Tailor-made: leveraging the tumor microenvironment to boost treatment for advanced liver cancer

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Chapter 1

General introduction and scope of the thesis

General introduction

Our understanding of cancer has dramatically improved over the centuries, evolving from early misconceptions to a clearer understanding of its causes, development, and overall impact on the body. For much of that time, cancer was primarily seen as the result of accumulating genetic and epigenetic changes due to aging or exposure to mutagenic agents such as cigarettes, ultraviolet radiation, and asbestos[1,2]. However, recent studies revealed that cancer-associated mutations are commonly present in "normal" tissues and benign conditions[3], where they remain non-malignant unless they bypass both cell-intrinsic and extrinsic tumor suppressive mechanisms[4]. Beyond intrinsic controls like cell-cycle checkpoints and programmed cell death, premalignant cells exist within a microenvironment that restrains their transformation[5,6]. Yet, despite its tumor-limiting role, the microenvironment can be reprogrammed to facilitate malignant conversion, support tumor initiation, and promote cancer progression[6]. In the liver, a powerful driver of milieu-mediated tumorigenic changes is chronic inflammation[7]. But how does chronic inflammation remodel the liver milieu, steering it from a healthy state to a cancerous one?

The liver, an immune-privileged organ, carries out a myriad of functions to maintain systemic homeostasis[8]. Among its roles, it regulates critical metabolic processes, including glucose and lipid metabolism[9]. To support these activities, the liver sinusoids are populated by specialized tissue-resident cells such as the liver macrophages-Kupffer cells (KCs), liver endothelial cells, and hepatic stellate cells[10]. In this dynamic environment, the liver is constantly exposed to a diverse array of self-antigens and foreign substances, necessitating a dual role in immune surveillance and the enforcement of a tolerogenic milieu. This balance is achieved through the suppression of adaptive T cell responses, ensuring immune tolerance while maintaining metabolic function[9,11].

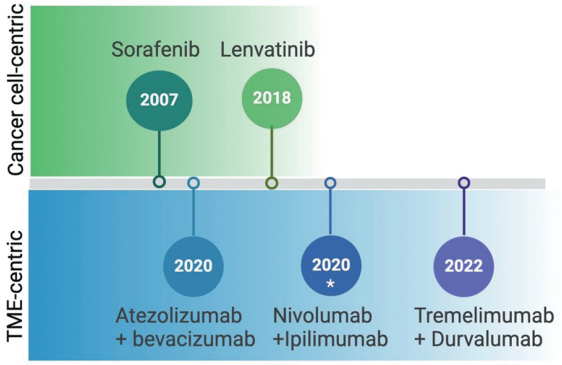
When these well-orchestrated regulatory processes are continuously disrupted by viral infections such as hepatitis B or C, along with metabolic imbalances caused by systemic conditions like obesity and type 2 diabetes, chronic inflammation ensues[12]. Defined by sustained immune activation and the infiltration of pro-inflammatory innate immune cells, including neutrophils and macrophages, chronic inflammation drives tissue damage and triggers persistent cycles of aberrant wound healing and repair, often leading to maladaptive tissue remodeling and functional impairment[13]. These inflammatory processes support the development of pathological conditions such as chronic liver injury, fibrosis, cirrhosis, and primary liver cancer. Notably, among the different cancer types that arise in the liver, such as intrahepatic cholangiocarcinoma, hepatoblastoma, hepatocellular carcinoma (HCC) is the predominant form of primary liver cancer[14].

Challenges in hepatocellular carcinoma: heterogeneity, therapy response and resistance

HCC is an increasingly growing global health problem, accounting for approximately 80% of liver cancer cases, and is often diagnosed at advanced stages[14]. This delayed diagnosis is largely attributed to insufficient screening practices within clinical settings, driven by complex socio-economic factors, suboptimal patient follow-up for individuals with co-morbidities such as obesity and substance abuse, and the diagnostic limitations imposed by cirrhosis and the lack of highly specific biomarkers[15-18]. Despite the steady decline in the proportion of virally-induced HCCs owing to the advancements in effective treatments of viral infections[19,20], there is a significant rise in HCC numbers annually primarily attributed to the emergence of metabolic dysfunction-associated steatohepatitis (MASH) cases in the western hemisphere[12]. To stratify these patients for optimal treatment management, the Barcelona Clinic Liver Cancer (BCLC) staging system was developed and is widely used to classify patients based on tumor size, number, and liver function[21].

While early and intermediate HCCs can be treated by surgical resection, transarterial chemoembolization (TACE), and transplantation based on the BCLC guidelines, advanced/unresectable HCCs have limited therapeutic options due to their persistent underlying chronic liver disease[21,22]. Despite the decade-long use of tyrosine kinase inhibitors (TKI) like sorafenib and the significant advancements in HCC management with the introduction of immunotherapy combinations that should help reinvigorate antitumoral T cell responses, only a small proportion of patients have benefitted from these treatments[23] (**Figure 1**). Furthermore, in MASH-induced HCC, T cell-centric immunotherapy not only failed to achieve tumor control but can also worsen tumor pathology, causing extensive tissue damage and immune surveillance hindrance due to abnormal T cell activation[24]. The variation in treatment response and therapy failure due to acquired drug resistance in liver cancer, stemming from diverse etiologies, is largely driven by significant intra- and inter-tumor heterogeneity. This includes the presence of “difficult-to-treat” driver mutations and a uniquely complex heterogeneous tumor microenvironment (TME), factors not accounted for in the BCLC staging system [25,26]. These challenges underscore the need for personalized therapeutic strategies for HCC patients, as the one-size-fits-all approach is ineffective.

Figure 1.1: Timeline of Food and Drug Administration (FDA)-approved treatments for HCC.



Treatments that received accelerated approval by the Food and Drug Administration (FDA) for unresectable HCCs.
*Nivolumab and Ipilimumab received approval for patients that were previously treated with sorafenib[27].

Myeloid cells as therapeutic targets in cancer treatment

The lack of broadly effective treatments has prompted the search for new strategies to overcome this challenge. One strategy, rooted in conventional cancer cell-centric approaches, aims to overcome resistance by combining pathway inhibitors that produce synergistic effects, inducing apoptotic cell death while also controlling tumor growth[28]. More original approaches, inspired by T-cell-centered immunotherapies, has prompted researchers to explore other cells that engage in crosstalk with cancer cells to enhance cancer treatment[29]. In the TME, immune cells, particularly myeloid cells, represent genetically stable targets that can be leveraged to boost therapeutic modalities[30].

Myeloid cells, such as macrophages, play crucial roles in regulating inflammation, fibrosis, and abnormal lipid retention, all of which are significantly influenced by TME-derived stimuli[31-33]. For example, KCs can differently respond to free fatty acids by adopting pro-inflammatory or anti-inflammatory states depending on the type of lipid they are exposed to, influencing liver conditions[34]. Moreover, monocyte-derived macrophages can take on a restorative phenotype upon hepatic injury and engage in crosstalk with stellate cells to drive tissue remodeling and wound-healing responses[35]. These functions, while context-dependent, are primarily coordinated to restore tissue homeostasis. However, as mentioned earlier, in chronic inflammatory conditions, their dysregulation generally contributes to cancer development.

Unfortunately, tumors often exploit macrophage functions to promote their own survival, by leveraging these cells to actively inhibit antitumoral T cell activity, promote cancer cell proliferation, metastasis, and angiogenesis[36-38]. As the immuno-oncology field aims to alleviate the

immunosuppressive burden that limits the effectiveness of treatments, reprogramming the TME emerges as a promising strategy to enhance durable antitumor responses.

Cancer cell-intrinsic and extrinsic features dictate myeloid cell function

As previously mentioned, one of the biggest challenges hindering therapeutic success is the intra- and inter-tumor heterogeneity observed in patients. Identifying optimal therapeutic targets that benefit the majority of patients is a mission that has proven more challenging than initially anticipated. This includes the search for myeloid-focused targets that could complement existing treatment strategies. Notably, single-cell RNA sequencing has uncovered a diverse spectrum of myeloid cell states, highlighting the functional heterogeneity among these subsets[39]. Additionally, therapeutic agents such as tyrosine kinase inhibitors (TKIs) that possess immunomodulatory properties can alter the TME in ways that can obstruct or enhance antitumor responses, adding to the multi-layered complexity of designing effective treatments[40-42].

Moreover, a multitude of other factors can influence the composition and functionality of myeloid cells. The multifaceted roles of myeloid cells are particularly evident in tumor-associated macrophages (TAMs), which are heavily influenced by their developmental origin, tissue, and microenvironmental niche, as well as the disease stage, as a result of their inherent plasticity.[43-45]. Further complicating matters, the genetic heterogeneity of tumors also plays a critical role in modulating TAM behavior within the TME, with the genetic landscape of cancer cells dictating their functional contributions to tumor progression[46,47].

Commonly mutated genes that drive the tumorigenic transformation of cancer cells substantially participate in the recruitment, activation, and immunosuppression of the immune system[48] as exemplified by mutant *Trp53* promoting the recruitment of monocytic subsets through overexpression of CCL2 in glioblastoma[49], a mechanism similarly observed in *Nras*^{G12V}-induced HCC, highlighting a convergent feature between these distinct genetic alterations and malignancies[32]. Moreover, it has been established that Myc and KRas^{G12D} synergize in accelerating tumor progression in lung cancer through the modulation of CCL9 and IL-23, promoting the recruitment of protumoral macrophages and simultaneously impeding the activation of the adaptive immune system, thereby obstructing effective antitumor immunity[50]. These findings highlight that reprogramming of the TME should be evaluated in a context-dependent manner.

In HCC and various cancer types, TAMs have been identified as the main contributors to the pro-tumorigenic microenvironment[51]. As a result, several strategies aimed at targeting TAMs are being explored in clinical trials[52]. This includes colony-stimulating factor 1 receptor (CSF-1R) that is under investigation as a single agent (NCT01316822)[52] in advanced / metastatic cancers or as a dual agent (NCT04301778)[52] in combination with chemotherapy, radio-embolization, or

immunotherapy for cholangiocarcinoma patients, but progress has been slow, and outcomes remain uncertain. Moreover, despite macrophage-targeting therapy achieving clinical approval for tenosynovial giant cell tumors in 2019[53], advancements in developing macrophage-specific treatments for liver cancer have remained unsuccessful.

While preclinical studies that employed macrophage targeting therapies have exhibited some potential, their translational applicability remains inconclusive. For instance, in transgenic glioma models, combining radiotherapy with continuous CSF-1R inhibition converged to reprogram TAM populations that delayed tumor relapse and enhanced overall survival benefit compared to standard-of-care[54]. However, whether the prolonged use of anti-CSF-1R can be used in liver cancer has raised concerns, as KCs' reliance on CSF-1 for their survival suggests that their depletion could precipitate liver damage[55,56].

Similarly, in head and neck preclinical cancer models, the administration of a fusion protein comprising anti-CSF-1R and IL-10 induced a substantial enhancement in antitumor efficacy. This effect was characterized by a robust increase in $CD8^+$ T cell recruitment and expansion, alongside a concomitant depletion of regulatory T cells and TAMs, thereby promoting a more favorable immune microenvironment[57]. Nevertheless, such experimental approaches remain speculative, as IL-10 is well-recognized for its pleiotropic effects[58]. Unlike the favorable outcomes observed in the head and neck cancer model, in the context of colorectal cancer-derived liver metastases, IL-10, by itself, attenuates the antitumor response through the promotion of PD-L1-expressing monocytic expansion, which, in turn, diminishes $CD8^+$ T cell infiltration[59].

The key takeaway from these trials is that the path to meaningful clinical outcomes for macrophage targeting is paved with challenges. While it is understood that factors such as the genetic profile of tumors influence TAM states, these trials often underutilize the potential of patient stratification, which likely plays a significant role in the observed therapeutic responses. Moreover, substantial efforts are still needed to uncover TME-mediated mechanisms that promote antitumoral responses before these strategies can be successfully translated into effective treatments for patients. These studies suggest that a potential strategy for controlling tumor outgrowth may involve targeting both intrinsic cancer cell mechanisms and extrinsic factors, such as macrophages, allowing for a dual targeting approach.

In this thesis, we aimed to investigate how distinct mutations within liver cancer cells differentially modulate both the local and systemic microenvironments. We hypothesized that this understanding could reveal unique, exploitable therapeutic opportunities that can be precisely tailored to the genetic landscape of HCC. To achieve this, suitable preclinical models were required, which we sought to implement for this disease.

HCC preclinical models: their advantages and disadvantages

Over the years, a range of preclinical tumor mouse models have been developed to study liver cancer. These include diet-induced, virus-induced, and chemically-induced, and xenograft models, as well as genetically engineered mouse models (GEMMs)[60-62]. These models provide valuable insights into some aspects of liver injury, inflammation, fibrosis, and the impact of various dietary factors and fasting patterns on the progression of MASH and the development of HCC. Nevertheless, they were limited in their ability to mirror the genetic and phenotypic diversity of human HCC, and the time needed for tumor formation and the quantity of tumors produced can vary significantly, potentially leading to inconsistencies in experimental results[63-68].

To delve into the intricately heterogeneous immune landscape of HCC and assess the therapeutic potential of myeloid cell immunomodulation, we adopted the hydrodynamic tail vein (HDTV) injection technique. This model allowed for the simultaneous investigation of oncogene overexpression and tumor suppressor gene inactivation through the combined application of the Sleeping Beauty transposon system and CRISPR/Cas9 technology. This versatile model has previously propelled *in vivo* mechanistic studies, providing critical insights into the influence of oncogene-induced senescence on the TME[32] and elucidating the molecular underpinnings of β-catenin-driven immune evasion and acquired resistance to anti-PD1 immunotherapy[46]. In contrast to other models, HDTV offers a non-viral, efficient, and rapid platform for hepatocyte-specific gene delivery in immunocompetent mouse cohorts with minimal latency periods[69]. This technique involves the rapid injection of a DNA solution, equivalent to 10% of the mouse's body weight, into the tail vein. The resulting hydrodynamic pressure transiently disrupts hepatocyte membrane integrity, significantly enhancing DNA uptake and subsequent gene expression. However, despite standardized vector dosing, transgene expression levels cannot be precisely controlled[70,71]. Additionally, tumor kinetics and penetrance are affected by variables such as mouse strain and gender, introducing an element of unpredictability to experimental outcomes[72]. With our overarching aim of elucidating the influence of cancer cell genetics on the TME and identifying actionable targets to reprogram protumoral myeloid cells, HDTV stood out due to its flexibility in manipulating genetic alterations. This adaptability enables the modeling of diverse genetic profiles and molecular subtypes representative of human HCC, making it particularly valuable for drug screening applications[72]. Consequently, this model is exceptionally suited to address the central research objectives of this thesis.

Scope of the thesis

Before investigating exploitable myeloid-cell-centric therapeutic targets tailored to the genetic landscape of HCC, I first sought to establish an in-depth understanding of the myeloid-driven mechanisms supporting malignancy and their impact on the HCC TME dynamics.

In **Chapter 2**, we provide a comprehensive review of the role of myeloid cells, specifically infiltrating and resident monocytes/macrophages, across various liver states. We explore how these cells evolve within the immune landscape, transitioning from homeostasis to pathological conditions that drive tumorigenesis. We highlight their diverse compositions and functions, shaped by cancer risk factors, genetic drivers, and evolutionary dynamics, and underscore their contributions to both tumor progression and resistance to treatment. Moreover, we explore the breakthroughs and limitations of therapeutic strategies currently under clinical investigation that target both the intrinsic factors within tumor cells and the extrinsic influences of the TME. These insights highlighted the need for *in vivo* models that can effectively trace the co-evolution of myeloid cells and cancer cells. Such models are crucial for capturing the intricate genetic and microenvironmental interactions that fuel tumor progression, a challenge further explored in **Chapters 3** and **4.**

Building on this framework, **Chapter 3** discusses the methods we use to study the development and characterization of four innovative HCC models in **Chapter 4**, designed to faithfully mimic distinct histopathological and molecular features of human HCC subclasses. These models incorporate the heterogeneity of liver cancer and provide a versatile platform for dissecting the immune landscape and evaluating therapeutic interventions. In addition to model generation, we detail the implementation of advanced tools, including MRI-based tumor monitoring, optimized tissue preparation, dissociation, and preservation protocols, comprehensive flow cytometry panels for immune profiling, and image-analysis pipelines. Together, these methodologies offer the research community with the knowledge and skills required to expand the repertoire of genetically-distinct mouse models and the analytical tools for unraveling the complex interactions between cancer cell genetics and immune cell dynamics during HCC progression.

Chapter 4 provides an in-depth analysis of the genotype-specific and stage-dependent remodeling of the TME across four genetically distinct HCC models, illustrating how diverse oncogenic pathways distinctly influence immune and stromal reprogramming. Remarkably, despite comparable tumor growth kinetics and histopathological features, *Myc*-driven tumors revealed divergent patterns of immune education when this genetic overexpression was combined with different tumor suppressor losses of *p53* or *Pten*. While both models displayed a predominance of the myeloid compartment over the lymphoid compartment, a feature often associated with Myc-overexpressing tumors[50], Myc^{OE}/$Pten^{KO}$ tumors were predominantly characterized by an immunosuppressive myeloid signature, whereas Myc^{OE}/$Trp53^{KO}$ tumors exhibited an immunosuppressive stromal profile. These results highlight the nuanced interplay between oncogenic heterogeneity in the TME. The differences were even more striking within the *Nras*-driven models, which differed by just a single point mutation. The $Nras^{G12V}$/$Pten^{KO}$ model exhibited hallmark features of MASH-HCC, including pronounced T cell infiltration and a less aggressive phenotype compared to its counterparts. Conversely, $Nras^{G12D}$/$Pten^{KO}$-driven tumors presented as aggressive, fibrotic tumors dominated by a myeloid-centric immunophenotype. The results presented in **Chapter 4** underscored

the critical importance of examining the activation states linked to specific oncogenic signaling pathways in order to elucidate the molecular underpinnings of inter-patient heterogeneity. Mechanistically, we uncovered a critical driver of myeloid recruitment and polarization that could be exploited therapeutically in a cancer cell genetics-dependent manner. This approach has the potential to be effective not only in liver cancer but also across a range of other cancer types. These findings highlighted the translational potential of leveraging TME-informed therapeutic strategies to modulate the tumor milieu and improve treatment efficacy.

To contextualize these findings within the broader landscape of macrophage-targeting therapies, **Chapter 5** reviewed various strategies aimed at modulating macrophages across cancers in the brain, liver, and lung, organs enriched with resident macrophages. This chapter critically evaluated the successes and limitations of existing approaches, highlighting the potential of personalized macrophage-focused therapies integrated into combinatorial regimens to enhance treatment outcomes.

Finally, **Chapter 6** leveraged the developed models and insights acquired in **Chapter 3** and **4** to evaluate novel therapeutic combinations. Given the significant influence of oncogenic pathways on the TME, molecular therapies targeting these pathways could profoundly impact the shaping of the TME and its role in therapy response or resistance. In collaboration with the group of Prof. dr. René Bernards, we explore the effects of mTOR and SHP2 inhibitors on the immune landscape of *Myc*^{OE}/Trp53^{KO} HCC. Both mTOR and SHP2 signaling are well-established pathways with immunomodulatory outcomes[73,74], and their inhibition may have important implications for therapeutic efficacy, as demonstrated in other preclinical cancer models.

Together, the work presented in this thesis advances our understanding of how genetic alterations in HCC shape TME dynamics, with a particular focus on myeloid cells as mediators of tumor progression and therapeutic response. By integrating advanced models, methodologies, and therapeutic evaluations, this research provides a robust foundation for the development of tailored therapeutic approaches aimed at overcoming the challenges of tumor heterogeneity and immune resistance in HCC.

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