overwhelmed by lipid overload, leading to oxidative stress and possibly ferroptosis. The loss of CD36$^+$ tolerogenic KCs, their replacement by CD36$^+$ MDMs, and the activation of downstream CD36 inflammatory pathways, may collectively fuel tumour-promoting inflammation. In parallel, TREM2$^+$ lipid-proficient monocyte-derived macrophages emerge, cells potentially superior in withstanding metabolic stress.
- **Overt tumorigenesis** (Phase III)**:** as tumour progresses, CD36$^+$ macrophages face increased lipid stress, which, combined with immunosuppressive cues in the TME, skews them to a less inflammatory phenotype. At this stage, immunosuppressive TREM2$^+$ macrophages become more prevalent. Blocking TREM2 may prevent their lipid-handling processes, leading to an influx and expansion of CD36$^+$ macrophages. VEGF blockade redirects these macrophages toward a more anti-tumorigenic phenotype, which, when combined with PD-L1 inhibition, fosters both direct, and indirect T cell-mediated, anti-tumour responses, ultimately contributing to tumour control.

Although this timeline of LAM evolving features requires full validation, various literature reports support its plausibility. From the perspective of CD36$^+$ macrophages, it seems that their function differs in homeostasis and disease. A discrete subset of KCs—termed as KC2—was recently identified to play a critical role in lipid homeostasis in normal or lipid-loaded livers[94]. These cells were identified as CD36^{high} and may thus represent the CD36$^+$ CD206^{high} KCs we identified in control livers. These cells likely require a close interaction with endothelial cells to maintain their function—as previously shown for KCs[61]—and express high levels of CD206, which is likely partially associated with the KC tolerogenic phenotype in homeostasis. In our lab, we were unable to consistently isolate KC2s with the markers originally described in the publication. This however does not rule out the existence of CD36$^+$ KCs at homeostasis.

In the context of inflammatory diseases and metabolic deregulation, CD36$^+$ macrophages cells indeed likely represent a more inflammatory subset. For instance, constitutive *Cd36* deletion in HFD-fed mice promoted influx of LAMs with lower inflammatory capacity in white adipose tissue[95]. Corroborating with CD36 expression on MDMs upon tissue entry, a 1996 report showed that *Cd36* mRNA expression increased exponentially during monocyte-to-macrophage differentiation, with gradual decrease in mature macrophages[96]. This suggests that CD36$^+$ macrophages are early

responders to tissue injury, in line with the response to paracetamol-induced tissue injury, where CD36^{+} macrophages peak at 24h and are later replaced by TREM2^{+} cells[56]. In vitro, CD36-mediated recognition of oxidized low-density lipoprotein (oxLDL) disengages oxidative phosphorylation and fuels ROS production from mitochondria, inducing NF-kB-mediated inflammatory signalling in macrophage [97], highlighting the role of CD36 signalling in fuelling inflammation.

In **Chapter 2**, I discuss the dynamics and role of TREM2^{+} macrophages in metabolic disorders and cancer, highlighting that these cells are particularly immunosuppressive in established tumours. Whether TREM2 is directly involved in inducing an anti-inflammatory, immunosuppressive program, or simply a surrogate for this type of cells, still remains under investigation. A recent study showed that TREM2 is not essential for the efferocytosis of lipid droplets but is needed to curb an inflammatory cascade through inhibition of inflammasome[98]. TREM2 was also shown to directly dampen macrophages' capacity to produce pro-inflammatory cytokines upon exposure to TLR agonists[82], overall suggesting a direct role for TREM2 in governing macrophage anti-inflammatory program.

What drives the expression of CD36 and TREM2? PPARγ promotes *Cd36* transcription, whose activation can enhance *Pparg* expression[79,99]. LXRα activation—as in *Nras*^{G12V}/*Pten*^{KO}-induced HCC evolution—can also upregulate *Cd36* mRNA[79]. However, these lipid-associated transcription factors have also been implicated in *Trem2* regulation[100], raising the prospect that *Trem2* and *Cd36* expression are temporally distinct, with *Cd36* being engaged earlier on, as hypothesized above in different stages of the diseased liver. Alternatively, additional regulatory factors may be required for CD36 and Trem2 independent expression. In fact, *Trem2* is restricted to macrophages, while *Cd36* is ubiquitous, including in hepatocytes and endothelial cells—the highest contributor to the *Cd36*^{high} pool in *Nras*^{G12V}/*Pten*^{KO}-induced HCC—suggesting distinct regulatory programs for these receptors. The presence of CD36 in different cell types may represent distinct, cell-specific functions. In hepatocytes, CD36 facilitates de novo lipogenesis and fatty acid uptake, leading to lipotoxicity and steatosis progression[101,102]. In endothelial cells, CD36 plays in lipid transfer to other parenchymal cells and angiogenesis modulation[79,103]. Beyond their difference in transcriptional regulation, interaction between TREM2 and CD36 is also expected can also occur. For instance, soluble TREM2 interferes with normal recycling of CD36 on microglia, highlighting one mechanism that cleaved TREM2, induced in inflammatory environments, can limit CD36 expression[104].

An essential hypothesis in my theory relies on a supposedly better lipid-handling capacity from TREM2^{+} macrophages, and an inherent vulnerability of CD36^{+} macrophages towards lipid overload and ensuing overt oxidative stress. For the latter, the direct connection between CD36 and ROS generation corroborates this hypothesis[97], albeit not necessarily pointing to CD36^{+} cells being unable to deal with metabolic stress. One paper suggests that CD206^{high} macrophages are more vulnerable to ROS-induced ferroptosis in rheumatoid arthritis[105], suggesting that early CD36^{+} CD206^{high} KCs might also be more susceptible to it. Our preliminary findings using scRNAseq suggest that *Cd36*^{high} in *Nras*^{G12V}/*Pten*^{KO}-induced HCC have downregulated response to oxidative stress. However, to my knowledge, no reports have yet decisively showed that CD36^{+} macrophages are more susceptible to lipid overload. Interestingly, this has been shown for effector CD36^{+} T cells, where CD36 expression was shown to promote fatty acid uptake, foster lipid peroxidation and ferroptosis, and hinder their anti-tumour capacity[106,107], suggesting that a similar mechanism is also plausible in macrophages.

Interestingly, *Trem2* deletion was shown to curb survival of foamy macrophage in atherosclerosis due to enhanced cellular stress, reduced proliferation, and augmented cell death in

response to a failure in upregulating cholesterol efflux machinery[108], suggesting a mechanism through which TREM2-proficient cells may better cope with lipid overload. *Trem2*-deficient microglia undergo extensive autophagy due to low mTOR activity[109], highlighting that TREM2 *per se* has influence on macrophage metabolic rewiring.

In summary, future research is needed to unravel whether $CD36^{+}$ and $TREM2^{+}$ constitute distinct macrophage populations with timely regulated functionalities. This will be crucial for the development of tailored, time-specific treatments targeting these distinct macrophage subsets.

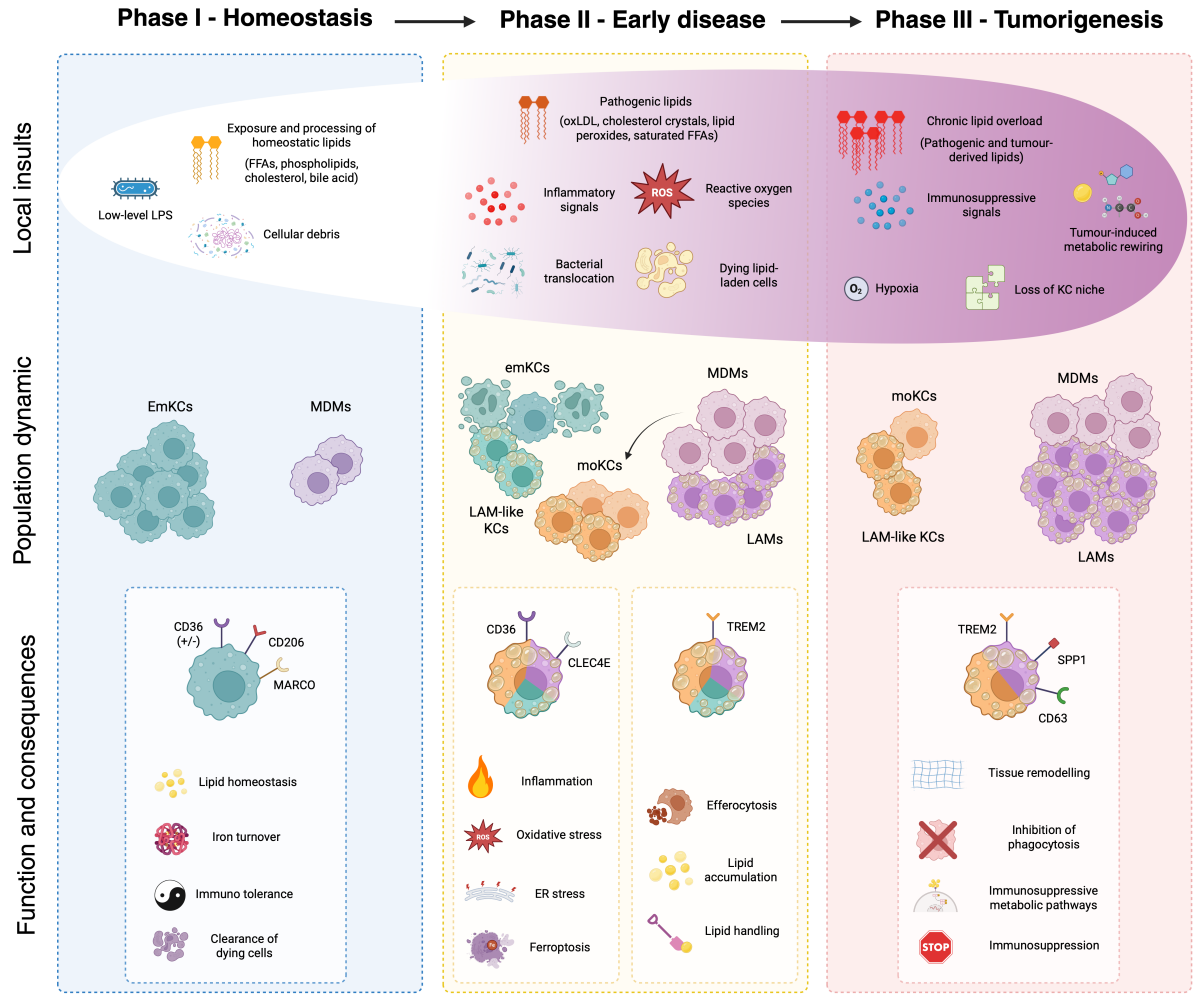

Fig. 1 | A three-phase model for hepatic LAM evolution and rewiring from homeostasis to tumours. This schematic illustrates a proposed timeline of lipid-associated macrophage (LAM) development across three distinct phases—homeostasis (phase I), early disease (phase II), and tumorigenesis (phase III)—highlighting local insults, population changes, and changes in function and consequences to macrophage fate over disease progression. In phase I (homeostasis), the liver is populated primarily by embryonically derived Kupffer cells (emKCs), which handle physiological lipid species (e.g., FFAs, phospholipids, cholesterol, bile acids) through receptors such as CD36 and MARCO. These cells promote iron turnover, immunotolerance, and efferocytosis, and express tolerogenic markers like CD206. In phase II (early disease), upon injury or metabolic stress, emKCs acquire a LAM-like phenotype or are replaced by monocyte-derived macrophages (MDMs) acquiring a KC phenotype, termed monocyte-derived KCs (moKCs). The participation of MDMs in the macrophage pool also increases. Macrophages are increasingly exposed to pathogenic lipids (e.g., oxLDL, cholesterol crystals, lipid peroxides, saturated FFAs), triggering inflammatory cascades, oxidative and ER stress, and cell death (e.g., ferroptosis). TREM2$^+$ macrophages emerge with enhanced lipid-handling capacity, while inflammatory CD36$^+$ CLEC4E$^+$ cells are prone to stress and dysfunction and likely die due to ferroptosis. In phase III (tumorigenesis), the metabolic and environmental challenges are heightened due to loss of KC niche signals, hypoxia, cancer cell-indued nutrient shifts, the presence of immunosuppressive cues, and chronic lipid exposure. In this phase, LAMs expand and are dominated by TREM2$^+$ immunosuppressive subsets. These cells display activation of immunosuppressive metabolic pathways, and express markers like SPP1 and CD63, have impaired phagocytosis, and promote T cell suppression. This model underscores the functional heterogeneity and temporal dynamics of LAMs, emphasizing the potential divergence between CD36$^+$ and TREM2$^+$ macrophage subsets. CLEC4E, C-type lectin domain family 4 member E. KCs, Kupffer cells; emKCs, embryonic KCs; ER, endoplasmic reticulum; FFA, free fatty acids; LAMs, lipid-associated macrophages; LPS, lipopolysaccharide; MARCO, macrophage receptor with collagenous structure; MDMs, monocyte-derived macrophages; moKCs, monocyte-derived KCs; oxLDL, oxidized low-density lipoprotein; SPP1, osteopontin; TREM2, triggering receptor expressed on myeloid cells 2.

Myeloid-derived inflammation driving tumorigenesis

In parallel with the immunometabolic adaptations undergone by macrophages, these cells can shape the oncogenic process via inflammation. Furthermore, as described in **Chapter 2** and expanded in the previous sections, the ontogeny of liver macrophages can also strongly influence their functional roles in tumour progression. Whether derived from embryonic progenitors or recruited monocytes, these cells help shape the tissue landscape by modulating inflammation, fibrosis, and metabolic stress. In particular, monocyte-derived macrophages—frequently dominating the macrophage pool in aggressive models—are potent drivers of inflammation, often fuelling tumour-promoting processes. Importantly, inflammation itself is a fundamental driver of tumorigenesis[110]—particularly in HCC, and regardless of the aetiology involved[1,17]. While inflammatory responses are widely recognized for their role in initiating cancer-promoting processes[110], they are also essential for mounting an effective anti-tumour response. However, chronic inflammation frequently coexists with immunosuppression, creating a complex interplay that can either promote or inhibit tumour progression[110,111]. Therefore, fine-tuning the inflammatory response is critical for achieving optimal anti-tumour response without further fuelling tumorigenesis[110,111].

As expected, myeloid cells are key orchestrators of the inflammatory response, particularly those infiltrating diseased tissues in response to alarmins, chemokines, DAMPs, and PAMPs[110,112]. However, the impact of myeloid cells on tumorigenesis is highly context- and time-dependent. As exemplified by our own work in **Chapter 5**, infiltrating IL1B+CCR2+ macrophages likely contribute to tumorigenesis by fuelling inflammation and oxidative stress in a MASH background. The massive influx of myeloid cells in *Nras*^{G12D}/*Pten*^{KO} tumours in **Chapter 4**, which already occur at early tumorigenesis, are likely boosting fibrosis development and accelerating tumour growth via release of inflammatory mediators, such as IL-1. As discussed in **Chapter 2**, similar conclusions are drawn in other cancer types, including in PDAC[113]. In a nutshell, blocking myeloid infiltration and inflammation at earlier stages, but heightening this response at established tumours, seems to be the desired strategy. Still, our results in **Chapter 4** suggest that, in a scenario where T cells are largely absent, hampering inflammation effectively curbed direct tumour proliferation, overall suggesting that modulating inflammation is highly context-dependent and must be considered carefully.

In contrast to a more obvious, somewhat "pure" inflammatory phenotype, the paradoxical co-existence of inflammatory and T cell-engaging response—mirroring the common overlap between chronic inflammation and immunosuppression—can also be observed within the same immune cell population. This was evident in the Ly6C^{low} cells described in **Chapter 4**, where GM-CSF-driven inflammatory signalling coincided with the upregulation of T cell-engaging factors, IL-1-related genes, but also immunosuppressive programmed death-ligand 1 (PD-L1) expression. Ultimately, these cells exhibited a predominantly pro-tumorigenic function, yet it remains unclear whether their T cell-stimulating properties would prevail in a more T cell-rich environment. A similar paradox is observed in chemokine (C-X-C motif) ligand 16 (*Cxcl16*)^{high} macrophages in MASH-driven *Nras*^{G12V}/*Pten*^{KO} HCC, which exhibit high *Trem2* and LAM signatures—widely associated with T cell suppression, as discussed in **Chapter 2**—yet also express high levels of T cell-activating and recruiting genes, including *Cxcl16* itself and key regulators of MHCII expression. Thus, the net effect of targeting such populations may yield unexpected and context-dependent outcomes in terms of tumour control, underscoring the need for careful consideration in therapeutic strategies.

In brief, targeting tumour-promoting, myeloid-mediated inflammation may be beneficial in controlling tumour growth. However, this remains a therapeutically challenging strategy, given its inherently context-dependent effects—as further discussed below.

Macrophages as therapeutic targets

Since the emergence of immunotherapy as a cornerstone of cancer treatment, its progress has been life-changing. However, most research has predominantly focused on directly enhancing T cell responses, given the strong association between T cell infiltration and improved prognosis in many cancer types[114]. Among innate immune cells, macrophages have emerged as key regulators of the tumour microenvironment and are the primary focus of this section, owing to their abundance, versatility, and prominent attention given throughout this Thesis work. However, other myeloid populations—particularly neutrophils—also contribute to tumour progression, often through mechanisms such as angiogenesis promotion, immunosuppression, or the formation of neutrophil extracellular traps (NETs)[115]. Although not the focus of the present work, the involvement of neutrophils in hepatocarcinogenesis and their potential as therapeutic targets—including in MASH-HCC[116]—should be further explored in future studies.

We and others have demonstrated that macrophages are the most abundant cells in most solid cancers, playing a pivotal role in promoting tumorigenesis through immunosuppression, inflammation, and metabolic rewiring[52,58,112], underscoring myeloid cells as promising therapeutic targets through boosting T cell response, controlling overt inflammation, or via direct anti-tumour activity[112,117]. In fact, the synergy underlying response to SOC for HCC (anti-VEGF plus anti-PD-L1) was shown to depend on controlling angiogenesis and myeloid cell-associated inflammation[118], reinforcing the feasibility of myeloid-targeting strategies in this disease.

As discussed in **Chapters 2**, **4** and **5**, the response of advanced HCC patients to standard treatment remains limited, emphasizing the need for further improvements. Here, I outline several strategies to modulate macrophages in cancer, their correlation with the findings emerging from my PhD work, and key considerations for achieving optimal therapeutic outcomes in future studies and trials.

Controlling macrophage-associated inflammation

Macrophage-induced inflammation can be targeted, for instance, through direct depletion of specific inflammatory subsets, or through macrophage repolarization, for instance. Curbing inflammation is often desired in early tumorigenesis but may be counterproductive in established tumours by limiting their anti-cancer activity. Therefore, any advance in this matter must consider the fine balance between detrimental and beneficial inflammatory response.

Nonetheless, in certain contexts, curbing macrophage-derived inflammation may be beneficial in established tumours. This is true for GM-CSF blockade in *Nras*^{G12D}/*Pten*^{KO}-driven HCC in **Chapter 4**, which acted primarily through hindering cancer cell survival through curbing myeloid cell-derived inflammatory signalling[58]. Lack of IL-1R secretion and rampant IL-1B signalling in *Myc*-driven tumours suggest that blocking this cytokine may be beneficial in these tumours, even though administration of recombinant IL-1Ra failed to avert tumour progression in this setting (data not shown). Other macrophage-derived inflammatory mediators with known role in HCC promotion include IL-6 and

tumor necrosis alpha (TNF-α), whose disruption in HCC—including MASH-HCC—yielded promising results in other reports[48,119] and may also be amenable myeloid-derived targets for therapy.

For MASH-HCC, controlling inflammation during the MASH step may avert its transition to HCC. One example of this was shown by the use of fibroblast growth factor 21 (FGF21) agonists. FGF21 is downregulated in MASH patients[120], and FGF21KO mice showed oxidized fatty acid-induced KC death, mobilization of MDMs transitioning towards moKCs, and transformation of free fatty acids into sphingosine-1 phosphate (S1P), leading to MASH-HCC transition via increased inflammatory signalling through the S1P-YAP pathway[120]. This latter finding highlights the role of inflammation, distinct ontogenetic roles of macrophages, and metabolic rewiring in MASH-HCC.

More systemic approaches to regulate inflammation could involve metabolic-rewiring drugs with anti-inflammatory properties. For instance, inexpensive drugs like aspirin and metformin have seen a recent surge of interest as cancer-modulating agents, partially due to their anti-inflammatory properties on myeloid cells[121]. However, their systemic anti-inflammatory effects may be counterproductive if robust anti-tumour immunity is required. To circumvent this potential drawback, a more targeted strategy could involve selectively counteracting specific pro-tumorigenic functions of pro-inflammatory macrophages. For instance, while pro-inflammatory macrophages produce ROS that can kill tumour cells, excess oxidative stress may be undesired due to higher chance of DNA damage and depletion of effector immune cells that are vulnerable to it. Therefore, selectively reducing ROS production, such as by targeting enzymes like NADPH oxidase 2 (NOX2), may prevent ROS-induced collateral damage while preserving beneficial pro-inflammatory functions.

Inhibiting monocyte infiltration

Considering monocyte-derived macrophages as more inflammatory and tumour-permissive, inhibiting their infiltration may figure as a more targeted therapeutic approach. Indeed, many studies have explored the blockade of myeloid-associated chemokine receptors and ligands in HCC. For instance, inhibition of the CCR2/CCL2 signalling axis unleashed T cell response in orthotopic HCC murine models[122]. In miR-122 KO mice—which induces hepatitis and eventually HCC—hepatocarcinogenesis and liver damage were curbed via reduced inflammation through CCL2 inhibition[123].

During my PhD, we tested whether disruption of the CCR2/CCL2 axis could alter tumour kinetics in the four genetically-distinct HCC models reported in **Chapter 4**. Induction of these models in *Ccr2*^{KO} mice showed no changes in survival compared to WT mice. Similarly, anti-CCL2 administration in *Myc*^{OE}/*Pten*^{KO}, *Nras*^{G12D}/*Pten*^{KO} and *Nras*^{G12V}/*Pten*^{KO} HCC also offered no survival benefit, although it significantly delayed tumour growth in *Myc*^{OE}/*Trp53*^{KO} in a mechanism potentially dependant on CD8^{+} T cell expansion. These unpublished findings suggest that *Ccr2*^{KO} and CCL2 inhibition led to different outcomes. It could be that *Ccr2*^{KO} is insufficient to completely deplete the classical monocyte pool, allowing increasing local proliferation of these cells to overcome, whereas CCL2 inhibition should, in theory, block any CCL2/CCR2 communication. It also highlights that other chemokines beyond CCL2 may modulate myeloid cell infiltration. For instance, GM-CSF-dependent CCL6—another monocyte-attracting chemokine—is upregulated in the *Nras*^{G12D}/*Pten*^{KO} TME. *Ccl5*^{high} T cells are enriched in the TME of *Nras*^{G12V}/*Pten*^{KO} and, although chemokine ligand 5 (CCL5) can attract tumour-fighting cDC1s and T cells[124], it is also involved in monocyte infiltration. These observations suggest that CCL2-independent venues are used by monocyte-derived macrophage to penetrate in these tumours.

One potential approach to regulate monocyte infiltration is to block several receptors at once. Dual blockade of CCR2 and C-C chemokine receptor type 5 (CCR5) has been extensively tested in liver diseases, showing capacity to curb inflammation, steatosis and liver damage in alcohol-fed mice[125]. When combined with FGF21 blockade, CCL2/CCR2 inhibition promoted reversal of MASH features in choline-deficient, high-fat diet (CD-HFD)-fed mice. As a standalone treatment, it improved MASH clinicopathological signs in patients[126]. Nevertheless, no study has explored its use in MASH-HCC. Whether this strategy is sufficient, whether therapeutic approaches are needed, or whether these are the most effective chemokines to target remains to be fully explored.

Macrophage depletion

Several studies have explored macrophage depletion through macrophage colony-stimulating factor receptor (CSF-1R) inhibition in solid cancers. Previous work in our lab showed that combining this pan macrophage-targeting approach with radiotherapy improved survival in glioblastoma-bearing mice[127]. Unfortunately, the therapeutic response is limited and resistance ensues, which may include the appearance of GM-CSF-dependent, pro-inflammatory tumour-associated macrophages (TAMs)—as shown in brain metastasis[128]—or via promotion of a fibrotic niche that supports tumour cell survival and glioblastoma relapse[129], for instance. Furthermore, CSF-1R inhibition depletes macrophages indiscriminately, regardless of their distinct roles, polarization states, or pro- and anti-tumorigenic activities. This broad depletion may disrupt beneficial immune functions, including antigen presentation and tissue homeostasis, potentially limiting the therapeutic benefit of this approach.

In findings not presented in this thesis, using three of the genetically-distinct murine HCC models described in **Chapter 4**—namely *Myc*^{OE}/*Trp53*^{KO}, *Myc*^{OE}/*Pten*^{KO} and *Nras*^{G12V}/*Pten*^{KO}—anti-CSF-1R inhibition efficiently depleted TAMs, but offered no survival benefit. We did not explore the mechanisms underlying this lack of response, but it is likely to differ between the distinct models, to depend on the appearance of CSF-1R-independent pro-tumorigenic macrophages, or to not be sufficient as a single-agent approach.

In my opinion, macrophage depletion must be more targeted to a specific subset, and/or combined with another immunotherapeutic approach. This could be accomplished, for instance, via combined depletion of SPP1^{+} macrophages with T cell-engaging immunotherapy. The identification of other pro-tumorigenic subsets will be crucial for the development of tailored approaches.

Enhancing macrophage anti-tumorigenic activity

Another attractive therapeutic venue involves the promotion of specific macrophage anti-tumorigenic functions. One involves inhibition of the “don’t eat me” CD47-signal regulatory protein alpha (SIRPα) axis, a checkpoint that prevents excessive phagocytosis[130]. Blocking this interaction licenses macrophage-mediated engulfment of cancer cells, an approach that has already shown efficacy in head and neck cancer[131] and preclinical models of HCC[132]. The therapeutic potential of the CD47 axis may depend on the mutational landscape of tumours. For instance, RAS and MYC have been reported to directly upregulate CD47 expression[133,134], allude to increased susceptibility of our more aggressive models to CD47 blockade. Interestingly, CD47 inhibition was also shown to alleviate MASH through macrophage-mediated clearance of dying hepatocytes[135], potentially preventing MASH-HCC and thus potentially benefiting *Nras*^{G12V}/*Pten*^{KO}-driven HCC.

An alternative direction is boosting CD40 signalling[136], which can induce a macrophage anti-tumorigenic phenotype[136]. CD40 agonism was shown to shift macrophage polarization to fuel response

to immunotherapy in cholangiocarcinoma[137], and to promote a T cell-independent regression of tumours via promotion of monocytes and macrophage infiltration, which subsequently degraded fibrosis and delayed tumour progression in KPC-driven PDAC-bearing mice[138]. This suggests that CD40 agonist as therapeutically relevant for fibrotic tumours, such as *Nras*^{G12D}/*Pten*^{KO}-driven HCC described in **Chapter 4**. Combining CSF-1R blockade with CD40 agonist was explored in two independent studies, which suggested a macrophage hyperactivation state prior to depletion, leading to enhanced T cell-mediated anti-tumour activity in preclinical orthotopic cancer models[139,140]. CD40 agonism can also directly alter macrophage metabolism—a topic discussed in more detail in the last section of this Thesis—to ultimately promote direct anti-tumour activity, including enhanced mitochondrial respiration, increased glycolysis, and reduced fatty acid oxidation—metabolic shifts that support a pro-inflammatory and antigen-presenting phenotype[141]. Whether CD40 activation could figure as a therapeutic approach in our HCC mouse models remains to be investigated. In this regarding, CD40 boosting on TAMs could license response to immunotherapy in the refractory MASH-HCC.

Lymphocytes in HCC: revisited

The influx of lymphocytes is widely regarded as beneficial in most solid cancers[114], including the majority of HCC cases[17]. However, as laid out in **Chapter 2**, the accumulation of these cells in the liver may be counterproductive in MASH-HCC, as T cell infiltration in MASH correlates with worse prognosis and predicts progression to MASH-HCC, where T cell enhancement has so far resulted in either neutral or worse outcomes[14,142,143]. This underscores the need to move beyond the simplistic view of T cells as universally "good guys" and instead adopt a more nuanced perspective that accounts for their context-dependent roles. Here, I lay down notions that I deem crucial when studying lymphocytes in HCC, with a particular focus on T cells.

Bystander *versus* tumour-reactive T cells

The analysis of T cell infiltration in solid tumours should include the assessment of their activation status, abundance, and phenotype. Beyond this, whether intratumoral T cells are truly tumour-reactive, or merely bystanders[144,145], remains an open question. These cells, which recognize non-tumour antigens, such as viral and bacterial peptides, are the majority of T cells in solid cancer, being recruited through non-cancer-specific chemokine signalling and contributing to local inflammation without necessarily mediating anti-tumour immunity[144]. Understanding whether T cells found in HCC are predominantly tumour-reactive or bystanders could have major implications for predicting patient prognosis and immunotherapy response. Interestingly, bystander T cells may also recognize noncanonical epitopes[144]—including those generated by oxidative stress[146]—which is relevant in MASH[14]. This suggests that MASH-HCC may be enriched in pro-inflammatory, but non-tumour-specific T cells.

Supporting this, as discussed in **Chapter 5**, *Nras*^{G12V}/*Pten*^{KO}-driven MASH-HCC exhibit progressive increase in CD8$^+$ T cell content; however, CD8$^+$ T cell depletion both before and after tumour onset had no impact on tumour kinetics, implying that most of these cells are bystanders throughout the evolution from MASH to MASH-HCC. However, response to SOC and TREM2 blockade in this model was accompanied by further expression of exhaustion markers in intratumoral CD8$^+$ T cells. This could indicate that increased T cell activation is indeed achieved in response to combination treatment through disruption of the tumor immunosuppressive burden, yet only short lived as resistance emerges and these cells become dysfunctional. In this context, CD8$^+$ T cells are likely

required for therapy response, and inhibiting specific immunosuppressive myeloid cell subsets enhances the presence and activity of tumour-reactive T cells. Future studies in our lab will explore TCR sequencing of *Nras*^{G12V}/*Pten*^{KO}-driven MASH-HCC scRNAseq dataset, to shed light on the anti-tumoral potential of infiltrating T cells.

In the more aggressive HCC models, such as *Myc*^{OE}-driven tumours, we did not assess whether the scarce T cells are predominantly bystanders or tumour-reactive. However, since CCL2 blockade extended the survival of *Myc*^{OE}/*Trp53*^{KO}-driven HCC-bearing mice accompanied by increased CD8^{+} T cell infiltration, it suggests a role in promoting T cell-mediated anti-tumour immunity. These results further support the potential of myeloid-targeting approaches in enhancing beneficial T cell subsets in solid cancers. However, this possibility is likely irrelevant to *Nras*^{G12D}/*Pten*^{KO}-driven HCC, which are devoid of T cells and where therapeutic strategies focusing on enabling T cell infiltration should be considered first.

Immune-deserted tumours — beyond immune checkpoint inhibitors

Immune-deserted cancers, such as PDAC—a collagen-enriched, largely inaccessible solid tumour that is resistant to immunotherapy[147]—are infamously refractory to immune-targeting cancer treatment[148]. As shown in **Chapter 4**, both *Myc*^{OE}-driven HCC models have reduced lymphoid infiltration, but *Nras*^{G12D}/*Pten*^{KO} is notoriously low in intratumoral and systemic lymphocytes. The mechanisms underlying these effects are likely multifactorial and influenced by genetic background but nonetheless underscore the limitations of therapies relying exclusively on immune checkpoint inhibitors.

Understanding how oncogenic signalling pathways govern this immune desert phenotype is paramount. For instance, the prevailing consensus is that MYC-enriched tumours are poorly infiltrated by lymphocytes[23,58,149]. MYC also profoundly reshapes cancer cell metabolism, driving glycolysis and leading to glucose depletion and lactate accumulation in the TME[9,150], which may impose nutrient limitations that directly impair lymphocyte survival, proliferation, and anti-tumour functions[9]. MYC also downregulates MHC-I expression on cancer cells and promotes the secretion of myeloid-supporting cytokines, collectively diminishing lymphocyte-mediated tumour cell killing[9]. Notably, data not shown here revealed an increase in prostaglandin-associated signatures in *Myc*^{OE}-driven HCC models, further supporting a role for lipid-mediated immunosuppression. Interestingly, NK cells are notoriously sensitive to prostaglandin signalling[151] and these cells are virtually absent in our HCC mouse models, suggesting that targeting this pathway may represent a viable strategy for lymphocyte-driven anti-tumour responses.

Interestingly, despite being less aggressive than *Myc*^{OE} tumours, *Nras*^{G12D}/*Pten*^{KO}-driven HCC display even less lymphocyte content than *Myc*^{OE}/*Trp53*^{KO} and *Myc*^{OE}/*Pten*^{KO} HCC. Mechanisms leading to this exclusion, in addition to the consequences of *Myc* overexpression discussed previously likely encompass MAPK overactivation and/or its distinctive histopathological features. The unique fibrotic nature of *Nras*^{G12D}/*Pten*^{KO}-driven HCC likely limits lymphocyte infiltration through physical hindrance, or sequestration of lymphocyte-attracting cytokines and chemokines. Fibrosis may also be a consequence of activation of hepatic stellate cells and presence of excessive immunosuppressive signalling, particularly transforming growth factor beta (TGF-β), which directly disrupt lymphocyte function[152]. In this setting, strategies aimed at increasing lymphocyte infiltration and potentially promoting tumour control could include anti-fibrotic agents, myeloid-depleting therapies, or administration of lymphocyte-inducing chemokines. This discussion has critical clinical implications,

given that most HCC patients present with advanced fibrosis[1], suggesting that lymphocyte-enriching strategies beyond PD-L1 blockade are essential to optimize responses to SOC treatment.

Overall, I postulate that further research must be geared towards understanding how we can revert an immune-deserted phenotype in patients that are currently not eligible for T cell-boosting therapy due to lack of lymphocyte infiltration. However, this strategy is not without caveats. However, given the negative impact of T cell infiltration in MASH-HCC, a more thorough approach is warranted. Indeed, as discussed in **Chapter 2** and **5**, increased T cell presence in MASH-related tumours has been associated with tissue damage, inflammation, and even accelerated tumour progression[143]. This paradox raises the possibility that, in certain metabolic contexts, enhancing lymphocyte infiltration could backfire unless their function is tightly regulated. Future therapeutic strategies must therefore not only focus on increasing T cell numbers, but also on rewiring their phenotype toward tumour-controlling rather than tissue-damaging states.

Tertiary lymphoid structures in HCC: context-dependent implications for tumour immunity

A polar opposite of an immune-deserted tissue, tertiary lymphoid structures (TLS) have gathered increased attention in tumour immunology[153]. As discussed in **Chapter 5**, TLS consist of various immune cells, depending on their maturity state. What qualifies as a bona fide TLS remains a subject of debate, but the prevailing consensus suggests that immature TLSs primarily comprise aggregates of B and T cells, whereas mature TLSs contain a germinal centre with a distinct B cell core surrounded by T cells, follicular dendritic cells (FDCs), high endothelial venules (HEVs), and specialized subsets such as follicular helper T cells[153].

These structures, which are usually associated with inflammatory disorders, are linked to better prognosis and response to therapy in most solid cancers[153]. However, how TLSs modulate carcinogenesis depends on several factors, including their maturity, the disease stage they are found, their own spatial localization, and their cellular composition[153]. In HCC, TLSs are controversial, as they were shown to promote premalignant progenitor cells[154], but also correlate with better prognosis when present intratumorally[155]. In MASH, TLSs are seen in 60% of patients, correlating positively with inflammation and fibrosis, suggesting a detrimental role in MASH-HCC[143].

In *Nras*^{G12V}/*Pten*^{KO} MASH-HCC, disruption of lymphoid aggregates did not affect tumour progression, suggesting that TLSs may arise primarily as a consequence of inflammatory signalling rather than serving as functional hubs for anti-tumoral T cell priming, potentially as a niche for bystander T cell proliferation. *Nras*^{G12V}/*Pten*^{KO} MASH-HCC TLSs also exhibit T_{reg} accumulation, cells that have been shown to disrupt DC:T cell crosstalk, hinder anti-tumour T cell formation, and correlate with worse patient prognosis in solid tumours when present within TLSs[153,156]. Depleting T_{regs} could remodel TLSs to enhance tumour control in this setting. Nonetheless, the enhancement of T cell activation and exhaustion following combined anti-TREM2 and SOC treatment suggests that alleviating the immunosuppressive burden might enable the generation of bona fide anti-tumoral T cell responses. It remains to be determined whether TLSs are critical for this positive treatment outcome.

Given the contrast between immune-deserted and TLS-rich tumours, inducing TLS formation in the immune desert scenario is particularly enticing. In theory, this could establish a hub for T cell priming and activation in more aggressive HCC, hopefully ensuring more lymphocyte infiltration and tumour control. Administration of recombinant LT-β, LIGHT or other TLS-modulating agents may thus confer therapeutic benefits. However, the expected collateral inflammatory response could lead to

unintended consequences, including exacerbation of inflammation, widespread immune activation, and even microbiome changes leading to metabolic diseases[157,158]. Overall, these findings contest the prevailing idea that TLSs are universally beneficial in cancer.

Beyond TLSs: the underappreciated role of B cells in cancer immunity

B cells and plasma cells are also largely neglected in cancer studies, despite their role in sustaining anti-tumour responses and their correlation with better therapeutic outcome and patient prognosis in solid cancers[159]. Even when B cells are considered, they are often bundled with TLSs[160,161], where they pose as secondary players. In HCC, B cells with immunosuppressive function have been posited to contribute to tumour progression in HCC via release of IL-10 and expression of PD-L1, collectively inhibiting $CD8^+$ T cell-mediated tumour control[162,163]. Yet B cell and plasma cell abundance were both linked to better HCC prognosis[164], overall suggesting that B cells have context-dependent roles in supporting or fostering HCC. Importantly, B cells serve as a functional bridge between innate and adaptive immunity, not only by producing antigen-specific antibodies but also through cytokine release and antigen presentation to T cells[159]. Moreover, B cells can modulate macrophage activity through the secretion of cytokines and growth factors such as GM-CSF and IFN-γ, of monocyte-attracting chemokines like CCL5, or via interaction with Fc receptors, further influencing the TME inflammatory landscape[159].

In all four HCC models described in **Chapter 4**, systemic and intratumoral B cells almost vanish as tumour progresses, including in the TLS-enriched, *Nras*^{G12V}/*Pten*^{KO} MASH-HCC (data not shown). This may suggest a common B cell-excluding program, although the underlying mechanism is likely distinct per model. Factors contributing to this likely include a lack of B cell-attracting chemokines, the presence of immunosuppressive molecules and immune cell subsets, physical restraint, and potentially low expression of B cell-promoting cytokines, such as IL-21. Importantly, it remains unclear whether B cells are actively excluded from the tumour core and instead accumulate in peritumoral regions, or whether they are systemically depleted altogether. Future spatial analyses will be required to distinguish between these possibilities.

In the more aggressive models, *Myc* and *Nras* overexpression likely block B cell infiltration, as RAS and MYC cooperation can induce B cell exclusion via curbing of type 1 interferon response[165], and through infiltration of immunosuppressive macrophages and increased IL-23 signalling[23]. Testing whether pan-RAS inhibitor treatment in *Nras*^{G12D}/*Pten*^{KO} HCC would lead to B cell influx and potentially better treatment outcome is an intriguing prospect that could be assessed.

In *Nras*^{G12V}/*Pten*^{KO} MASH-HCC, the absence of intratumoral B cells is puzzling. Although TLSs are detected by tissue analysis, most of these structures are localized peritumorally and may therefore be absent from material used for flow cytometry. Still, scRNAseq analysis of *Nras*^{G12V}/*Pten*^{KO} MASH-HCC in **Chapter 5** showed plasma cells at pre-tumour stage, but not at end-stage samples, highlighting a possible activation and later exclusion or death of B cells. Intriguingly, B cells responding to oxidative stress-derived antigens are present in MASLD[166], which may explain the formation of plasma cells in our model and alluding to bystander or detrimental B cell subsets. B cells also undergo metabolic shifts during activation, increased reliance on oxidative phosphorylation and fatty acid oxidation[167], possibly making them susceptible to ER and oxidative stresses in a lipid-enriched environment—factors that may underlie their demise in MASH-HCC. Considering reports of immunosuppressive B cells in MASH, boosting their infiltration or activation carries significant risks.

Revisiting CD4$^+$ T cells as critical regulators in tumour immunity

CD4$^+$ T cells play a critical role in orchestrating immune responses through multiple mechanisms, including immune licensing, the release of immunomodulatory cytokines, the promotion of long-lasting CD8$^+$ T cell immunity, and even direct cytotoxic activity[168]. Yet despite their fundamental role, most cancer immunology literature remains heavily focused on CD8$^+$ T cells, often downgrading CD4$^+$ T cell-related findings to ancillary observations rather than considering them as key players in disease control and potential therapeutic targets.

Admittedly, this thesis largely followed the same trend of overlooking CD4$^+$ T cells. A major reason for the limited focus on CD4$^+$ T cells in this work—and arguably in much of the field—is their inherent complexity. CD4$^+$ T cells encompass a diverse array of phenotypic subsets, including Th1, Th2, Th17, T_{regs}, and lesser-characterized populations, each of which can exert distinct and sometimes opposing effects on tumour progression and immune regulation[168]. Any meaningful conclusions regarding CD4$^+$ T cell dynamics in cancer must, therefore, consider their subset-specific contributions to tumour control or relapse. Analyses of CD4$^+$ T cells in this study were primarily limited to distinguishing between T_{regs} and conventional CD4$^+$ T cells, without further delineation of specific Th subsets or their functional changes across distinct HCC models and treatment conditions. Future studies should incorporate a comprehensive flow cytometry panel capable of distinguishing Th subsets. This could provide novel insights into how combinatorial strategies integrating myeloid-targeting and T cell-boosting therapies may be further refined by assessing underlying shifts in CD4$^+$ T cell phenotype and function with greater precision.

Future of T cell targeting in HCC

The approval of immunotherapy as a first-line treatment in advanced HCC represents a major breakthrough. Yet, patient response is limited and dependent on the underlying aetiology, thereby advocating for more granular approaches to boost lymphocyte anti-tumour response in these patients. Several recent clinical trials focusing on ICB response in unresectable HCC took place, yet key limitations persist. One trial evaluating combined anti-CTLA4 and anti-PD-1 still used sorafenib as comparator rather than the current SOC and failed to stratify patients by aetiology—a critical parameter[169]. Another high-profile trial showed that anti-T cell immunoreceptor with Ig and ITIM domains (TIGIT)—another T cell-associated checkpoint inhibitor—synergizes with SOC to improve HCC patient survival[170]. In this study, while HCC aetiologies were reported, they were not accounted for in the results stratification. These examples highlight the slow pace at which clinical research is integrating aetiology-based, molecular class stratifications. Furthermore, virtually all lymphocyte-targeting approaches in HCC target T cells[171], highlighting that investigations into reshaping other lymphoid cells remain unexplored while potentially fruitful.

While lymphocyte-modulating strategies remain central, targeting metabolic dysfunction, myeloid immunosuppression, or oncogenic pathways may offer valuable alternatives to simply layering additional T cell–centric checkpoint inhibitors. Growing evidence supports the feasibility of such interventions. For instance, RAS inhibition reverts tumour immunosuppressive burden through, in part, reducing the tumour cell-derived production of myeloid-attracting chemokines and augmented interferon response, rendering tumours more likely to respond to ICB approaches[172]. This may hold promising results in MAPK-enriched tumours, such as the *Nras*^{G12D}/*Pten*^{KO} HCC. For still-undruggable targets like MYC, mitigating downstream immunosuppressive effects of oncogene activation offers an alternative strategy. This was shown in MYC-overexpressing triple negative breast cancer-bearing mice,

where OX40 receptor engagement combined with anti-PD1 synergized with Toll-like receptor 9 (TLR9) agonists, restoring interferon signalling and reverting low MHCI expression on cancer cells induced by overactive MYC[173]. A similar approach could be achieved in HCC driven by *Myc* overexpression. In **Chapter 4**, we showed that the effect of anti-GM-CSF was independent of T cell response in *Nras*^{G12D}/*Pten*^{KO} HCC, where *Myc* is upregulated. Combining a TLR-engaging approach in combination with ICB—as shown for triple negative breast cancer—as well as ICB and T cell-attracting chemokines, could potentially revert the immune desert phenotype of *Myc*-overexpressing *Nras*^{G12D}/*Pten*^{KO} HCC, likely leading to a greater therapeutic response.

For MASH-HCC, strategies aimed at enhancing T cell activity must be carefully considered due to the potential risk of expanding auto-aggressive T cell subsets[174]. Similarly, achieving a durable response to ICB in this context likely requires combinatorial approaches incorporating myeloid-targeting and metabolic rewiring—as discussed in the next section. Evidence supporting the former includes the enhanced response observed in MASH-HCC-bearing mice treated with a combination of CXCR2 inhibition—suppressing pro-tumorigenic neutrophils—and PD-1 blockade[116]. Similarly, the potentially increased T cell activity seen in *Nras*^{G12V}/*Pten*^{KO} MASH-HCC-bearing mice receiving SOC therapy in combination with anti-TREM2 further highlights the role of myeloid modulation in restoring anti-tumour immunity. As discussed in **Chapter 2**, the use of GLP-1R agonists in combination with ICB may hold promising results for these patients. At the time of this writing, work is underway to test semaglutide and ICB in *Nras*^{G12V}/*Pten*^{KO} MASH-HCC-bearing mice.

Overall, lymphocyte-modulating approaches should extend beyond the common paradigm of pairing anti-PD-1 with arbitrary agents. Instead, a more tailored strategy should be pursued, incorporating other checkpoint inhibitors, such as TIGIT or OX40, to optimize patient outcomes.

Targeting cancer cell and immune cell metabolism in HCC

Cancer metabolism is a cornerstone of the oncogenic process[175], as briefed upon in **Chapter 2**. However, this field is far more complex than the simplistic view that cancer cells merely undergo metabolic shifts, such as increased aerobic glycolysis, during carcinogenesis[175]. Importantly, immune cells—especially T cells and macrophages—also undergo extensive metabolic rewiring in the TME, often competing with cancer cells for key nutrients such as glucose, glutamine, and lipids[176]. These shared metabolic constraints must be considered when designing therapeutic strategies.

Disarming cancer cells through metabolic intervention

In terms of cancer cells, specific oncogenic pathways uniquely modulate their metabolic profile[177], indicating the need for a tailored therapeutic approach dependent on the genetic background. Furthermore, cancer cells may also alter their metabolic phenotype in light of nutrient availability within the TME, rather than due to intrinsic changes in their mutational landscape[178]. Cancer can also arise from seemingly innocuous mutations under metabolically stressed conditions. This concept was recently reported in hepatocytes exposed to metabolic stress in alcohol- and nonalcohol-induced fatty liver diseases, where convergent accumulation of mutations linked to lipid processing facilitated the oncogenic process[179]. Collectively, these notions underscore the complexity of targeting cancer metabolism.

In **Chapter 5**, we attempted to use lipid-altering strategies to extend survival of *Nras*^{G12V}/*Pten*^{KO}-induced, HCC-bearing mice, but none of the chosen therapeutic approaches

succeeded. This may be attributed to compensatory metabolic adaptations via alternative pathways—highlighting the need for combinatorial strategies—incorrect timing (pre-tumour versus post-tumour intervention) or targeting of pathways that may not necessarily influence either cancer or immune cell function in this context. Several other metabolic-centric treatment options could be considered for *Nras*^{G12V}/*Pten*^{KO} MASH-HCC, or MASH-HCC more broadly. A non-exhaustive list includes inhibitors of de novo lipogenesis, such as fatty acid synthase (FASN) blockade[180], or interventions to curb lipid desaturation, for instance, by blocking stearoyl-CoA 9-desaturase (SCD1)[181]. Carnitine palmitoyltransferase I (CPT1) inhibitors could reduce fatty acid oxidation[180], thereby potentially mitigating excessive oxidative stress under lipotoxic conditions and preventing evolution to MASH-HCC. Inducers of AMPK, including metformin[182], could prevent lipid accumulation[183] and even promote autophagy. Preventing lipid scavenging by cancer cells through CD36 inhibition may also modulate tumorigenesis[180], although this approach carries the risk of affecting other CD36^{+} cells in the tumour microenvironment. Another promising but yet unexplored approach in MASH-HCC is the use of thyroid hormone receptor beta (THRB) agonists, a class of drugs that modulate glucose and lipid metabolism and ameliorate mitochondrial function in hepatocytes and that are being used in the clinic for MASH patients[184]. Similarly, FGF21R agonists, known to induce lipolysis, improve systemic metabolic parameters like insulin resistance while also exerting direct anti-fibrotic effects[185], warranting investigation in this context and in fibrotic MASH-HCC models. Using strategies to break ferroptosis response, such as via glutathione peroxidase 4 (GPX4) inhibitors, could selectively eliminate lipid-laden cancer cells by impairing their ability to manage oxidative stress[186]. Finally, given the widespread clinical use of GLP-1R agonists and the growing body of evidence supporting their role in modulating MASH pathology[182], their potential benefit in MASH-HCC also merits further exploration.

One critical unknown factor is the source and composition of lipids in tumour cells in MASH-HCC. Addressing this diversity in lipid species is particularly challenging due to the liver's constant exposure to diverse lipid sources, unlike more isolated organs such as the brain. Lipid acquisition likely involves a combination of de novo lipogenesis, exogenous uptake of lipoproteins, transfer from lipid-laden cells, and other mechanisms. This complexity could significantly impact treatment efficacy—blocking endogenous lipid synthesis, for instance, may be ineffective if extracellular lipids are the predominant source. Additionally, a key confounding factor in this analysis is the chronic nutrient overload in patients with metabolic disorders, which could alter the lipid landscape to which pre-cancerous and cancerous cells are exposed. Disentangling these variables through extensive and targeted lipidomic analyses in relevant models may provide crucial insights, thus hopefully guiding therapy development.

Importantly, targeting cancer metabolism does not necessarily have to be restricted to tumours with overt metabolic dysfunction. In *Myc*-overexpressing tumours, the activation of glycolysis and glutaminolysis pathways suggests that their modulation may be beneficial, a strategy therefore relevant in *Myc*^{OE}-driven HCC models[150]. This could potentially be combined with immunotherapy approaches, as reduced utilisation of glucose and glutamine, along with decreased lactate accumulation within the TME, may alleviate metabolic competition and reduce immunosuppressive metabolites like lactate, thereby supporting T cell infiltration, survival, and effector function[187]. However, challenges will lie in achieving targeted metabolic approaches, as immune cells also depend on glycolysis, and in certain cases glutaminolysis, for optimal function[178].

In *Nras*^{G12D}/*Pten*^{KO} tumours, MYC-associated metabolic vulnerabilities are also likely to arise, alongside metabolic alterations unique to this model. Bulk RNA sequencing analyses comparing

Nras^{G12V}/*Pten*^{KO} and *Nras*^{G12D}/*Pten*^{KO} cancer cells indicate that the latter is associated with reduced cholesterol homeostasis, oxidative phosphorylation, bile acid metabolism, and fatty acid metabolism. Additionally, we observe an increase in transcripts linked to myogenesis. Given that muscle cells typically rely on high glycolytic rates during rapid contraction and growth phases, the activation of myogenic pathways in *Nras*^{G12D}/*Pten*^{KO} cancer cells may reflect an increased glycolytic flux that could represent a targetable vulnerability. These observations closely align with reports of MYC-driven cancer metabolism[150], suggesting that glycolytic-modulating agents could further enhance cancer cell death and necrosis induced by the combination of GM-CSF blockade and SOC treatment. As the effect of this combinatory treatment described in **Chapter 4** is T cell-independent, any potential adverse effects of glycolysis inhibition on T cell activity may be less of a concern in this context, although modulation of potentially anti-tumorigenic myeloid cell subsets may also occur.

Metabolic reprogramming and consequence to macrophage function

As described in **Chapter 2**, cells residing in the TME of solid tumours—including macrophages— undergo significant metabolic reprogramming in response to nutrient and metabolite imbalances[182]. Metabolically-challenged macrophages may subsequently support tumorigenesis, depending on the exact metabolite involved[182]. Lipid overload can lead to LAM formation, worsening the immunosuppressive burden within the TME. Amino acid depletion may lead to the upregulation of enzymes such as indoleamine 2,3-dioxygenase (IDO) and ARG1, further impairing T cell function[182]. Glucose restriction and lactate accumulation can shift macrophage metabolism toward oxidative phosphorylation, promoting a tumour-permissive phenotype[182]. These observations suggest that targeting metabolic reprogramming could modulate macrophage function and tumour progression.

Metabolic reprogramming could be modulated through interference with the mTOR pathway, the master regulator of nutrient sensing[188], since mTOR impacts macrophage polarization and function[188]. mTOR inhibition triggers NF-kb activation and subsequent release of pro-inflammatory cytokines[188]; however, in tissue-resident macrophages—such as in the liver and adipose tissue—mTOR deletion alleviated metabolic dysfunction and inflammation in high fat diet-fed mice[188]. Promotion of autophagy through mTOR inhibition may also alter the LAM phenotype by fostering lipid processing[188,189]. Intriguingly, dietary approaches involving fasting and caloric restriction are accompanied by lower mTOR activity[189], suggesting that these interventions may also modulate macrophage function through this pathway.

Another strategy of metabolic reprogramming through signalling pathway modulation is via PPARs—transcription factors involved in response to lipid signalling[83]. PPARγ activation on macrophages has been shown to promote an anti-inflammatory phenotype[83], but is required for CD36 expression[79], which may thus promote inflammation if engaged. The PPARγ-CD36 axis has been implicated in promoting lipid-laden cells[190], thus serving as an upstream target to limit LAM accumulation. PPARα activation also tilts macrophage polarization to a wound-healing, immunosuppressive state[83], suggesting that its inhibition in solid cancers, but activation in pre-tumoral states, may be relevant. As with other metabolic-rewiring agents, non-specific targeting may yield unwanted outcomes. PPARα has been implicated in limiting diet-induced MASH and MASH-HCC in the context of intermittent fasting diet through augmented fatty acid oxidation and ketogenesis[191], thereby unspecific inhibition of PPARα could limit these functions and exacerbate disease progression.

Activation of AMP-activated protein kinase (AMPK), an energy sensor that restores metabolic homeostasis by promoting catabolic pathways and inhibiting anabolic processes, represents another

strategy to enforce metabolic reprogramming. Similarly, targeting metabolic pathways—such as inhibiting glycolysis or blocking lactate transporters—may further limit the metabolic flexibility of macrophages in the TME, thereby curbing their tumour-promoting functions.

Metabolic disorder drugs, like metformin and GLP-1R agonists, may also impact macrophage function and consequently shape tumorigenesis. For instance, metformin can activate AMPK, which has been shown to reprogram macrophages into a less tumour-supportive phenotype—as discussed in detail in **Chapter 2.** However, as hinted previously, targeting macrophage metabolism through systemic treatments may be counterproductive. Therefore, specifically targeting metabolically-challenged macrophages is of utmost interest. For LAMs, the use of lipid nanoparticles or liposomes carrying immunometabolic-altering agents is under investigation in our lab as potential techniques to preferentially target these cells via selective uptake mechanisms. Since not all metabolically challenged macrophages are lipid-rich, the development of additional strategies to target different macrophage states—like hypoxic, glucose- or amino acid-deprived— will be crucial for advancing this field.

Reshaping T cell-associated metabolic challenges to foster anti-tumour response

In the TME, T cells face harsh metabolic constraints and undergo extensive metabolic rewiring that can limit their anti-tumour potential. As discussed in **Chapter 2** and in previous sections, tumour-infiltrating T cells often exhibit an exhausted phenotype, marked by impaired effector functions, reduced proliferation, and mitochondrial dysfunction[192]. These features are tightly coupled to the metabolic stress imposed by the TME, including hypoxia, the accumulation of immunosuppressive metabolites, and the metabolic competition between tumour cells, immunosuppressive myeloid cells, and effector T cells for critical nutrients like glucose and glutamine[192]. For the latter, as discussed previously, cancer cells can deplete glucose through enhanced aerobic glycolysis, while macrophages may consume amino acids such as arginine and tryptophan, impairing T cell proliferation and cytokine production via enzymes like ARG1 and IDO[112,192]. Targeting these pathways in cancer cells and macrophages is thus expected to concurrently reinvigorate T cell function.

One important consideration for T cell function is the presence of immunosuppressive metabolites in the TME—such as lactate and lipid accumulation, as well as extracellular adenosine—which can collectively hinder T cell function and impede tumour clearance. For instance, targeting of squalene epoxidase on cancer cells—and thus reducing cholesterol accumulation in the TME—in MASH-HCC-bearing mice was sufficient to restore anti-PD1 treatment response[193], highlighting one potential therapeutic avenue in *Nras*^{G12V}/*Pten*^{KO} HCC. Adenosine has recently garnered increased attention. This nucleoside can impair T cell proliferation and activation, production of IFN-γ, and induce exhaustion via modulation of the A2A receptor. Blockade of ectoenzymes producing adenosine, such as CD39 and CD73, can restore T cell function[194]. In HCC, hypoxia reduces adenosine kinase (ADK)-mediated adenosine breakdown, being associated with reduced $CD8^+$ T cell infiltration and increased T cell exhaustion[195]. Combining adenosine receptor antagonists with anti–PD-1 therapy significantly improved survival and delayed tumour growth in HCC mouse models[195]. Adenosine can also be taken up by T cells through equilibrative nucleoside transporter 1 (ENT1) and converted intracellularly to adenosine monophosphate (AMP), inhibiting pyrimidine synthesis and impairing T cell proliferation[196]. ENT1 blockade in combination with anti-PD1 enhanced tumour control in breast cancer-bearing mice[196]. Collectively, these findings suggest that targeting adenosine metabolism and signalling may be a viable route to re-establish effective T cell immunity in HCC. However, whether adenosine or other

T cell-dampening metabolites are upregulated in our preclinical models remains to be fully determined.

Additionally, targeting T cell metabolism—both directly and indirectly—has been explored in several recent studies and may be extended to the HCC models described in this Thesis work. For instance, depletion of intracellular nicotinamide adenine dinucleotide (NAD) has recently been shown to drive T cell dysfunction in aging and cancer, which can be observed in T cells expressing CD38—an ectoenzyme which blockade restores T cell function[197,198]. This marker is upregulated in T cells accumulating in end-stage *Nras*^{G12V}/*Pten*^{KO} HCC, highlighting an underexplored therapeutic venue to revive their function in MASH-HCC. Interestingly, excessive use of cholesterol by CD36^{+} T cells has also been linked to ferroptosis and impaired anti-tumour capacity. This is also relevant for lipid-enriched *Nras*^{G12V}/*Pten*^{KO} tumours, although in this case, CD36 may be counterproductive if CD36^{+} macrophages are indeed important for driving anti-tumour response in MASH-HCC—as discussed in **Chapter 5**. Several markers linked to increased lipid metabolism are upregulated in dysfunctional CD8^{+} T cells accumulating in end-stage *Nras*^{G12V}/*Pten*^{KO} HCC, suggesting that its modulation could be fruitful to unleash their activity, as highlighted in recent studies[199,200]. If indeed T cell metabolic adaptations underlie their poor infiltration and hinder anti-tumour capacity in *Myc*^{OE}- and *Nras*^{G12D}-driven tumours, exactly which metabolic features are altered, and whether they can be therapeutically targeted for better anti-tumour response in these aggressive models remains to be explored.

As discussed for macrophages, one way to potentially support T cell function in the face of nutrient deprivation, metabolic maladaptation or suppressive metabolites, may be through metabolic reprogramming via modulation of metabolic signalling pathways. mTOR activation, often hyperactive in effector T cells, supports glycolysis and short-term function but can drive terminal differentiation and exhaustion[201]. Conversely, partial mTOR inhibition has been shown to promote the development of anti-viral memory-like T cells with superior persistence[202] that could translate into better anti-tumour capacity for tumour-specific T cells. In the context of excessive nutrient exposure—such as lipid-enriched *Nras*^{G12V}/*Pten*^{KO} tumours—the critical nutrient-sensing role of mTOR may therefore be an interesting pathway to modulate[203]. Another nutrient sensor, AMPK, is activated in T cells following TCR stimulation under low-glucose or hypoxic environments, and is necessary to support mitochondrial respiration, ATP production and fatty acid oxidation, suggesting AMPK activation as another mechanism to support anti-tumour T cell activity[204]. Similarly, PPAR-α is upregulated in CD8^{+} T cells under glucose- and oxygen-deprived conditions and can be pharmacologically activated to enhance fatty acid oxidation, preserve mitochondrial function, and bolster effector responses, thereby sustaining T cell activity within a nutrient-poor tumour microenvironment[205]. Whether these nutrient-deprived conditions are similar to those observed in the TME of our distinct HCC mouse models is currently unknown and warrants investigation. If applicable, these metabolic rewiring strategies may thus represent orthogonal therapeutic levers to boost T cell infiltration, persistence, and cytotoxic function in HCC. Targeting such pathways could be particularly relevant in *Myc*^{OE}- and *Nras*^{G12D}-driven tumours, where T cells fail to accumulate. Whether these interventions can reinvigorate T cells and synergize with immunotherapies in MASH-HCC will be relevant to investigate.

Overall, these observations underscore the potential of relieving extrinsic metabolic constraints and modulating intrinsic metabolic signalling pathways to reinvigorate T cell function in cancer. Whether the targeted blockade of immunosuppressive metabolites, restoration of mitochondrial fitness, or tuning of key metabolic sensors like mTOR, AMPK, and PPARs is a strategy that holds potential to metabolically reprogram dysfunctional T cells and restore their anti-tumour

capacity in HCC remains to be determined. Nonetheless, these approaches may pave the way for potentially better anti-tumour responses in T cell-enriched *Nras*^{G12V}/*Pten*^{KO} HCC and augmented infiltration and activation in T cell-poor *Myc*^{OE}- and *Nras*^{G12D}-driven counterparts, supporting the rationale for integrating metabolic interventions with immunotherapy in genetically distinct HCC subtypes.

Concluding remarks

This thesis highlights the complex interplay between oncogenic mutations, immune cell dynamics, and metabolic dysfunction in shaping the TME and cancer cell phenotype in HCC. Using genetically-distinct preclinical models, we demonstrated how distinct oncogenic drivers influence myeloid inflammation and invasion, lymphocyte exclusion and infiltration patterns, and metabolic rewiring—factors that collectively affect tumour progression and response to therapies in a cancer cell mutation-dependent manner. These findings underscore the need for immunotherapeutic strategies that are tailored to the specific immune evasion mechanisms, metabolic profiles, aetiologies and oncogenic signalling pathways of different HCC subtypes, particularly in the context of the growing prevalence of metabolic dysfunction in MASH-HCC.

While myeloid-targeting approaches, including TREM2 blockade, show potential in reshaping the immunosuppressive microenvironment, their efficacy is likely to be highly context- and time-dependent. A deeper understanding of macrophage heterogeneity and metabolic adaptations will be essential to refine these strategies, moving away from analyses that segregate macrophages into extreme phenotypes and instead focusing on the broad spectrum of polarization states they adopt in cancer[206]. Future research should integrate (spatial) multiomics analyses, preclinical ex vivo and in vivo models faithful to human disease, personalised immunotherapies and metabolic-modulating agents to develop more precise and effective treatments. Taken together, this work supports the need for combinatorial, patient-tailored approaches to improve outcomes in HCC, advancing beyond the limitations of current immunotherapy strategies.

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