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The hepatic immunological pattern shaped by dominant-subdominant cellular interactions creates a collective function beyond the function of each cellular constituent to orchestrate progression of hepatocellular carcinoma

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The hepatic immunological pattern shaped by dominant-subdominant cellular interactions creates a collective function beyond the function of each cellular constituent to orchestrate progression of hepatocellular carcinoma

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

BMP: Bone morphogenetic protein

CAR T: Chimeric antigen receptor T cells

CCL: C-C motif chemokine

CCR: C-C motif chemokine receptor

CD: Cluster of differentiation

CSF: Colony stimulating factor

CTLA-4: Cytotoxic T lymphocyte-associated antigen 4

CXCL: C-X-C motif chemokine

DAMPs: Damage-associated molecular pattern molecules

DCs: Dendritic cells

DIAMOND: Diet-induced animal model of NAFLD

FLT3: FMS-like tyrosine kinase 3

FN1: Fibronectin-1

GITR: Glucocorticoid-induced tumor necrosis factor receptor

HCC: Hepatocellular carcinoma

ICI: Immune checkpoint inhibitors

IFN: Interferon

IGF: Insulin-like growth factor

IL: Interleukin

IPA: Ingenuity pathway analysis

KCs: Kupffer cells

LIGHT: Lymphotoxin inducible glycoprotein for herpes virus entry mediator receptor on T cells

MDSCs: Myeloid-derived suppressor cells

NAFLD: Nonalcoholic fatty liver disease

NASH: Nonalcoholic steatohepatitis

NK: Natural Killer

NKT: Natural Killer T cells

OSM: Oncostatin M

PAMPs: Pathogen-associated molecular pattern molecules

PARs: Protease-activated receptors

PCA: Principal component analysis

PD-1/PD-L1: Programmed cell death protein 1/programmed cell death protein ligand 1

PDGF: Platelet derived growth factor

RANKL: Receptor activator of nuclear factor kappa-B ligand

RORγt: RAR-related orphan receptor gamma

snRNA-seq: single nuclei RNA sequencing

STAT: signal transducer and activator of transcription

TAMs: Tumor-associated macrophages

TCR: T cell receptor

TGF-β: Transforming growth factor-beta

TLR: Toll-like receptors

TNF: Tumor necrosis factor

TSLP: Thymic stromal lymphopoietin

UMAP: Uniform manifold approximation and projection

VEGF: Vascular endothelial growth factor

VTN: Vitronectin

WD: Western Diet

Abstract

The hepatic immunological pattern shaped by dominant-subdominant cellular interactions creates a collective function beyond the function of each cellular constituent to orchestrate progression of hepatocellular carcinoma

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Abundance of data on the role of inflammatory immune responses in the progression or inhibition of hepatocellular carcinoma (HCC) has failed to offer a curative immunotherapy for HCC. This is largely because of taking reductionist approaches and missing the collective function of the hepatic immune system by focusing on specific immune cell types. Reductionistic approaches has enabled discovery of cellular components of the immune system, understanding the function of each immune cells, and developing targeted immunotherapies and vaccines for human diseases. This approach has also contributed to the development of theoretical models of immunity, such as the self-nonself/infectious nonself (SNS/INS) model and its revolutionary

versions, the danger model as well as the adaptation model of immunity. The strength of such targeted approaches lies in a focused and precise analysis of the individual components in isolation, yet missing the mutual interconnection and feedback loops between the various components which create a dynamically changing system, as well as failure to understand the emergent properties of the immune response as a system. Therefore, there is an urgent need to balance reductionism with a systems immunology approach for a comprehensive understanding of the immune function. A systems immunology approach suggests focusing on cellular interactions and analyzing the feedback loops between them, understanding the emergent properties or collective function of the immune responses, considering the immune cell interactions with hepatic structural cells, and lastly, adopting a holistic perspective that considers the immune responses as a collective function, rather than only focusing on the individual components. To this end, we propose that focusing on the dominant-subdominant patterns of the immune cells would allow understanding of the mechanism by which a collective immune function emerges. To identify the collective immune function through a systems immunology perspective, we performed high-throughput analysis of snRNAseq data collected from the liver of DIAMOND mice during the progression of nonalcoholic fatty liver disease (NAFLD) to HCC. Assessment of the immune and non-immune cell patterns throughout disease progression, along with the expression of molecules involved in intercellular signaling provides insight to pattern-specific functions of the hepatic immune system. We report that mutual signaling interactions of the hepatic immune cells in a dominant-subdominant manner, as well as their interaction with structural cells shape the immunological pattern manifesting a collective function beyond the function of the cellular constituents. Changes in the position of immune cell types from subdominant to dominant resulted in changes in their function by producing new ligands or

targeting different cells by the same ligands, in which innate immune cells play a major role in promoting fibrosis and the development of HCC. These data suggest that discovery of the immune pattern not only explains dynamic changes in immune cells during the course of disease, but also offers immune modulatory interventions for the treatment of NAFLD and HCC.

Introduction

NAFLD as a Chronic Inflammatory Disease that Leads to the Development of HCC

Hepatocellular carcinoma (HCC) is the third most common cause of death worldwide [1], in which its prevalence has been increasing in Western countries, as well as other parts of the world [2]. The rise in the number of HCC cases has been linked to the increasing incidence of obesity and the development of nonalcoholic fatty liver disease (NAFLD) [3,4]. NAFLD characterizes both nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH), which are induced through metabolic dysregulation by consuming high fat and high sugar diets, alongside microbiome dysbiosis [5]. Dietary sugar intake is a critical factor inducing hepatic metabolic syndrome, especially fructose, which can hinder the metabolism of glucose through JNK activation and its association with proteins involved in the development of insulin resistance, resulting in decreased phosphorylation of IRS-1/-2 [6], along with promoting the expression of inflammatory genes through NF-kB [7]. Further, adipose tissue derived factors released during fat accumulation like free fatty acids and TNF-α reinforce inflammatory signaling in conjunction with hepatic IL-1β [8]. Novel murine models such as diet-induced animal model of NAFLD (DIAMOND) mice have been established to recapitulate progression of NAFLD and NASH in humans by the consumption of high-fat and high-sugar diets in a longitudinal fashion over time [9].

The global prevalence of NAFLD is estimated to be approximately 25% in the general population, while NASH prevalence in NAFLD patients is estimated to occur in over 60% of the cases [10,11]. A characteristic feature of NASH is lipotoxic injury within the liver, which activates innate immune cells and subsequently adaptive immune cells, both of which are seen in disease progression towards cirrhosis and eventually HCC [12,13]. Patients with NASH have a higher

chance of developing HCC compared to those with NAFLD, although the latter is more prevalent in the general population [3,14]. Males have a greater susceptibility to developing NAFLD and HCC, while elevated risk is only seen in postmenopausal women [15,16]. This is also depicted by more frequent development of HCC in male NAFLD patients with non-cirrhotic livers, and females exhibiting better survival rates [17–20]. Nevertheless, the common driving event seen in liver disease is hepatic inflammation, which is considered as a major factor in the progression of NAFLD to HCC [21]. Acute inflammation is transient and typically induced by innate immune cells via pattern recognition receptors that establish feedforward loops of inflammatory responses during metabolic stress, which can become chronic over time and cause damage to hepatic tissues [22,23]. Such transient inflammation is required for the activation of the adaptive immune response as a defense mechanism to protect the host from tissue-damaging events.

It is well established that approximately 90% of HCC cases develop due to chronic inflammation in the liver, which is typically coupled with fibrotic and cirrhotic features during disease progression [14,24]. Chronic inflammation triggers carcinogenic events and induces transformation in hepatic cells [15,25]. Inflammation stems from necroinflammation via constitutive cell death and hepatic regeneration due to NASH-induced stresses and metabolic syndrome, alongside exposure to PAMPs and DAMPs that further contribute to hepatocarcinogenesis [24,26]. IL-6 and TNF signaling molecules induce hepatic inflammation during obesity to activate STAT3, in which continued consumption of a high-fat diet contributes to the generation of chronic inflammation through these cytokines and promotes HCC development [27]. Some studies have also implicated altered cytokine profiles in the progression of NASH to HCC, namely IL-1β

and IL-18 that activate hepatic inflammasomes [14,28]. However, the exact cell type and mechanisms attributable to causing NAFLD-driven HCC are yet to be discovered.

Dual Roles of the Hepatic Immune Responses during the Progression or Inhibition of NAFLD and HCC

Our classic understanding of the immunobiology of the liver is dominated by duality of immune functions, i.e., pro-inflammatory and anti-inflammatory immune responses, in attacking the target tissue or tolerating the tissue [29]. According to this model, the hepatic immune system under homeostatic conditions is typically tolerogenic and regulated by myeloid cells amidst constant exposure to environmental and dietary antigens delivered from the portal vein, which must be tolerated to maintain the ability to elicit immunosurveillance functions, but this balance can quickly shift towards pro-inflammatory and become chronic through constitutive activation of innate immune cells via tissue damage, excess fat consumption, and tumor growth [30,31]. The liver has a robust innate immune cell population mainly comprised of Kupffer cells (KCs), NK and NKT cells, and both recruited circulating monocytes and tissue-resident macrophages that are implicated in NAFLD pathogenesis, partially through the binding of free fatty acids by TLRs that induce immune activation [32,33]. Among innate immune cells, the liver-resident macrophages or KCs were suggested to play an anti-inflammatory function, while monocyte-derived macrophages recruited into the liver derive inflammatory immune responses. To this end, KCs were reported to participate in clearance of cellular debris and metabolic waste [34], phagocytosis of red blood cells and recycling of iron [35,36], regulation of cholesterol homeostasis through the production of cholesteryl ester transfer protein, which is important for reducing high-density lipoprotein (HDL) and increasing low-density lipoprotein (LDL) [37], clearance of infection [38], and promotion of immune tolerance by activating Tregs [39]. A rapid loss of KCs occurs during NASH

and HCC [40], along with increased recruitment of monocyte-derived macrophages into the liver during NASH and HCC [41,42]. The latter is classified into pro-inflammatory M1 and anti-inflammatory or inflammation resolving M2 macrophages. Inflammatory M1 macrophages were reported to become dominant during NAFLD/NASH [43,44], showing both anti-tumor and tumor-promoting functions by inducing apoptosis in malignant cells and inducing carcinogenesis events through liver injury and NF-kB activation [45–48]. Anti-inflammatory M2 macrophages were also reported to attenuate liver injury and inhibit NAFLD and HCC by promoting tissue repair [49,50], as well as promoting tumor growth by producing IL-10 [51]. These reports challenge the concept that characterizes M1 inflammatory and M2 anti-inflammatory macrophages as anti- and pro-tumor immune responses, respectively, and thus, calls for revisiting our understanding of immunobiology of chronic liver diseases, NAFLD/NASH and HCC.

When it comes to an adaptive immune response, inflammatory CD8+ T cells, CD4+ Th1 cells, NK and NKT cells were suggested to have anti-tumor efficacy, and cancer immunotherapies are relied on promoting such immune responses. On the other hand, anti-inflammatory immune responses such as Th2 and Tregs were considered as pro-tumor cells by modulating inflammatory immune responses. Although some reports support this concept, many other reports suggest tumor-promoting function of the inflammatory immune responses. For instance, CD8+ T cells act as the primary effector cell eliciting antitumor immunity [52,53]. On the other hand, CD8+ T cells and NKT cells have also been implicated in promoting HCC development by their crosstalk with one another and interactions with hepatocytes through LIGHT signaling [54]. Some studies have even demonstrated a dual role for CD8+ T cell response in inducing liver injury and HCC [55]. On the other hand, some studies have suggested that CD4+ T cells could inhibit HCC [56,57], while another study demonstrated that restoration of CD4+ T

cells alone was unable to prevent HCC [58]. Various reports have suggested Th17 cells inhibit HCC [59,60], whereas others implicate Th17 cells promote liver injury and HCC [61,62]. In addition, Tregs typically act to inhibit effector T cells through secretion of the suppressive cytokines IL-10 and IL-35 in HCC [14,63], but other studies have shown Tregs to produce TNF-α and express features of Th17 cells like RORγt, CCR6, and IL-17 [64], and even detected enrichment of immune-inflammatory processes and lymphocyte regulation from Treg-related genes [65]. Also, IFN-γ producing Tregs were reported to manifest anti-tumor function [66]. Although, Th1 cells foster an inflammatory immune response that promotes CD8+ T cell activation and antitumor immunity [67], the balance between Th1 and Th2 cells and their cytokines influences HCC progression, whereas a dominance of Th2 cytokines like IL-4, IL-6, and IL-10 induce a suppressive environment favoring tumor growth [68,69].

Hepatic immune cells respond to metabolic stimuli during NAFLD progression that alters their functional phenotypes [70]. Fat and lipid accumulation directly induce hepatic inflammation when taken up by innate immune cell receptors, like macrophage scavenger receptor 1 [71]. Macrophages are a key cell type involved in NAFLD and NASH due to their activation by microbial-related factors, metabolites, lipids, and hepatocyte-derived DAMPs, which can be polarized to proinflammatory phenotypes during ongoing or chronic hepatic inflammation [72]. Recent advances in single cell sequencing technologies have generated large amounts of data suggesting the macrophage dichotomous classification into M1 or M2 is oversimplified [73], especially for classifying these cells as such in the tumor microenvironment [74,75]. Single cell sequencing methods have also characterized the plasticity of other immune cells within HCC by showing heterogenous and unique transcriptional states in T cells, macrophages, and dendritic cells, in which tumor-associated macrophages had a more complex phenotype than just M1 and

M2 [76]. This is also seen in HCC patients by macrophages not following the classical polarization pattern, and M1 macrophages that gained M2 traits maintained their antitumor capabilities [77], and the coexistence of M1 and M2 tumor-associated macrophages (TAMs) in the microenvironment identified similarities between the two that depends on the balance of inflammatory and suppressive factors, in which M2 TAMs produced TNF-α [78,79]. There is also a growing acknowledgment of unique transcriptional populations of CD8+ and CD4+ T cells via multidimensional flow cytometry and similar high throughput sequencing techniques [80,81], showing distinct subpopulations of CD4+ and CD8+ T cells that can be differentiated into functional classifications like cytotoxic, exhausted, effector, and even their clonality and lineages of development [82]. Further, in one study on glioblastoma, immune checkpoint therapies targeting GITR and PD-1 converted suppressive Tregs into effector-like phenotypes that produced IFN-γ and augmented antitumor immunity [83]. Analyses of human bladder cancer identified multiple unique functional expression states of Tregs and cytotoxic CD4+ T cells [84]. Thus, the hepatic immune system is beginning to be understood at a more comprehensive level, but this necessitates an understanding of the interactions between immune cells modulating the balance of inflammation. For example, the accumulation of macrophages during NAFLD diminishes antigen-specific immunity mediated by CD8+ T cells [85], along with MDSCs in HCC, which hinder antigen-specific T cell proliferation and modulate KCs expression of functional molecules like CCL2, IL-18, IL-1β, and IL-10, as well as components involved in antigen presentation [24,86]. MDSCs are canonically defined as potently immunosuppressive in many cancers, but there is also evidence showing their expression of inflammatory genes such as TNF-α and IL-1β [87,88]. The number of contradictory reports in the field makes understanding the balance of inflammation and the transition of NAFLD to HCC difficult to fully comprehend, thus

warranting further studies examining the hepatic immune system and its interacting components as a system with many axes of interaction via high throughput sequencing techniques, ensuring the plasticity of immune cells are not masked as they are in other methods.

Challenges in a curative immunotherapy of HCC

The anti-tumor function of the immune system was first noticed by reporting spontaneous regression of cancer after erysipelas infection, but attempts to repeat it failed [89]. Similar observations were made by William B. Coley, and he developed the first cancer immunotherapy in 1891 by injecting mixtures of live and inactivated pathogens, Streptococcus pyogenes and Serratia marcescens into patients' tumors [90]. He achieved successful regression of sarcoma, lymphoma, and testicular carcinoma, but because of lack of understanding of the mechanism of immune-mediated tumor regression and risk of infecting patients with pathogens, his immunotherapeutic approach was abandoned. With the discovery of T cells, NK cells and DCs, immunotherapies were focused on inducing immune responses by means of vaccines, cellular therapies or antibody therapies, resulting in prolonging patients' survival. To this end, the self-nonself (SNS) model of immunity has been guiding major immunotherapies by suggesting that expression of mutant peptides by malignant cells make them a good target for immunotherapy if we can overcome immune suppressant tumor microenvironment by immune checkpoint blockers [91] and make immune cells strong killers by means of CAR T or NK cells or engineered TCR for a specific tumor antigen. Nevertheless, a curative immunotherapy remains elusive. Immune checkpoint molecules are exploited to target co-inhibitory proteins such as PD-1, which are expressed on effector lymphocytes, to block their engagement with cognate ligands like PD-L1 on antigen presenting cells, stromal cells, and tumor cells, in order to prevent T cell inactivation and inhibition of effector cell functionality [92]. Similar principles apply to other immunotherapies

promoting T cell activation like the use of the Tremelimumab targeting CTLA-4, which is another immune checkpoint molecule that inhibits T cell activation by outcompeting the CD28 co-receptor for binding CD80 and CD86 that showed promising results in patients with advanced HCC [93–95]. Therapeutic regimens combining immunotherapies have been recognized as a necessary approach to achieve more effective immune responses against HCC [96], although therapies inducing T cell responses are promising, there is increasing evidence suggesting the inadequacy of these approaches in attaining beneficial outcomes [97].

Immune checkpoint inhibitors (ICI) were claimed to be a breakthrough for the treatment of cancers and immune-mediated diseases, but much work is still needed to ensure better patient end points, more rational combinatorial methods of treatment, and identification of comprehensive biomarkers [98,99]. Monoclonal antibody applications for ICI molecules such as PD-1/PD-L1 and CTLA-4 have been used to target and augment adaptive immune cell responses in HCC patients, however long-lasting responses are still lacking due to objective response rates only being approximately 15-20% [99–101]. Nivolumab, an anti-PD-1 immunotherapeutic, has been noted as a candidate option for a subset of patients with advanced HCC, although it did not improve overall survival rates [102]. Other reports have also highlighted the failure of anti-PD-1 treatment options to offer a cure for HCC and succumb to therapeutic resistance [25,103], as well as increasing the incidence of liver cancer by augmenting CD8+ T cell-induced injury and hepatic carcinogenesis [104]. Other monoclonal immunotherapies like Atezolizumab and Bevacizumab, which target PD-L1 and VEGF respectively, have been able to increase patient survival by a few months in those with advanced stage HCC, but many patients given both therapies discontinued treatment more frequently due to adverse events and side effects [105]. Sorafenib, a small molecule inhibitor of receptor tyrosine kinases such as VEGFR-1/-2/-3 and RET has not been effective in

modulating HCC development via inflammation-induced carcinogenesis and only offer prolonged survival as well [106], and this first-line treatment for advanced HCC commonly succumbs to resistance [107]. Similar to other studies conducted with receptor tyrosine kinase inhibitors Sorafenib and Lenvatinib, current treatment options only prolong patient survival and still do not offer a curative therapy for HCC [108,109].

Advancements in knowledge have been generated through the use of immune checkpoint inhibitory molecules and these methods have increased the number of treatment options for patients, but they cannot repair a debilitated immune system, nor tumor-mediated inactivation of immune cells [110]. Immunotherapies such as those targeting CTLA-4 and PD-1 are fixated on augmenting T cell responses and bypassing exhaustion, while a major challenge remaining is the common occurrence of adverse events such as cytokine release syndrome and neurotoxicity [111]. However, other novel treatments like CAR T cell therapies have been promising for hematological cancers, but they still are considered exploratory and need more work to be effective in solid tumors [112,113]. Some groups have even conceived ways to refine CAR T cell activity by combining immunotherapies to block the PD-1/PD-L1 signaling axis and utilizing PD-1 deficient CAR T cells [114], or CAR T cells that carry a PD-1 fusion molecule to engage PD-L1 expressed on HCC tumor cells [115]. However, CAR T cell therapies inherently display high risks of off-target toxicity through excess cytokine production, along with issues being able to target HCC due to the lack of specific antigens such as alpha fetoprotein [116–118]. Next generation sequencing methodologies like whole-exome and RNA-sequencing can identify and predict neoantigens in HCC that may be experimentally validated [119], in which neoantigen discovery in HCC patients through these methods can discover HLA binding molecules to prompt the induction of polyfunctional T cells to mount a response against tumor-specific or tumor-

associated antigens [120]. Strategies such as neoantigen identification and modifications of immunogenicity have been employed to increase the efficacy of neoantigen vaccines, but this approach remains dependent on antigen presenting cells being able to uptake the antigen and present it efficiently, along with the end result of the immune response mounted [119,121]. After all, antigenic profiles of malignant cells are in dynamic change which could result in tumor escape. Endogenous TCR editing can also improve biological activity, but this approach is susceptible to T cell exhaustion just like CAR T cell therapies because of the immunosuppressive properties within the tumor microenvironment, alongside issues trafficking to tumor sites [122,123]. Immunotherapeutic treatment options are thus increasing and becoming more encouraging in terms of extending patient survival, however, the lackluster effectivity implicates many of these so called "breakthroughs" are just stepwise incremental improvements without generating a cure for HCC.

Reductionist Approaches in the Understanding and Treatment of HCC and Future Perspectives

Biomedical research is dominated by reductionist approaches based on the assumption that a system can be understood by breaking it down into smaller components, and that the function of a system is nothing more than the sum of the function of its constituents interacting in a cause-effect direction. Therefore, mechanistic studies are focused on the discovery of a causative component for targeted therapy. To this end, inflammatory TNF, STAT3 activation, or defective autophagy were reported to be driving factors for HCC [124]. However, some other reports suggest a hepatoprotective role of STAT3 activation [125] or pro-tumor effects of autophagy [126]. Also, a single cell type or pathway, such as anti-CTLA4 immunotherapy [127], anti-PD1 immunotherapy combined with targeting FGFR4 [128], CAR T cell therapy [129], NK cell therapy [130], tyrosine kinase inhibitors [131], and a dual CCR2/CCR5 antagonist targeting monocyte and

lymphocyte recruitment in nonalcoholic steatohepatitis (NASH) [132] exemplify the downfall of these methods, as none of which are the sole driving mechanism of NAFLD or HCC, and thus cannot offer curative treatment to patients. Various suppressive immune cells have been implicated in the failure of PD-1/PD-L1 immune checkpoint therapies, such as peripheral Tregs upregulating checkpoint molecules during HCC, and higher levels of peripheral PD-L1+ MDSCs in HCC patients [63,133], but some reports indicate the presence of Tregs may be required for modulating hepatic inflammation via co-stimulatory molecule inhibition [134]. Although, depletion of suppressive cell types like Tregs, MDSCs, and PD-1+ exhausted T cells restored CD8+ T cell production of granzyme B in patients with advanced HCC [135], promising results from ICI therapies targeting CD8+ T cells in cancers still fall short, with technologies like RNA-seq generating an appreciation of unique transcriptional states of immune cells that are dynamically changing during tumorigenesis, and thereby manifest as dysfunctional immune responses [136].

One of the root issues in reductionistic approaches are the lack of dynamic assessments, seen by snapshot studies at a certain time point and only evaluating a single component or cell type out of many in an isolated fashion. We have recently reported that the pattern of inflammatory cytokines did change and fluctuate over the course of disease following distinct patterns in male and female mice [137]. Snapshot studies conducted with next-generation sequencing methods to characterize the HCC tumor microenvironment have exemplified this, as this manner of research could not sufficiently interrogate the development and evolution of HCC [138]. Live cell imaging can even identify dynamic features hidden in snapshot analyses, although snapshot studies with high throughput sequencing data extract an immense amount of information, phenotypic transitions and heterogeneity in cell populations occurs in temporal fashions [139]. To this end, even the timing of cytokine expression is critical in modulating

inflammatory diseases, seen by fluctuations in microbial species coinciding and influencing cytokine expression throughout tumor progression [140].

Systems Immunology: discovery and modulation of immunological patterns

Systems immunology is becoming increasingly feasible, and it can be applied to multiple immunologically relevant diseases like cancer and autoimmunity, in order to understand the complexity of immune cell interactions and develop novel treatment strategies [141,142]. System-level approaches in biological research have still only shown a fraction of their potential for elucidating disease pathogenesis mechanisms, but recent events have shown systems biology as able to characterize immune responses in COVID-19 patients and identifying potential target molecules implicated in disease progression [143,144]. Systems immunology has even begun to be employed for understanding the tumor microenvironment and devising immunotherapies [145], in addition to big data and system-level approaches in hepatology and gastroenterology to find biomarkers, promote drug discovery, and undertake molecular diagnostics [146]. Therefore, understanding the tumor immune microenvironment has become a reality with high throughput sequencing methodologies, hence why these approaches are needed to optimize treatment efficacy through understanding the cell types orchestrating immune responses [147].

The advent of novel "omics" technologies is prompting a resurgence in holistic research approaches, rather than reductionistic ones [148], in which the use of "multilayered omics" has already shown its capability to serve as a useful tool in evaluating metabolic and signaling networks in HCC patients, in order to stratify them into groups to receive the most effective treatment options for their specific tumor network [149]. Even advances in proteomics using mass spectrometry coupled with integrated technological approaches have shown the complexity of innate immune cell communication interactions and their mechanistic roles in processes like host

defense and tissue homeostasis [150]. There are an endless number of components that can be evaluated with a systems approach integrating various "omics" techniques, as well as evaluating aspects like microbiome dysbiosis. Modulation of the microbial species present can alter various metabolites produced and possibly serve as a preventative therapy for the development of NAFLD and HCC [151]. In addition, immune profiling of cancer patients receiving immunotherapies have even shown that beneficial microbial species are found in patients responding well to treatment by augmenting components of immune responses [152]. Therefore, systematic methods and considering alternatives to immunotherapy like microbiome modulation and immune pattern recognition could present a very novel manner of devising treatment options that could exceed current immunotherapies, or even be utilized together.

Recently, there have been multiple reports that the immunological pattern, rather than each immune cell alone, can better explain the immunobiology of NAFLD and HCC [44,58,153]. Pattern discovery approaches in the tumor microenvironment have identified diverse patterns of immune and non-immune cells in HCC patients, as well as implementation of the most suitable treatment option based on the characteristics of their microenvironment to optimize therapeutic responses [154]. In addition, machine learning methods are becoming increasingly available and optimized for prognosis and predicting therapeutic outcomes in HCC patients, with the major drawbacks being the lack of external validation and acceptance of a common predictive model [155,156]. Computational modeling has also been applied to other complex inflammatory diseases like multiple sclerosis [157], as well as its use to model and understand interactions between immune cells and cancer, where a key balance between inflammation and metabolic-immune responses are needed to maintain lower risk of cancer development [158]. Further, pattern discovery and computational modeling have already been utilized in the fields of neuroscience and physics

to understand highly complex and dynamic systems through top-down models, which show great potential for their application to understand patterns and dynamics within biological processes [159]. Pattern discovery and machine learning approaches in conjunction with novel computational methods available today need to stop being viewed as future perspectives, but rather implemented and integrated into current research efforts to facilitate our understanding of diseases as a system of interactions and patterns, in order to have a chance at devising a real cure.

Materials and Methods

Mice and specimens

Six snap-freeze liver samples collected from DIAMOND mice (Diet-induced animal model of non-alcoholic fatty liver disease; 129A1/SvIm and C57BL/6J cross mice) were subjected to single nuclei RNA sequencing (snRNAseq). All mice were males and stratified into three groups, each including two samples. The control group was put on a standard chow diet (CD) for 40 weeks (Ctrl), two experiment groups were put on a WD either for 40 weeks when they were yet to develop HCC; thus, being classified as the pre-tumor (Pre-T) group, and those who were on a WD for 48 weeks by the time they have developed tumors (Post-T). WD consists of a high-fat food diet, coupled with sugar water (23.1 g/L of Fructose and 18.9 g/L of Glucose adjusted to 1L in distilled water) to promote the diet-induced phenotype in a longitudinal fashion over time. We utilized these specific time frames on a WD based on a previous study that found male DIAMOND mice consistently developed liver tumors after 48 weeks [44]. Lastly, the snap-freeze liver samples underwent snRNAseq through the 10x Genomics Chromium system. Specifically, the Singulomics Corporation conducted library construction with Next GEM v3.1, then libraries were sequenced with approximately 200 million paired-end and 150 base-pair long reads per sample on an Illumina NovaSeq sequencer. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University on animal protocol number AD10001306. All methods were performed in accordance with the relevant guidelines and regulations.

ARRIVE Guidelines

These studies were also conducted in accordance with ARRIVE Guidelines, in which more detailed information can be found in the ARRIVE Essential 10 (Table 1). Previous reports demonstrated validity of deep sequencing results in small cohorts of even 2 mice per group by presenting aggregate data [160–162].

ARRIVE Essential 10		
Study design	1	All mice were male and stratified into three groups housed in different cages: those on a standard chow diet (CD) for 40 weeks served as a control, while the rest of the mice were fed a high-fat diet in a longitudinal fashion. Mice in the Pre-T group were on this diet for 40 weeks, while those in the Post-T group were on the diet for 48 weeks and developed HCC, as shown in the development of this DIAMOND mice model [44,153].
Sample size	2	The total number of DIAMOND mice used was six, with two mice stratified in each group (Ctrl n = 2, Pre-T n = 2, and Post-T n = 2) in order to capture cellular changes in the liver during HCC progression. Sample size was decided based on cost of sequencing services and our ability to perform a base level of statistical analyses.

Inclusion and exclusion criteria	3	No animals were excluded from this study, nor was an exclusion criterion established.
Randomization	4	No randomization occurred, as all mice were housed in cages on the same rack, and their cage location remained the same throughout the study.
Blinding	5	No blinding was performed, as knowledge of which cage the mice were in was required to provide them with the correct experimental diet and water (CD and regular water compared to high-fat diet and sugar water).
Outcome measures	6	Any behavioral changes in mice were monitored throughout the study. The outcome measure assessed was examination of the livers from our experimental groups of mice via snRNA-seq.
Statistical methods	7	DESeq2 processed data was filtered on a p-value < 0.01, followed by subsequent filtering in IPA of only molecules with a z-score > 2. Quantification of specific molecules of interest was performed in each replicate (n = 2 per group) in order to generate an average and standard error mean (SEM) for plotting. CellChat analyses only presented predicted signaling

		pathways deemed significant by the program (p-value < 0.05).
Experimental animals	8	DIAMOND mice (129A1/SvIm and C57BL/6J cross mice) were all males in this study and started on their respective diets at two months of age for 40-48 weeks.
Experimental procedures	9	No experimental procedures were conducted during the time on high-fat diet. At the end of the time course mice were humanely euthanized to snap-freeze resected livers and subjected to snRNA-seq.
Results	10	Analyses of snRNA-seq data assessed both replicates (n = 2 per group) pooled together for visualization of data in Seurat and cell type annotation. Replicates were separated in R for statistical measures during analysis with DESeq2 and quantification of cells expressing specific molecules of interest

Table 1. Experimental design. Detailed methods and procedures utilized for obtaining and analyzing in vivo experimentation in mice

Hematoxylin and eosin staining

Formalin-fixed paraffin-embedded liver (FFPE) liver tissues were subjected to hematoxylin and eosin (H&E) stain using Tissue Tek Prisma Autostainer.

Data Pre-processing, Quality Control, and Visualization

After sequencing, reads were aligned to the mm10 version of the *Mus Musculus* reference genome provided by 10X Genomics with CellRanger v6.1.1 to generate our single nuclei sequencing results. All the sample data was processed in the Seurat R package by first giving group identifying labels to each sample, which were all merged to undergo quality control and normalization by filtering on nFeature_RNA > 200 & nFeature_RNA < 5000 and the percentage of mitochondrial gene expression < 5% (percent.mt). Too few or too many features may be indicative of dead cells or multiple cells in a single run; while increasing amounts of mitochondrial associated genes corresponds with dying cells. Initial visualization through dimensional reduction was performed on all cells within sample groups by using marker genes from SingleR cell type annotations in principal component analysis (PCA), followed by uniform manifold approximation and projection (UMAP) (Figure 1B; arXiv:1802.03426). Graphical visualization of cell clusters was performed with both PCA (Figure 2B) and UMAP (Figure 2C-D) in Seurat, followed by functional interrogation of cell types through additional R packages. Also, the Viridis package was utilized for improving heatmap coloration when using Seurat's DoHeatmap function to assess any genes of interest [163]; along with the pheatmap package for the reference annotation probability score heatmap (Figure 1C).

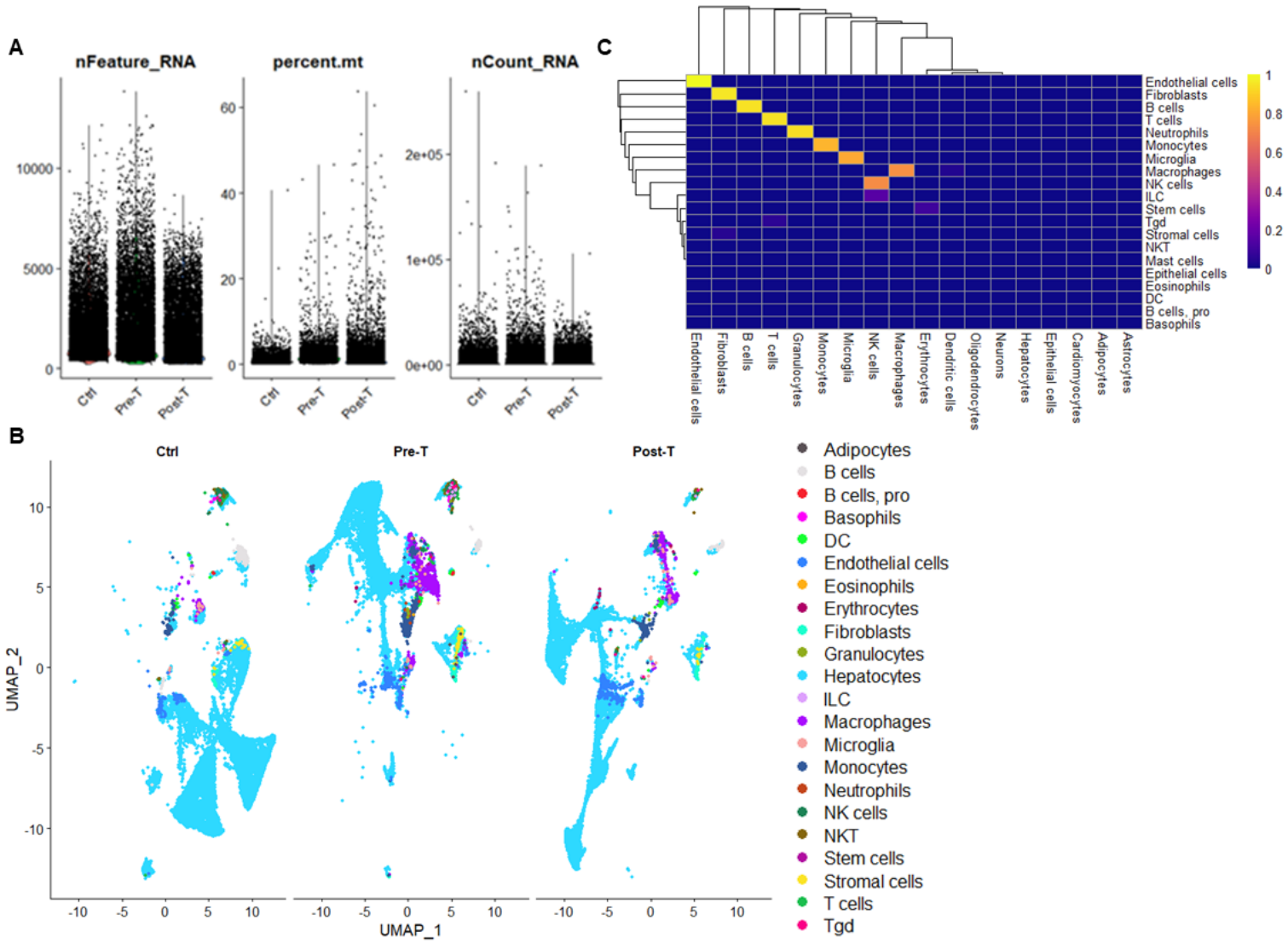


Figure 1. Normalization and cellular annotation of the liver: A) Quality control metrics employed to use only high-quality cells (cells were filtered on nFeature_RNA > 200 & nFeature_RNA < 5000 & percent.mt < 5. B) UMAP portraying cell type clusters in experimental sample groups separately after SingleR annotation of cell types. C) Heatmap portraying all matched cell type annotation probability scores based on the two murine reference databases (Immgen and mouse RNAseqdata) of annotated cells (probability score close to 1 indicates exact matches, whereas cell type annotations that are not shared between both databases were not compared).

Reference Database Cell Type Annotations and Cellular Subset Quantification

Cell typing was performed using a 2-pass method. First, we used SingleR [164], which enables access to reference databases of annotated cell types from a multitude of different experiments, in order

to annotate cell types globally by using both ImmGen and MouseRNAseqData databases. This was performed to ensure accuracy of cell type annotations, which was confirmed through matched probability scoring of annotated cells by both databases (Figure 1C). Only matched cell types with the same nomenclature were compared, as combining the use of both databases enabled us to identify cell types unique to one database or the other, such as Hepatocytes or NKT cells (http://bioconductor.org/books/release/SingleRBook/using-multiple-references.html). The ImmGen database contains 830 microarray samples focused on the classification of 20 main cell types and various subtypes of hematopoietic and immune cells, while the MouseRNAseq database contains 358 RNA-seq samples focused on annotating 28 specific cell types. All cell types annotated with both databases had the top 20 markers of each cell type recorded, so we could pass all gene markers excluding duplicates to PCA to optimize downstream visualization through dimensional reduction methods. All marker genes can be found in an Excel file listed in the Table 2 (SingleR Annotated Cell Markers xlsx file). Second, we used scSorter [165], which performs a "semi-supervised" machine learning approach, to further interrogate subsets of our singleR annotated cell types, such as T cells, Dendritic cells (DCs), B cells, Monocytes, and Macrophages. All cells were sorted based on known marker genes, along with non-marker genes using the scSorter R package, which employed a machine learning approach to differentiate the subsets of interest for downstream analyses. DCs were sorted into DC1, DC2, and pDC; Macrophages were sorted into M1, M2, M1-like, and Kupffer cells; Monocytes were sorted into monocytic-MDSCs (mMDSC) or remained classified as monocytes; and T cells were sorted into CD4 and CD8 subsets. The CD4 T cell subset was further interrogated by classifying helper T cell subsets (Th1, Th2, Th17, and Treg) with scSorter to assess their functional pattern in each sample group. We initially sorted CD4 and CD8 T cells with marker genes from "GeneList18", which recapitulated

patterns seen in previous works showing different ratios in the spleen compared with the liver [44], so we reported it in Figure 3D. However, we sorted CD4 and CD8 T cells differently with "GeneList14" in order to interrogate helper T cell subsets, as we needed to acquire a higher number of cells with the algorithm to sufficiently capture the pattern of helper T cells on WD. These specific sorting results were only included in the UMAP visualization on Figure 2D, demonstrating their pattern in Figure 3D. All sets of marker genes to sort cell type subsets can be found in an Excel file within Table 2 (IC Patterns scSorter genes and PMIDs xlsx file). All cells classified by SingleR annotations and scSorter were first quantified in R to understand the exact number of each cell type per sample group, and further recorded in Microsoft Excel for normalization and graphing. Any "Unknown" cell populations from the use of scSorter occur because they do not fit into any of the subsets of interest based on gene expression, so these were removed from analyses if there were few cells ($n<10$ in any of the three groups). If there were sufficient cells, they were interrogated for any specific differentially expressed genes that may implicate an identity. M1-like cells were classified based on their similar expression profile to M1 cells, especially their high expression of Itgad, a marker of pro-inflammatory macrophages retained in inflamed sites [166–168] (Figure 4). When sorting CD4+ Helper T cell subsets, we sorted differently with "GeneList18" markers to optimize the number of CD4+ T cells to sort out the helper T cells. This was performed to find the proportion of the pattern that helper T cells constitute on WD and to identify potential sources of signaling with the CellChat R package downstream. Notably, this group did have significantly more than 10 cells classified as unknown in one group (Ctrl: n = 98, Pre-T: n = 21, and Post-T: n = 9). However, because the Post-T had less than 10 cells and differential expression analysis in Seurat did not reveal any distinguishing genes compared with other helper T cell subsets, these cells were removed to ensure only accurate annotations were included downstream.

All known classified cellular subsets were normalized to 100% based on the total cells within that cell type category excluding any "Unknown".

Differential Gene Expression

Differential gene expression analysis was performed with DESeq2 [169] to identify differentially expressed genes across sample groups to compare expression signatures by uploading data containing the calculated log fold change and statistics of group comparisons to Ingenuity Pathway Analysis (IPA). Specifically, three groups were stratified to assess disease progression: first comparing Pre-T cells to those in the Ctrl (Ctrl vs. Pre-T), Post-T cells against those in the Ctrl (Ctrl vs. Post-T), and finally the Post-T group compared against the Pre-T (Pre-T vs. Post-T). A pooled or bulk immune cell analysis was performed by consolidating all immune cells into a single Seurat object within each experimental group to assess any differences we see in a collective immune signature, compared to the individual SingleR annotated cell populations we performed DESeq2 on alone. Certain cell types were combined to ensure sufficient counts were present for statistical comparison, such as NK and NKT cells (NK/NKT), along with the remaining innate immune cells such as Microglia (microglia-like), Basophils, Eosinophils, Granulocytes, Neutrophils, ILC, and Tγδ cells (other innate cells). Since we detected microglia-associated gene expression signatures in the liver, we used the term microglia-like cells for microglia. The pooled immune cell group had counts of greater than or equal to 10 when running DESeq2, whereas investigation of individual cell populations used a filter of greater than or equal to 3 to accommodate for sample size differences. Figure 8 took another pooled approach by merging all annotated and sorted cell type subsets to analyze the hepatic microenvironment in each group (includes: B1, B2, pro-B cells, CD8 T, Th1, Th17, Th2, Treg, NK, NKT, Tγδ, DC1, DC2, pDC,

m-MDSC, Monocytes, Kupffer, M1, M2, M1-like, Microglia (microglia-like), Basophils, Eosinophils, Granulocytes, Neutrophils, ILC, Fibroblasts, and Hepatocytes).

Identification of Intercellular Signaling Networks

The recently developed R package called CellChat [170] employs an online manually curated database based on KEGG and various published articles to generate a comparative framework for detecting intercellular communication across biological conditions. It characterizes and compares inferred signaling networks using a novel approach that analyzes through looking at social networks, pattern recognition, and manifold learning. This approach enables evaluation of all predicted signaling networks between specific cell types, while also identifying sources and targets of signaling interactions based on gene expression of the ligand-receptor pairs between cell populations in a sample group. We performed this analysis with just our SingleR annotated immune cells, along with the annotated non-immune cells of interest such as hepatocytes and structural cells (Figure 9). This was followed by a second perspective of analysis, in which only SingleR annotated immune cells were included to assess any immune-immune cell signaling interactions during disease progression (Figure 16). Cells were also filtered so only groups with more than 10 cells were assessed to ensure accurate representation of intercellular communication networks between cell types. Further, we performed multiple layers of this analysis with CellChat by adjusting the parameters in the "computeCommunProb" function to a "truncatedMean", in which we were able to examine the ligand-receptor pairs detected in a specific percentage of cells within each SingleR annotated cell type, such as 50%, 25% (Default "trimean" approach), 5%, and 2.5% to detect even lowly expressed interactions in immune cells.

Enrichment Analysis

Results from DESeq2 were uploaded to IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis), which enabled an in-depth interrogation of functional differences based on counts between cell populations of interest compared across sample groups. All cell type populations analyzed through DESeq2 were filtered on a p-value less than 0.01, and subsequently a z-score greater than 2 in the IPA analysis results. Enrichment was primarily assessed for terms such as cancer and disease related functions, signaling pathways, immune response, cytokines, microRNA (mir-RNA), and metabolism.

Quantification and Statistical Analysis

All cells (SingleR annotated and scSorter identified subsets) were quantified in R and recorded in Microsoft Excel, in order to normalize the data for immune and non-immune cells, respectively. Each group was treated as a single sample, as n=2 for each experimental group. However, all DESeq2 results were filtered in IPA to only assess genes with a p-value < 0.01, followed by subsequent filtering of IPA analysis results for detected functions and molecules with z-scores > 2. All cell types expressing specific genes of interest identified in our CellChat analyses (TNF-α, ADAM17, TNFR1, TNFR2, and double positive cells) were identified in Seurat and quantified to differentiate how TNF signaling is occurring in a more mechanistic fashion within these cell types (Figure 11). Further, extracting each set of predicted signaling interactions from our CellChat analyses enabled us to focus on signals stemming from structural cells in Figure 14, and the probability scores of those interactions with other cells in the liver were graphed for each signaling interaction to see which cell type was highly predicted to be receiving the signal.

Marker Genes Table	
SingleR cell type PCA genes	SingleR Annotated Cell Markers xlsx file
scSorter cell type subsets	IC Patterns scSorter genes and PMIDs xlsx file

Table 2. Marker Genes Table. SingleR Annotated Cell Markers contains the top 20 marker genes for each cell type based on the SingleR reference databases, in which all unique and duplicate genes were compiled to pass these genes to principal component analysis. IC Patterns scSorter genes and PMIDs contains marker genes for sorting cell type subsets out of the SingleR annotated population, in which the "GeneList# file for R" column shows the set of markers used for identifying subsets in each annotated cell type; along with PMIDs supporting the use of specific marker genes. All files can be found in the repositories listed in the data and code availability section.

Data and code availability

All code has been deposited on GitHub (https://github.com/koelschnj/Hepatic-Immune-Cell-Patterns-Code) and is publicly available on the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225381. The datasets generated and/or analyzed during the current study are available in the GEO repository, GSE225381.

Results

Progression of NAFLD and HCC are associated with remodeling of the hepatic structural cells, fibroblasts and endothelial cells, along with shifts from predominant T and B cells to macrophages and monocytes

In order to determine the hepatic immune regulation during progressive NAFLD and HCC, livers of DIAMOND mice, being on a regular Chow Diet (CD: Ctrl), as well as those from animals, being on a Western Diet (WD) during progressive NAFLD, either prior to the development of tumor (Pre-T) or after tumor development (Post-T) (Figure 2A), were subjected to single nuclei RNA sequencing (snRNAseq). First, we employed quality control metrics with Seurat to ensure only high-quality cells were used, followed by a dual reference database annotation of cell types through SingleR, in which matched probability scoring implicated accurate cell type annotation (see materials and methods section; Figure 1A-C). Marker genes from annotated cell types were passed to principal component analysis (PCA) to optimize visualization of immune and non-immune cells (Figure 2B), after which we visualized both compartments separately with UMAP (Figure 2C-D). In order to detect crosstalking networks or patterns of the hepatic cells and immune cells interacting with each other, the proportions of the hepatic and immune cell types were analyzed. Such analyses revealed a sustained predominance of hepatocytes comprising 95% of all the non-immune cells, and an increased frequency of fibroblasts during progressive NAFLD shifting it from subdominant to dominant compared with endothelial cells, and returning back to subdominant status during HCC (Figure 3A). Enrichment analysis with the Ingenuity Pathway Analysis (IPA) tool detected carcinogenesis processes as well as steatosis only in hepatocytes preceding the formation of HCC (Figure 3B). Quantifying the number of cells within the immune cell compartment detected a shift from predominant T and B cells (adaptive

immunity) to predominant macrophages and monocytes (innate immunity) (Figure 3C). Such a shift in the hepatic immunological pattern from T and B > macrophages and monocytes toward T and B < macrophages and monocytes was associated with alterations in the proportion of immune cell types. This included a reduced proportion of CD8+ T cells > CD4+ T cells and a shift from predominant Th2 to Th1 and then Th17 cells, as well as a shift from predominant Kupffer cells to M1 macrophages, predominant mMDSC, and increased ratio of NKT > NK cells (Figure 3D). Unknown cells within the macrophage population were classified as M1-like cells based on their similarity with M1 cells, notably their high expression of CD11d (Itgad) (Figure 4), which is expressed by M1 macrophages during chronic inflammation [166–168].

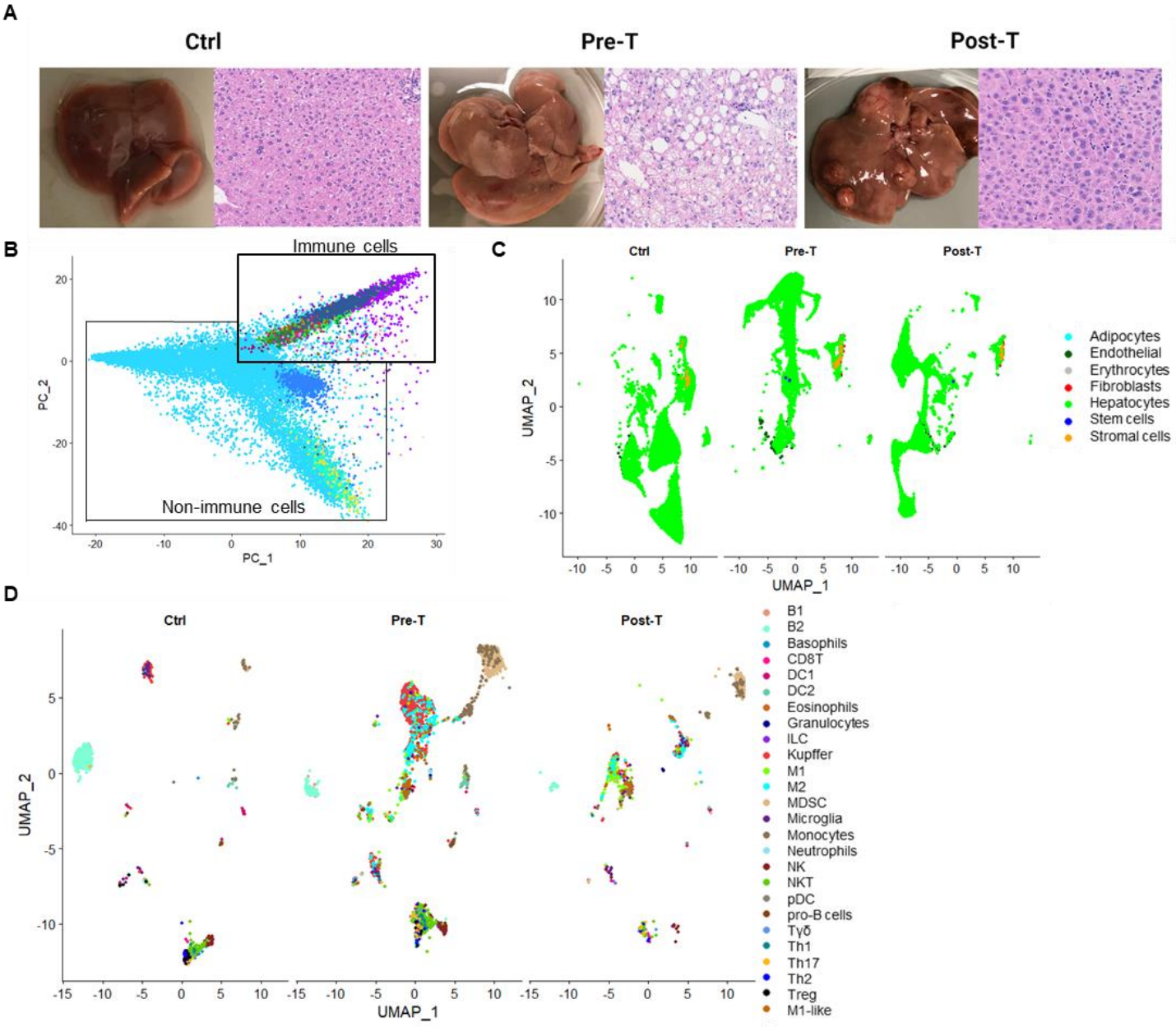


Figure 2. Visualization of the hepatic immune and non-immune cells: A) Livers as well as hematoxylin-Eosin staining of liver specimens collected from the control group (Ctrl) as well as from animals being on a WD prior to tumor development (Pre-T) or after the development of HCC (Post-T). IHC pictures were cropped from 20X images to visualize fatty liver as well as tumor cells clearly. B) SingleR annotated cell types after all cells were classified by the ImmGen and MouseRNAseq reference databases. After annotations were complete and compared, the top 20 marker genes of each cell type (immune and non-immune) were passed to PCA to optimize visualization, indicated by separate clustering of immune and non-immune cells due to the use of these genes. C-D) UMAP of pooled samples split to show group specific non-immune cells (C) and immune cells (D). All SingleR annotated cell types and scSorter identified subsets were included in data visualization.

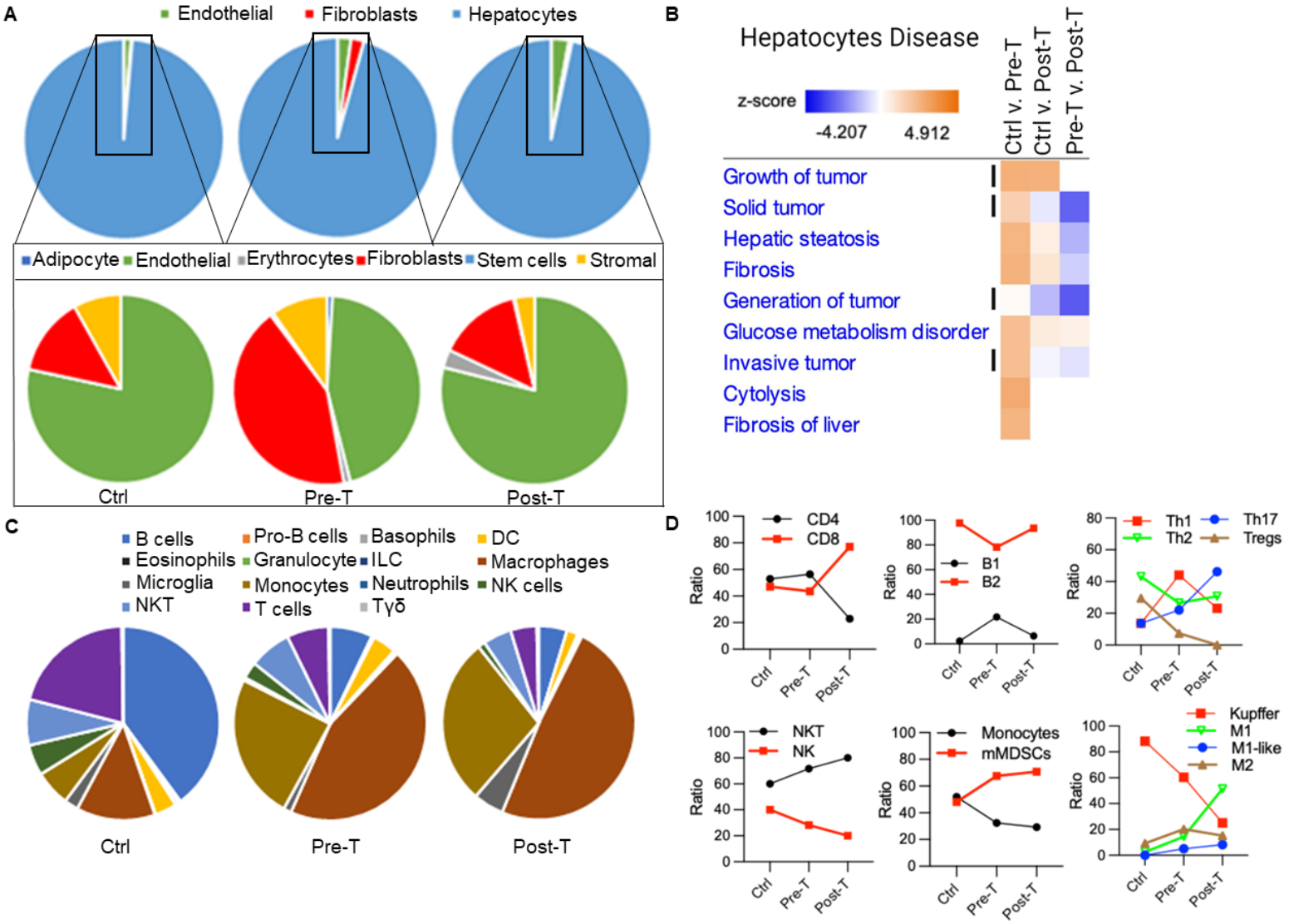


Figure 3. Quantitative assessment of hepatic immune and non-immune cell patterns: A) Pie graphs displaying non-immune cell components in each sample (hepatocytes accounted for over 95% of non-immune cells in each sample, while fibroblasts and endothelial cells primarily comprised the rest. B) IPA analysis of the hepatocyte population portraying disease-related functions and activation z-scores (blue and orange bar) based on DESeq2 results after filtering on a p-value < 0.01 and z-scores > 2. Carcinogenesis events are shown using vertical lines. C) Pie graphs showing the composition of immune cells annotated in each sample by ImmGen and MouseRNAseq databases accessed through SingleR. Panels A & C are based on the percentage of all cells in each compartment (innate and adaptive immune cells and non-immune cells) normalized in each for a total of 100%. D) Ratio of immune cell subsets within each population was identified using scSorter (D only includes cells classified as specific subsets of interest from scSorter to normalize each subset of cells to 100%, while removing any "Unknown" cells that were not classifiable to ensure accuracy).

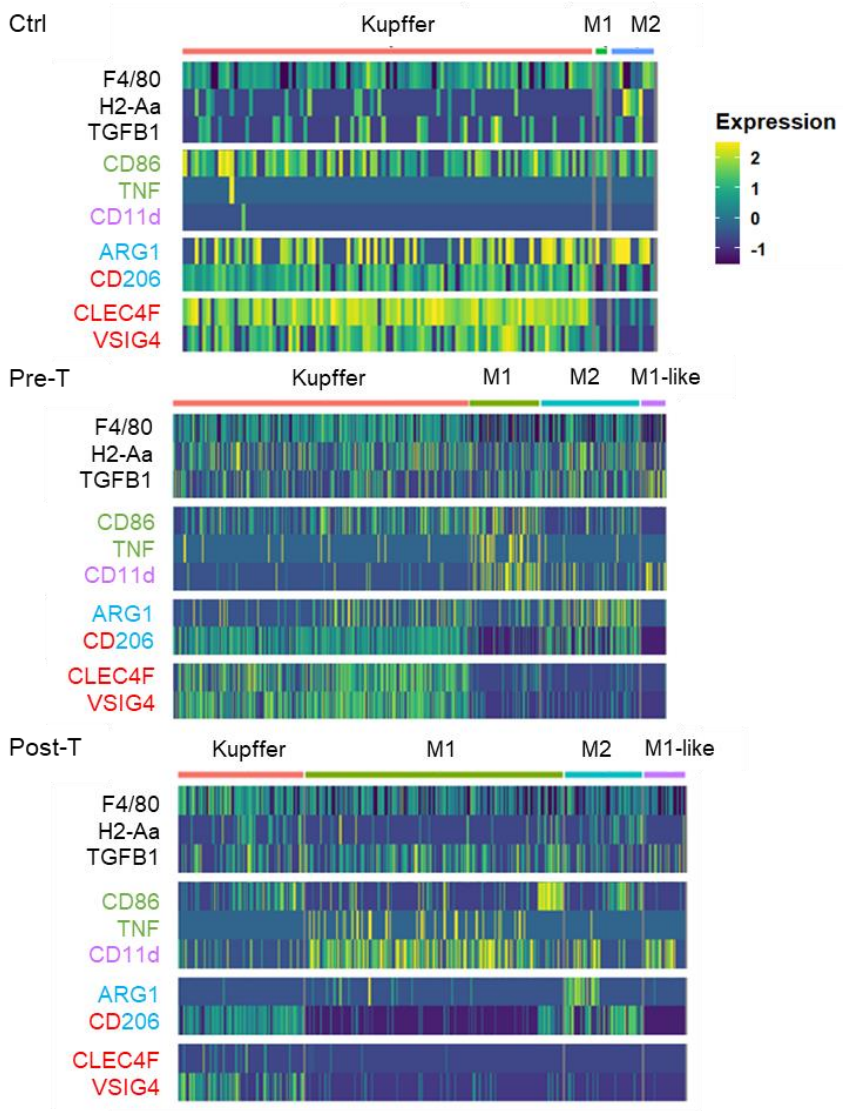


Figure 4. Macrophage functional profiles: Heatmaps showing the gene expression profile of major macrophage subsets (Kupffer cells, M1, M2, and M1-like) across groups (size of heatmap bands corresponds to the proportion of cells found with macrophage population by scSorter algorithm, where font color of genes on left of heatmaps corresponds to color above heatmaps and markers used to identify the subsets). All heatmaps in this figure were generated through the DoHeatmap function in Seurat version 4.3.0 (https://cran.r-project.org/web/packages/Seurat/index.html) and the viridis package version 0.6.2 for coloration (https://cran.r-project.org/web/packages/viridis/index.html).

The hepatic immunological pattern dominated by macrophages and monocytes creates a collective function that orchestrates the transition from tissue-protective to liver-damaging and tumor-promoting immunity

Results from differential gene expression analysis of all immune cells, and individual immune cell types were uploaded to IPA to determine whether the collective immune function is the sum of individual immune cell functions. As shown in Figure 5 and 6, the collective immune function (column 1) was beyond and independent from its cellular constituents (column 2-8). The collective immune function, but not the immune cell constituents, detected specific inflammatory cytokines being present or absent (Figure 5A, marked rows including IL1RN, TNFSF12, IFNL1, IFNB1, IL36A, EDN1, FASLG, IL37, CCL2, CXCL8, IL27, CCL20, IFN type I, CXCL3, CXCL2, SCGB1A1, C10orf99, IL7, TNFSF10, IL33, IL18, IL20). Also, it detected increased phagocytosis and antigen presentation, as well as cell death of lymphocytes associated with decreased quantity of T and B cells preceding the formation of HCC (Figure 5B, marked rows). Also, genes that affect the quantity of lymphocytes were found to be decreased while genes associated with cell death increased collectively, though they were not detectable for each immune cell type (Figure 5B, marked rows). Alterations in the collective function of the hepatic immune response were associated with significantly reduced cellular metabolism during the progression of NAFLD and HCC, which again, were detected only as collective immune function but not for each immune cell type (Figure 5C, marked rows). In particular, oxidative phosphorylation and glycolysis, which were reported to be associated with tumor immune surveillance [171], were found to increase prior to but not after tumor development only when collective functions of the immune cells were analyzed (Figure 5C, marked rows). Nevertheless, immune cell metabolism was collectively higher in the Post-T compared with that in the Pre-T

group (Figure 5C, marked rows), which was reflected by the dominance of M1 macrophages, as well as a higher ratio of mMDSCs to monocytes and shifts from predominant CD4+ T cells to CD8+ T cells (Figure 3D). Such alterations in the hepatic immune patterns increased their hepatotoxicity (Figure 6A), as well as their liver-damaging and tumor-promoting functions during the progression of NAFLD (Figure 6B, marked rows). The functional transformation of the hepatic immune response was further confirmed by the analysis of non-immune cells predicting carcinogenesis events only in hepatocytes (Figure 6C), as well as the pathways linked to mir-802 being associated with HCC [172,173] increased only in hepatocytes (Figure 7A). The predicted significance of mir-802 was detected through the pattern of gene expression changes interacting with mir-802 (Figure 7B).

In order to determine whether the tumor-promoting collective immune function preceding HCC in hepatocytes, we assessed the pooled SingleR annotated cell types and detected a similar set of events, such as highly upregulated hepatotoxicity and carcinogenesis preceding tumor development (Figure 8A-B, Figure 7C). These events were associated with inflammatory immune responses and cell death of immune cells altering the hepatic immune system towards tissue-damaging and tumor-promoting functions [174] (Figure 8C).

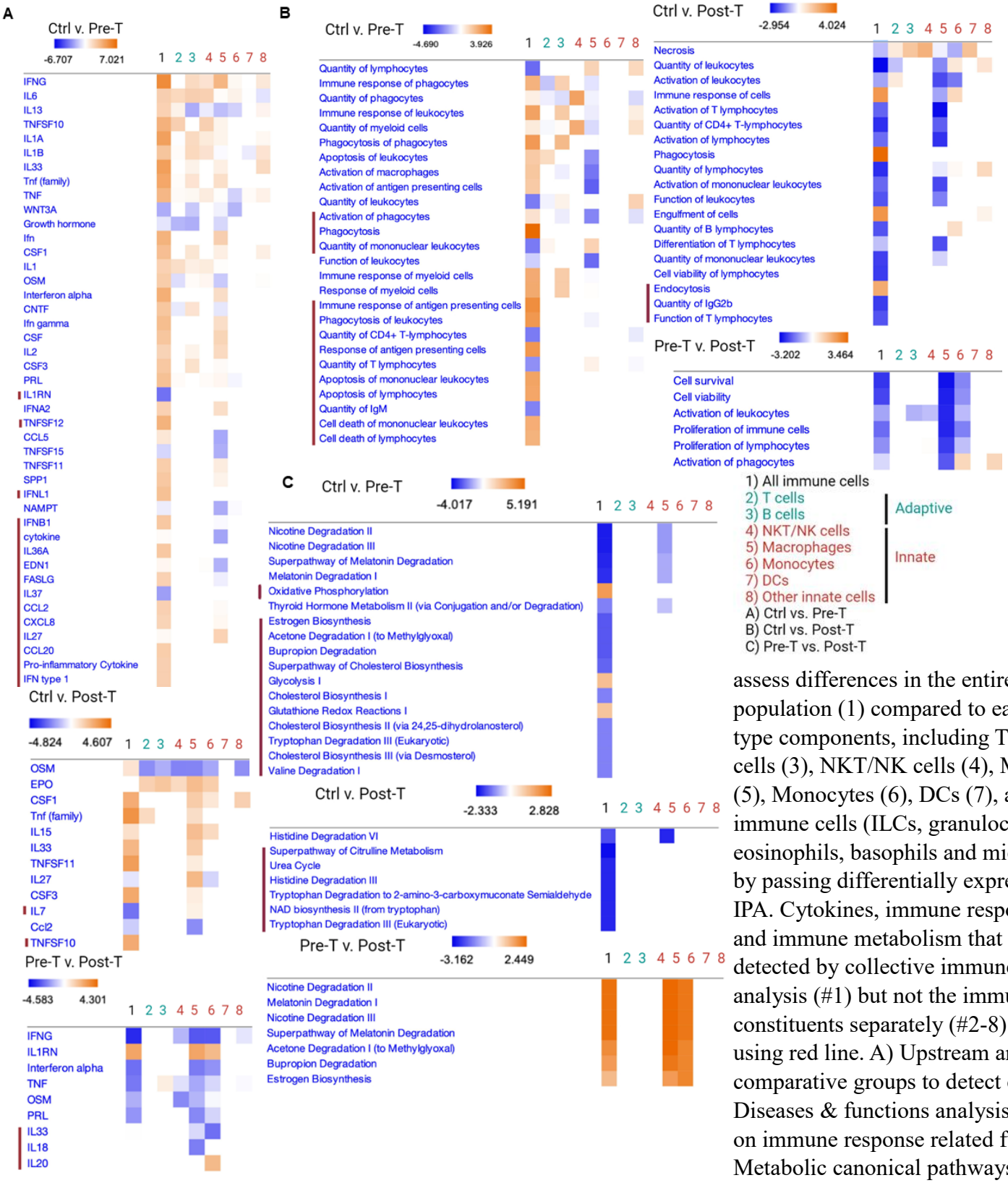


Figure 5. Shifts from dominant adaptive immunity to innate immunity alters the immune cells cytokine, immune response, and metabolic functions, while manifesting a collective function beyond its cellular constituents: DESeq2 was utilized to assess differences in the entire immune cell population (1) compared to each immune cell type components, including T cells (2), B cells (3), NKT/NK cells (4), Macrophages (5), Monocytes (6), DCs (7), and other immune cells (ILCs, granulocytes, eosinophils, basophils and microglia-like) (8) by passing differentially expressed genes to IPA. Cytokines, immune response pathways and immune metabolism that were only detected by collective immune function analysis (#1) but not the immune cell constituents separately (#2-8) are marked using red line. A) Upstream analysis of comparative groups to detect cytokines. B) Diseases & functions analysis was focused on immune response related functions. C) Metabolic canonical pathways were analyzed to detect immune cell metabolism across groups. Results from DESeq2 were filtered on a p-value < 0.01 and z-score > 2 in IPA for analysis. Carcinogenesis events are shown using vertical lines.

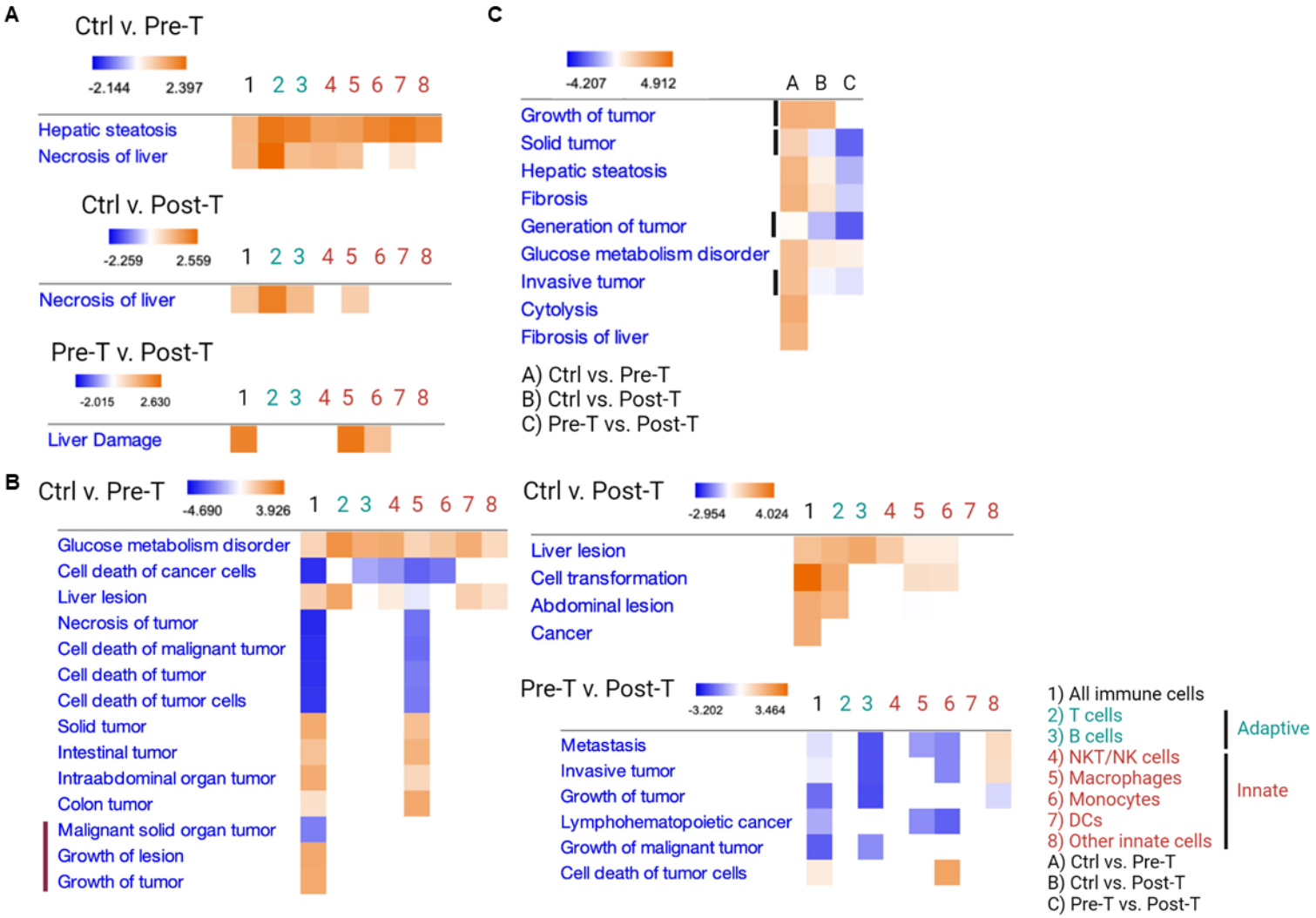


Figure 6. Shifts in the hepatic immunological pattern modulates the immune cells function and creates collective functions of the hepatic immune system: DESeq2 was utilized to assess differences in the entire immune cell population (1) compared to each immune cell type components, including T cells (2), B cells (3), NKT/NK cells (4), Macrophages (5), Monocytes (6), DCs (7), and other immune cells (ILCs, granulocytes, eosinophils, basophils and microglia-like) (8) by passing differentially expressed genes to IPA. Cytokines, immune response pathways and immune metabolism that were only detected by collective immune function analysis (#1) but not the immune cell constituents separately (#2-8) are marked using red line. A) Toxic functions were analyzed for the detection of hepatotoxicity related functions when comparing immune cells in each group. B) Diseases & functions analysis was focused on carcinogenesis events to detect tumor immunosurveillance functions in immune cells. C) Hepatocyte populations were subjected to diseases & functions analysis focused on carcinogenesis events; columns represent comparisons, in A) Ctrl vs. Pre-T, B) Ctrl vs. Post-T, and C) Pre-T vs. Post-T. Results from DESeq2 were filtered on a p-value < 0.01 and z-score > 2 in IPA for analysis. Carcinogenesis events are shown using vertical lines.

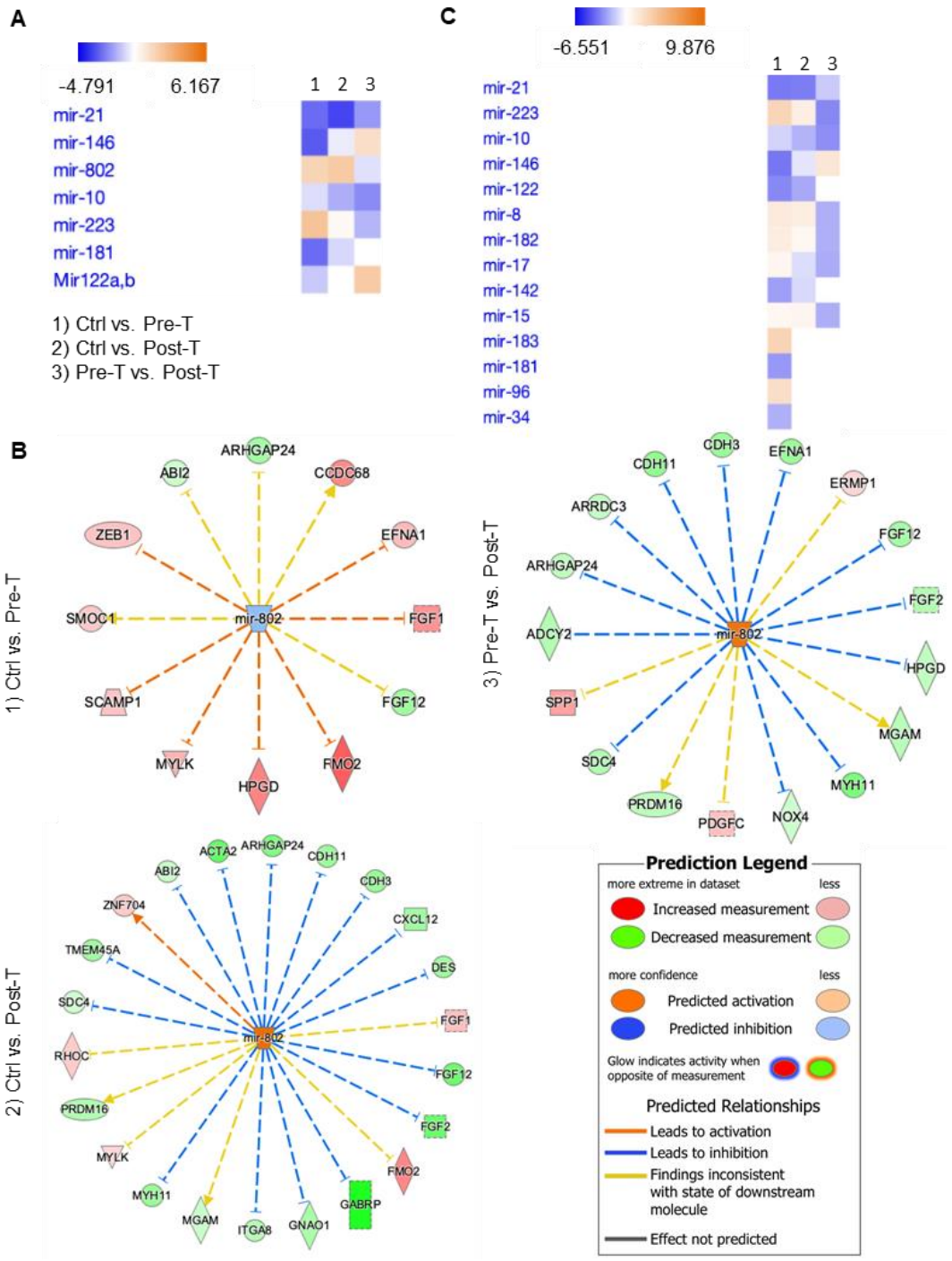


Figure 7. Detection of significantly changed micro-RNA expression in hepatic cells: A) Significantly upregulated or downregulated mir-RNA species detected in hepatocytes comparing three groups. B) Significantly changed pathways linked to mir-802 in hepatocytes. C) Significantly upregulated or downregulated mir-RNA detected in pooled SingleR annotated and scSorter identified subsets across groups.

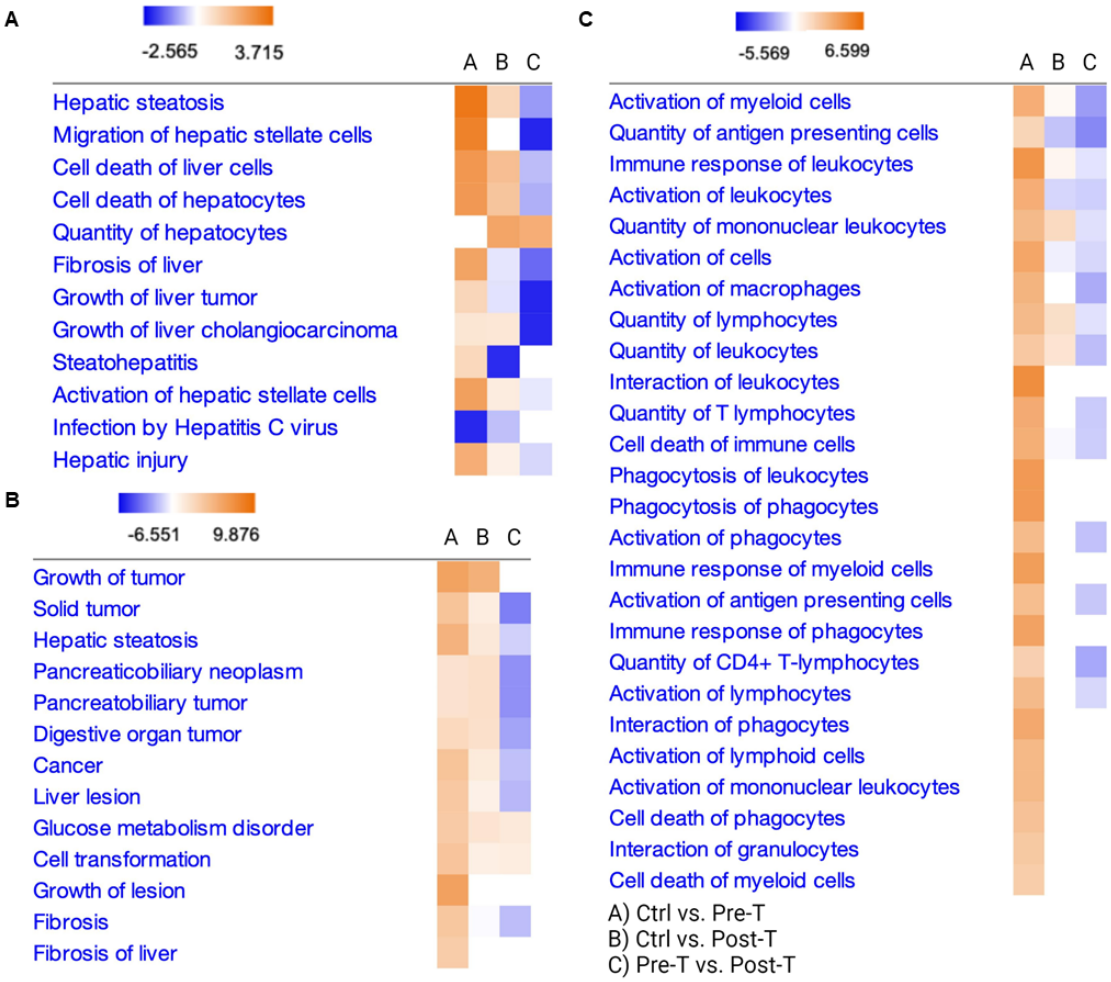


Figure 8. Collective immune function indicates increased inflammatory immune responses associated with liver damage and carcinogenesis events during a WD: All liver cells including the immune [B1, B2, pro-B cells, CD8+ T cells, CD4+ T cells (Th1, Th17, Th2, Treg), NK, NKT, Tγδ, DC1, DC2, pDC, MDSCs, Monocytes, Macrophages (Kupffer, M1, M2, M1-like), Microglia (microglia-like), Basophils, Eosinophils, Granulocytes, Neutrophils, ILCs] and non-immune cell population [Fibroblasts and Hepatocytes] and subsets from CellChat analysis containing SingleR annotated cell types and scSorter identified subsets were pooled to undergo DESeq2 and analysis in IPA. A) Toxic functions were analyzed for the detection of hepatotoxicity related functions in each group. B) Diseases & functions analysis focused on carcinogenesis events detected across groups. C) Diseases & functions analysis was focused on immune response related functions. Contrasts are as followed and noted in the bottom right legend: Ctrl vs. Pre-T uses Ctrl group as reference, and Pre-T as test; Ctrl vs. Post-T tests the Post-T group against the Ctrl; and Pre-T vs. Post-T compares the Post-T group to the Pre-T as its reference.

Structural cells and innate immune cells dominate the functional signaling network in the liver

Because of major shifts in the pattern of innate immune cells and structural cells during a WD (Figure 3), we sought to determine their contribution in the hepatic ligand-receptor signaling network compared with those of adaptive immune cells. First, we focused on the ligand-receptor pathways in which 50% of the cells within each cell type were involved. We found that in all cohorts, fibroblasts and hepatocytes appear to send the majority of signals while fibroblasts, endothelial cells, and macrophages dominated the incoming signals (Figure 9). In the Post-T group, monocytes also dominated the incoming signals (Figure 9). Some of these pathways such as FN1 and PARs remained active in all groups, but by targeting different cells in each group (Figure 10). Tenascin, CCL, BMP or CSF were uniquely involved in the Ctrl, Pre-T, and Post-T groups, respectively (Figure 10). IGF1 remained active in the Ctrl and Pre-T groups while VTN was active in the Ctrl and Post-T groups, yet, targeting different cells in each group (Figure 10). A default program analysis focusing on 25% of the cells within each cell type being involved in the hepatic signaling network revealed the appearance of adaptive immune cell (B and T cells) contributing in the signaling network, to a lesser extent than innate immune cells (Figure 11). Analysis of the ligand-receptor signaling interactions showed different functional signaling or receptor targeting of the same ligands in each group. For instance, TGF-β showed modulatory effects in the Ctrl group by promoting and inhibiting TGF-β signaling through Tgfbr1/Tgfbr2 and Acvr1b/Tgfbr2, respectively (Figure 12). In the Pre-T and Post-T groups, no TGF-β inhibitory signal (Acvr1b/Tgfbr2) [175] was detected (Figure 12). A strong inhibition of complement activation by B cells through CR2 interaction with C3 or C4b was evident in the Ctrl group, and it was switched to complement activation during a WD by the involvement of Itgam/Itgb2 and Itgax/Itgb2 receptor [176] (Figure 12). In the Ctrl group, IL-1β was produced by

macrophages and microglia-like targeting fibroblasts, microglia-like, NK cells and hepatocytes, while it shifted towards targeting only hepatocytes in the Pre-T group or hepatocytes and fibroblasts in the Post-T group (Figure 12).

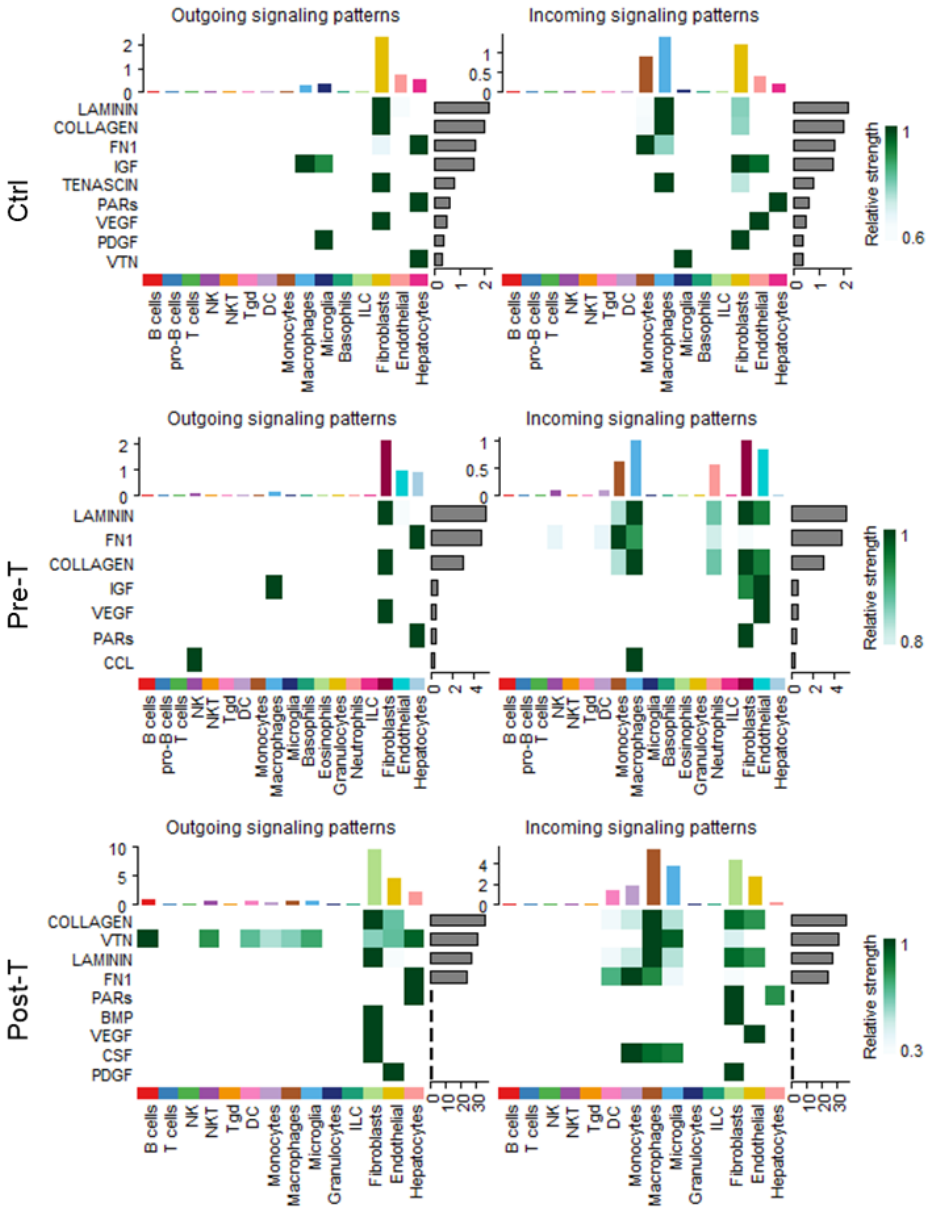


Figure 9. Structural cells and innate immune cells dominate functional signaling networks in the liver: Heatmaps portraying all signaling pathways found to be significant by the CellChat R package across SingleR annotated cell types, encompassing immune cells, structural cells, and hepatocytes. CellChat analysis parameters were adjusted to use a truncated mean of 50 % in order to detect pathways with ligand and receptor genes expressed in at least 50% of cells within annotated cell types, while only mapping significant pathways (p-value < 0.05) for each sample to show the cellular sources of signals (outgoing) and those receiving signals (incoming) based on the CellChat database of known ligand-receptor pairs in Ctrl (upper panel), Pre-T (Middle panel), and Post-T (lower panel). All figures were made through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat), and heatmaps in A and C used the dependent software ComplexHeatmap version 2.15.1 (https://github.com/jokergoo/ComplexHeatmap), while the chord diagrams in B and D used the dependent software circlize version 0.4.16 (https://github.com/jokergoo/circlize).

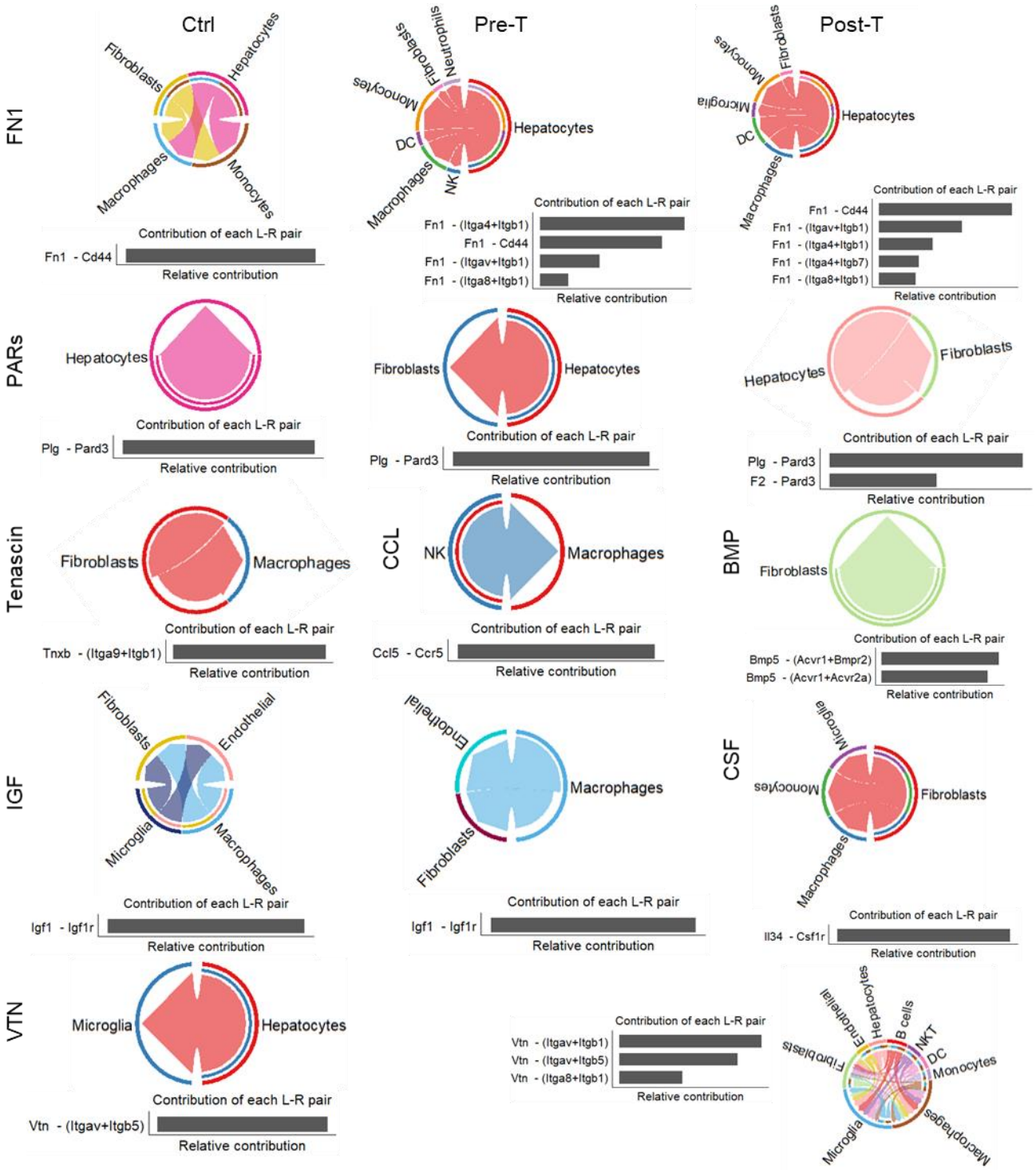


Figure 10. Differential signaling networks mainly stem from structural and innate immune cells: Chord diagrams of shared signaling pathways from the analysis using a truncated mean of 50% in all groups (FN1 and PARs), unique signaling pathway in each group (Tenascin, CCL, BMP, CSF) as well as shared signaling pathways in the Ctrl and Pre-T (IGF) or Ctrl and Post-T (VTN) groups. All figures were made through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat), and the chord diagrams used the dependent software circlize version 0.4.16 (https://github.com/jokergoo/circlize).

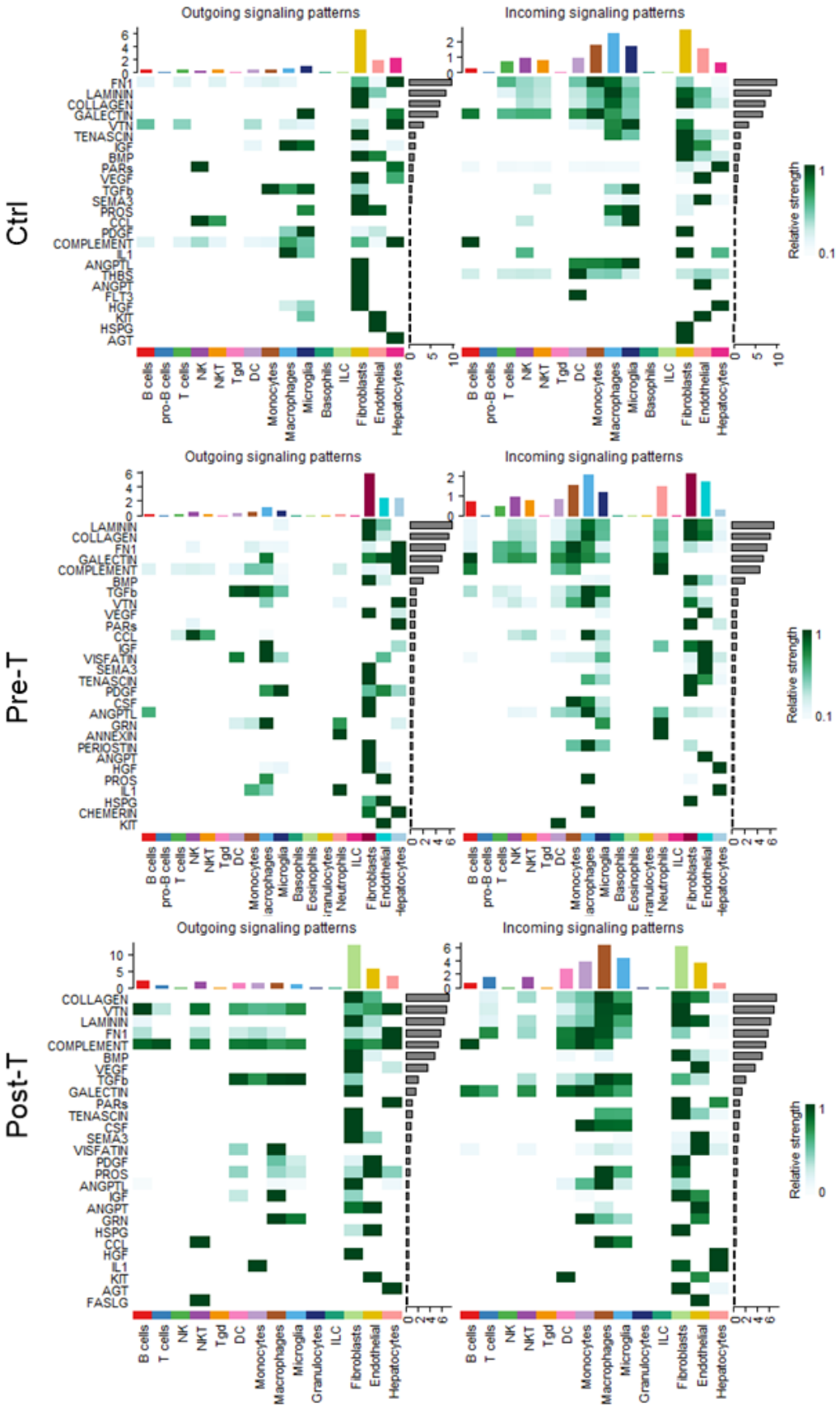


Figure 11. CellChat continues to detect structural cells and innate immune cells exerting dominance in the hepatic signaling networks: Heatmaps portraying all identified significant signaling pathways by CellChat using default parameters (25% of cells expressing genes in each cell type). Grouping shows Ctrl in the upper panel, Pre-T in the middle panel, and Post-T in the lower panel. All figures were made through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat), and heatmaps used the dependent software ComplexHeatmap version 2.15.1 (https://github.com/jokergoo/ComplexHeatmap).

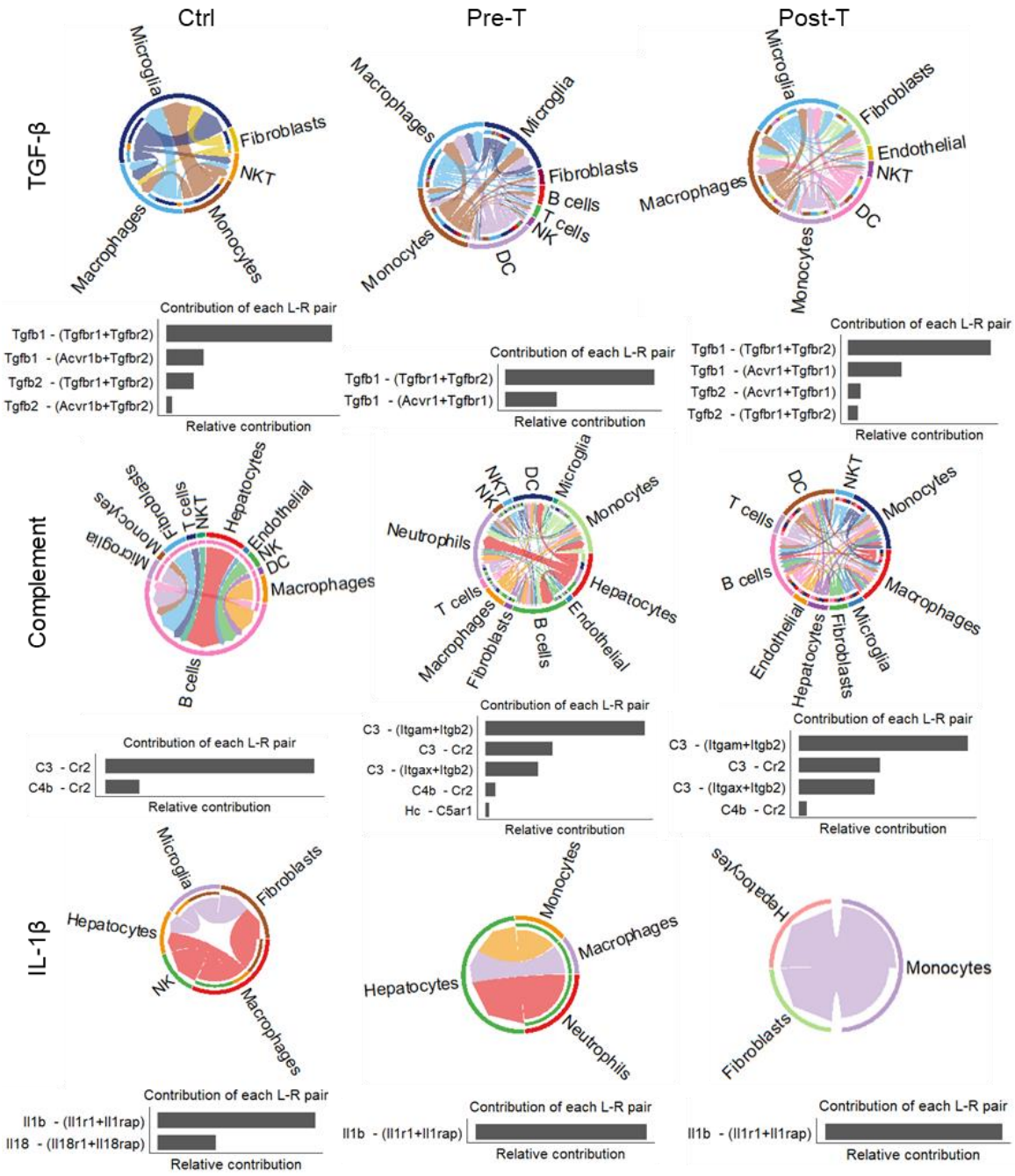


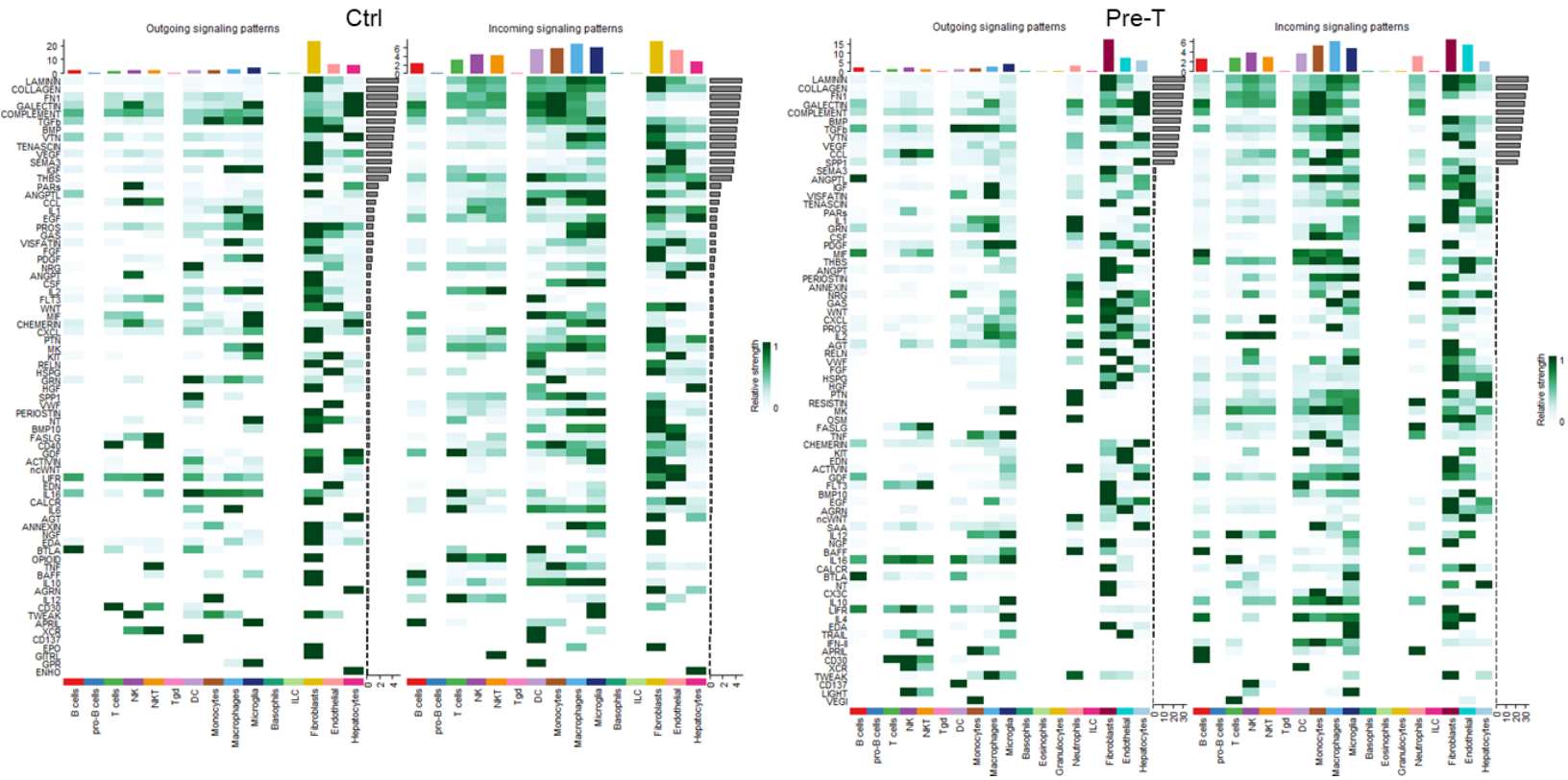
Figure 12. Shared functional signaling networks in the hepatic structural and innate immune cells: Chord diagrams showing three signaling pathways shared by all groups (Ctrl in the first column, Pre-T in the middle column, and Post-T in the right column) from the default analysis (25% of cells expressing genes in each cell type). All figures were made through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat), and the chord diagrams used the dependent software circlize version 0.4.16 (https://github.com/jokergoo/circlize).

The same immune cell types and cytokines manifest different functional signaling on immune cells and structural cells depending on the dominance of the innate or adaptive immune response

In order to determine whether distinct immunological pattern shaped by dominant-subdominant relationship of immune cell types may change functional signaling of the immune cells, all known ligand-receptor interactions were analyzed for each group by including even low frequency cells to cover for the majority of adaptive immune cells as well (Figure 13). Then, we focused on immune-related pathways, including those shared among three groups or unique to each group. For the shared pathways (Figure 14), all functional signaling molecules (TGF-β, TNF-α, IL1, IL2, IL10, IL12, IL16, FASLG) and regulatory molecules (CD137, BTLA, FLT3) were involved in immune cell-cell interaction, though manifesting different signaling patterns in each group. Only four pathways were similarly involved in interactions of immune cells with hepatocyte target cells in all groups (Figure 15: TGF-β, TNF-α, IL1, FASLG). Even these four cytokines were dominantly involved in immune cell interactions with one another and with structural cells compared with their interaction with hepatocyte target cells (Figure 15). The dominance of immune cell-cell interactions in all groups was clearly visualized when only immune cells were analyzed (Figure 16). Since TNF-α, which was detected in all groups, can manifest opposing functions depending on its receptors and being soluble or membranous (sTNF-α or mTNF-α), we looked at the expression of ADAM17 for increasing sTNF-α in TNF-α-producing cells [177] as well as TNFR1/TNFR2 expression [178] on target cells. We found that macrophages and monocytes were the main target of TNF-α with predominant expression of TNFR2 (Figure 17A). For the unique cytokines for each group identified in the heatmaps (Figure 13), IL6 (from macrophages and DCs) and GITRL (from fibroblasts) were detected in the Ctrl group with dominant T and B cells; OSM (from neutrophils), IL4 (from microglia-like), and

IFN-γ (from NKT cells) were detected in the Pre-T group; and RANKL (from T cells) was detected in the Post-T group during the dominance of macrophages and monocytes, interacting mainly with other immune cells as well as with structural cells and hepatocytes (Figure 17B).

Given the role of structural cells in regulating organ-specific immune responses [179,180], we found endothelial cells and fibroblasts affecting mainly the hepatic immune cells rather than hepatocytes (Figure 18). TGF-β and Flt3 affected mainly innate immune cells while homeostatic cytokines (IL-2, IL-7, IL-15) affected mainly NK or NKT cells, as well as T cells. Dominant ratio of fibroblasts over endothelial cells in the Pre-T group not only changed cytokine-mediated communication of fibroblasts with immune cells, but also resulted in the production of TSLP by fibroblasts affecting cells of the innate and adaptive immune system in the liver (Figure 18, bottom rows). IL-33 was engaged with DCs or monocytes in the Ctrl group, as well as with T cells and microglia-like in the Pre-T group, or only with T cells in the Post-T group. Restoration of endothelial cells dominance in the Post-T group, did not restore their functional signaling pattern compared with those in the Ctrl group (Figure 18). IL-1α was the only inflammatory cytokine from endothelial cells which mainly affected hepatocytes and structural cells in the liver.



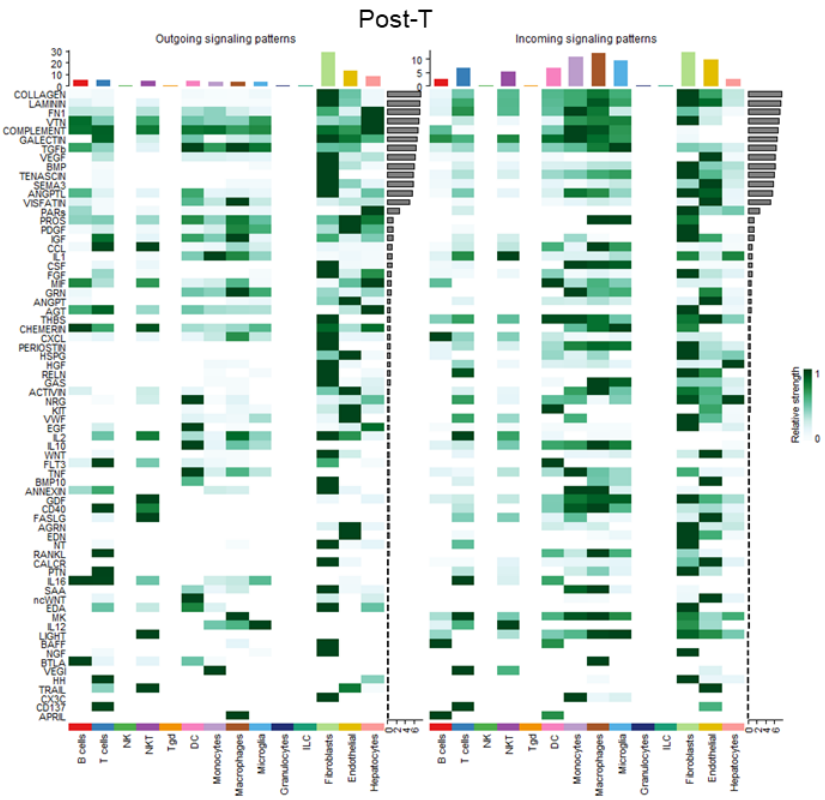


Figure 13. CellChat signaling analysis of hepatic cells at lower detection frequencies: CellChat analyzed cell type specific ligand-receptor signaling interactions in our SingleR annotated immune cells, structural cells, and hepatocytes. The analysis was conducted with truncated mean = 2.5% in order to detect even lowly expressed critical immunologically relevant signaling pathways. These heatmaps show all significantly detected signaling pathways in the Ctrl (upper panel), Pre-T (lower left panel), and Post-T (lower right panel).

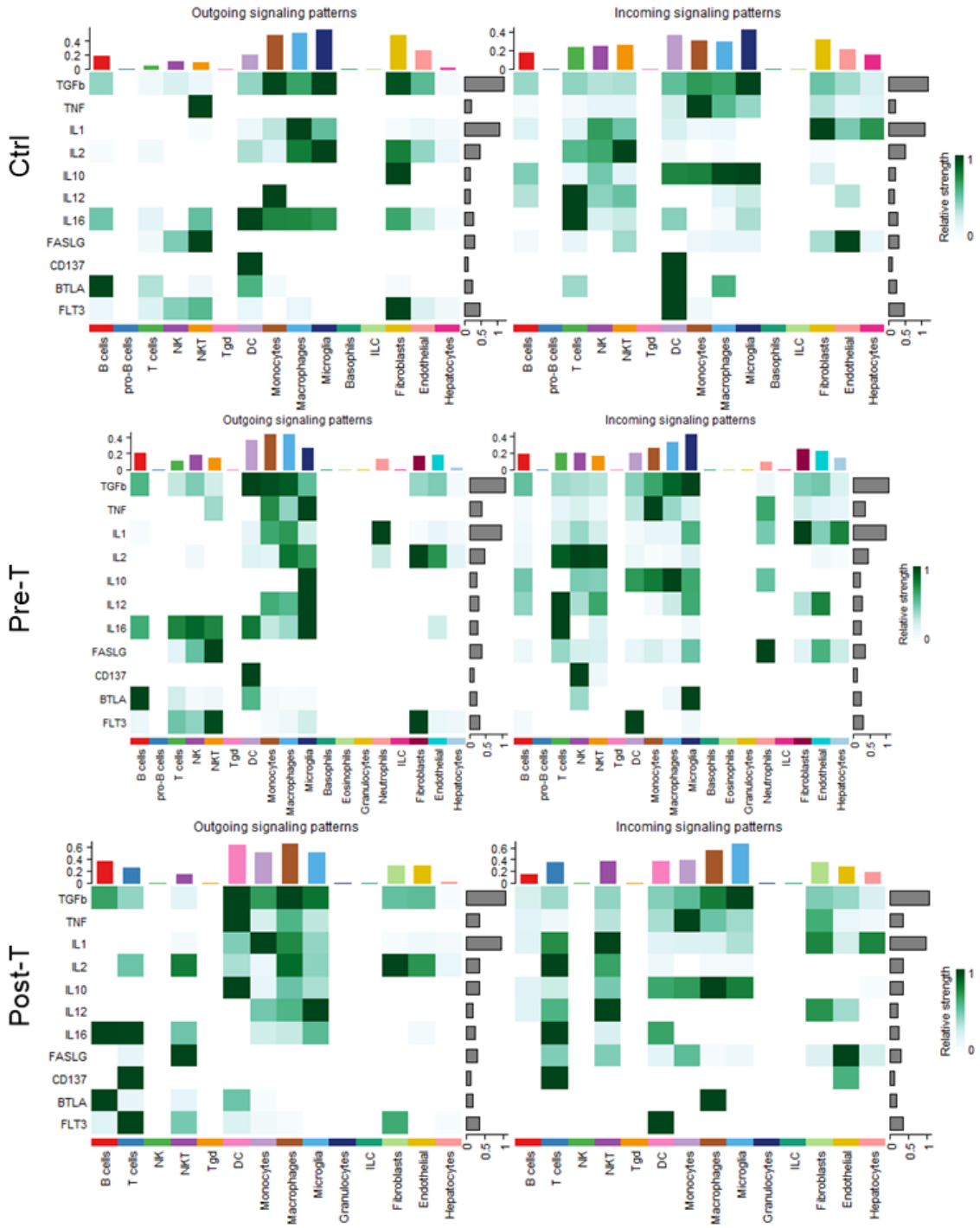


Figure 14. Functional signaling molecules present in all groups exhibit different patterns of interactions during HCC progression: Heatmaps portraying only selected immunologically relevant signaling and regulatory molecules identified by CellChat with analysis parameters adjusted to use a truncated mean of 2.5%, in order to detect functional signaling pathways in reduced numbers of adaptive immune cells during a WD. Groups are ordered from Ctrl (upper panel), Pre-T (middle panel), and Post-T (lower panel). Figures were made through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat) and heatmaps used the dependent software ComplexHeatmap version 2.15.1 (https://github.com/jokergoo/ComplexHeatmap.

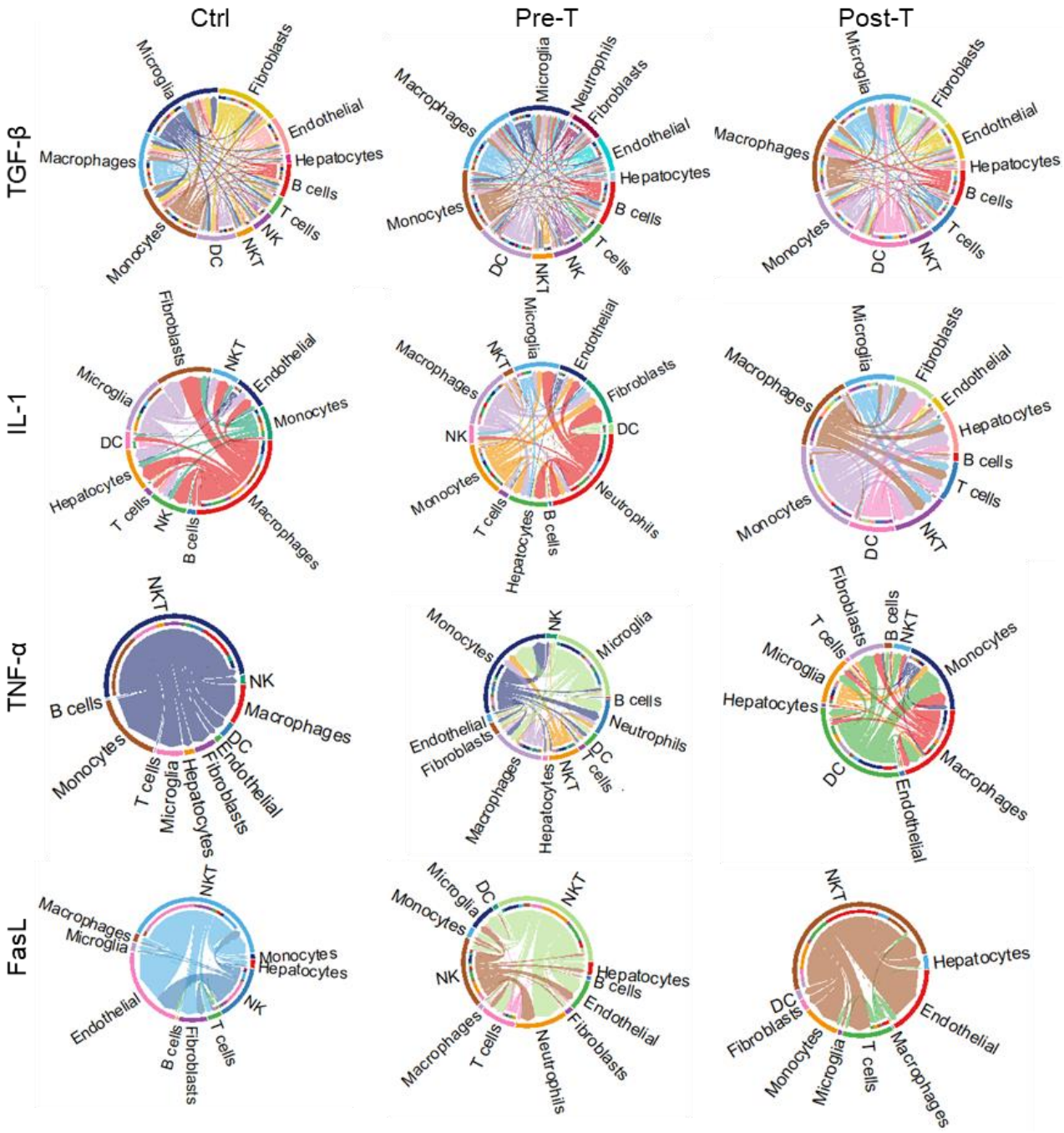


Figure 15. Chord diagrams of functional signaling molecules and their differentially expressed patterns of interactions during HCC progression: Chord diagrams showing the directionality of functional cytokine signaling targeting hepatocytes (TGF-β, IL-1, TNF-α, and FASL), as well as structural cells, and immune cells from the adjusted analysis parameters using a truncated mean of 2.5%%, in order to detect functional signaling pathways in reduced numbers of adaptive immune cells during a WD. Groups are ordered by Ctrl (left column), Pre-T (middle column), and Post-T (right column). Figures were made through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat) and the chord diagrams in used the dependent software circlize version 0.4.16 (https://github.com/jokergoo/circlize).

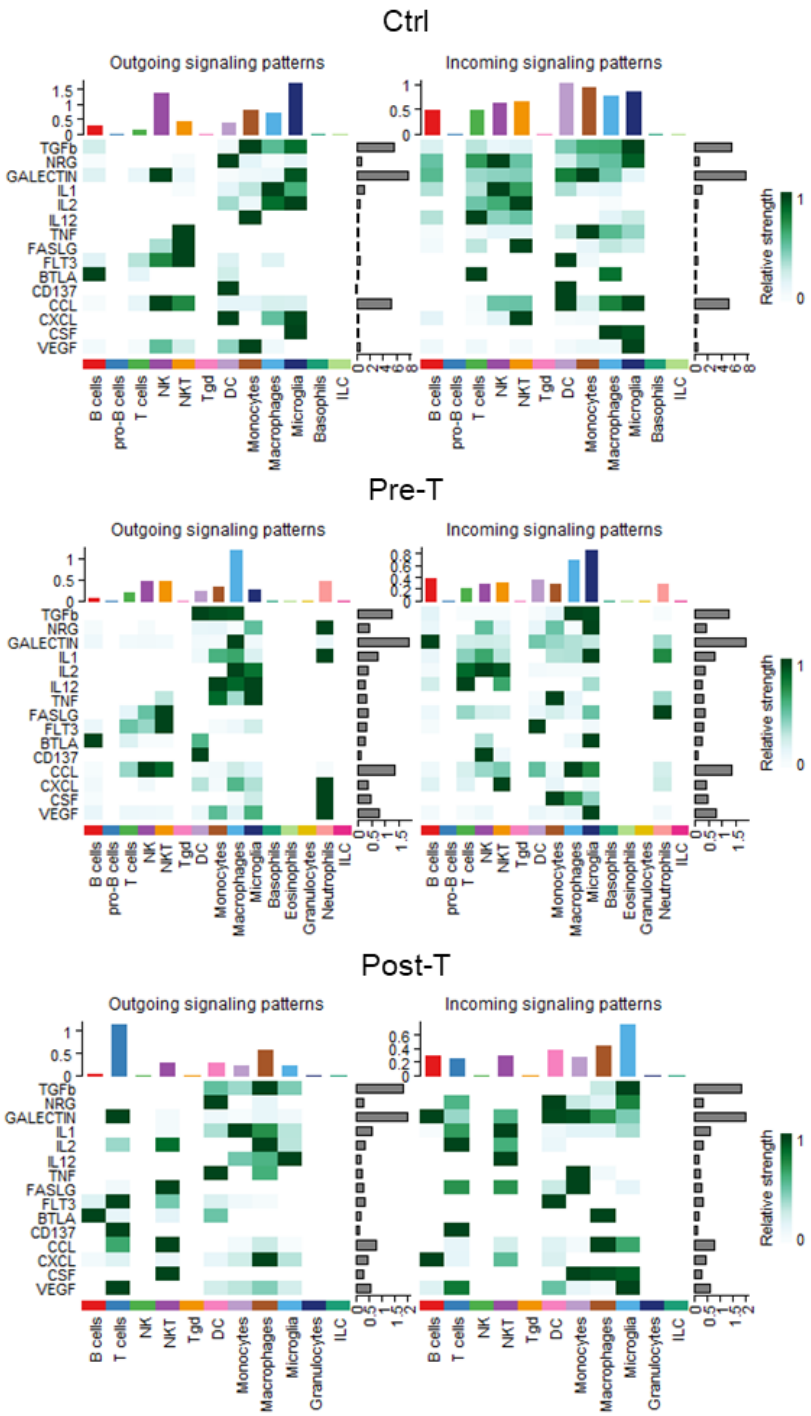


Figure 16. CellChat signaling analysis of immune cells in the liver: Another perspective of signaling was assessed by only using SingleR annotated immune cells, rather than including structural cells and hepatocytes. Once again, we adjusted CellChat parameters to use a truncated mean = 2.5% to detect additional pathways of interest. Here we show key shared immune cell pathways (TGF-β, NRG, Galectin, IL-1, IL-2, IL-12, TNF-α, FASLG, FLT3, BTLA, CD137, CCL, CXCL, CSF, and VEGF) across the Ctrl (upper panel), Pre-T (middle panel), and Post-T (lower panel) groups. All heatmaps were generated through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat), and the dependent software ComplexHeatmap version 2.15.1 (https://github.com/jokergoo/ComplexHeatmap).

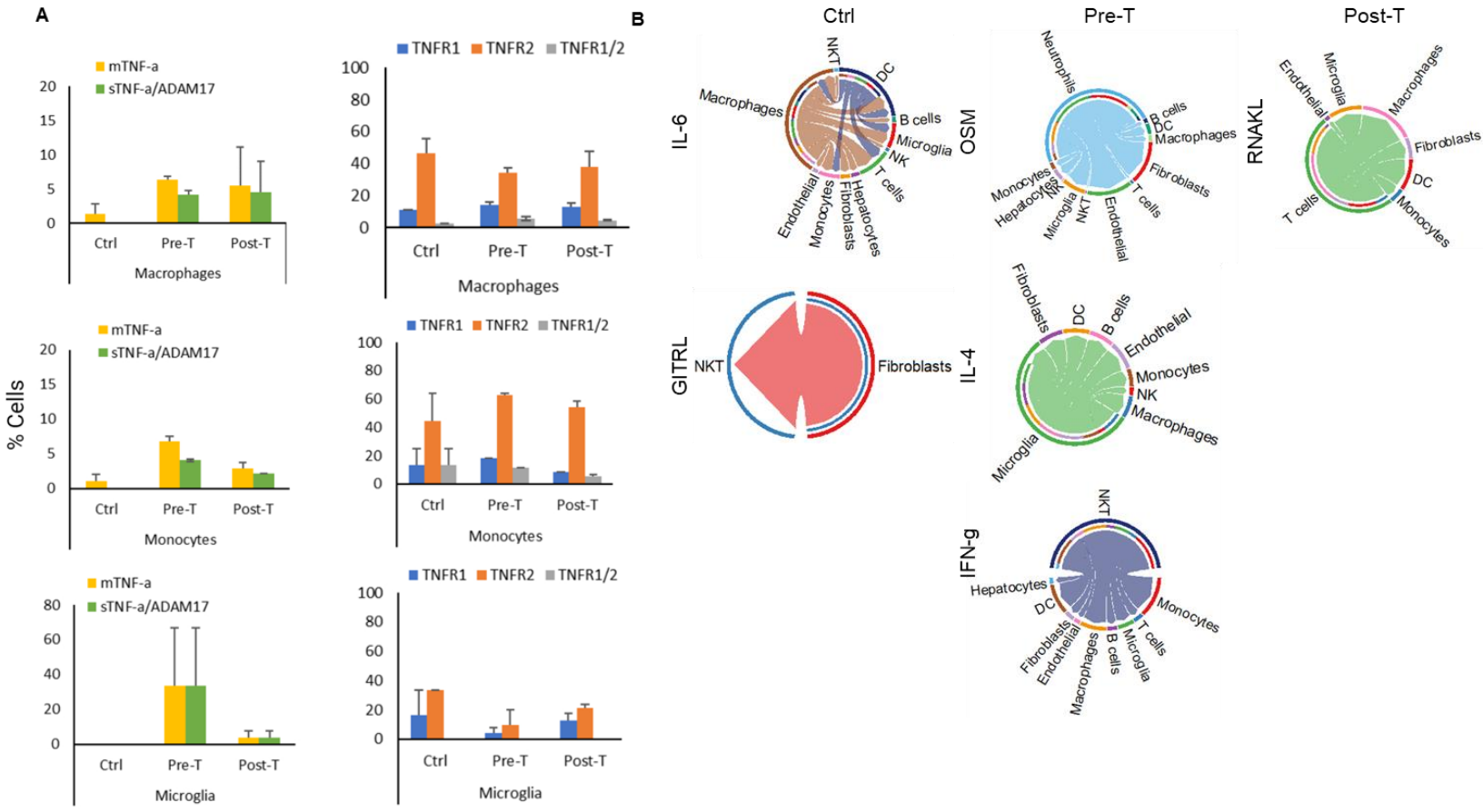


Figure 17. TNF-α and distinct signaling interactions within each unique cellular pattern: A) Functional patterns of the TNF-α signaling pathway were quantified by assessing the number of cells in each detected cell type involved in the pathway that were TNF-α+ (Tnf > 0) , TNF-α+/ADAM17+ (double positive and sTNF-α; Tnf > 0 & Adam17 > 0), TNFR1+ (Tnfrsf1a > 0), TNFR2+ (Tnfrsf1b > 0), and TNFR1+/2+ (double positive; Tnfrsf1a > 0 & Tnfrsf1b > 0); all cells expressing genes are presented as a percentage of the cell type population in each sample. B) Chord diagrams using CellChat for evaluating unique ligand-receptor in the Ctrl (IL-6 and GITRL), Pre-T (OSM, IL-4, and IFN-γ), and Post-T (RANKL) through the use of lower threshold analysis parameters as in Figure 13-14. Figure B was generated through the use of CellChat version 1.5.0 (https://github.com/sqjin/CellChat) and the dependent software circlize version 0.4.16 (https://github.com/jokergoo/circlize)

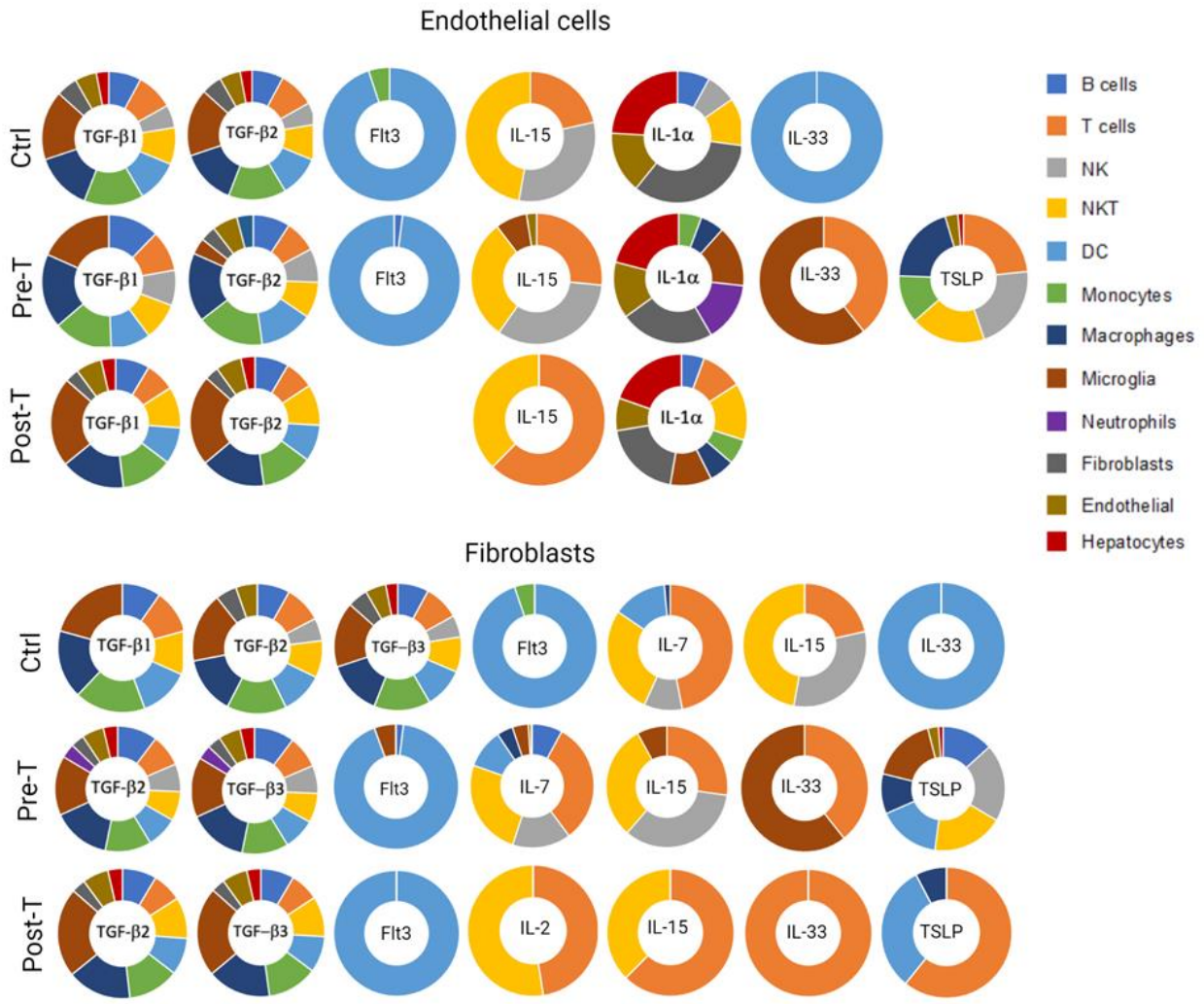


Figure 18. Structural cells are highly influential in the signaling interactions with other immune cells and non-immune cells in the liver: CellChat analyses enabled us to investigate signaling pathways based on the exact ligand and receptor pairs detected in each cell type, using the truncated mean of 2.5% analysis results. We focused on signaling coming from structural cells to immune cells, in which many functional signaling molecules were detected in endothelial cells (upper panel) and fibroblasts (lower panel) across groups.

Innate immune cells producing IL-1β are associated with the promotion of HCC

Given the dominance of the innate immune cells and structural cells in the signaling network in the liver, we sought to analyze the signaling pathways while focusing on different innate immune cell subsets interacting with hepatocytes and structural cells (Figure 19). We detected the expression of PDGF c and d isoforms being involved in fibroblasts proliferation and survival in all groups, while PDGF b isoform being involved in fibroblast activation and fibrinogenesis process was detected only during a WD (Figure 20). The innate immune cell recruiting chemokine, CSF, was produced by fibroblasts only during a WD recruiting macrophages and monocytes into the liver (Figure 20). Also, IL-1β was produced by Kupffer cells in the Ctrl group, whereas it was produced mainly by the innate immune cells targeting hepatocytes in the Pre-T group, as well as targeting hepatocytes and fibroblasts in the Post-T group (Figure 20). The IL-1 family cytokine, IL-18, targeted NK cells in the Ctrl and Pre-T group, while targeting NKT cells and $CD8^+$ T cells in the Post-T group (Figure 20). TNF-α was detected only in the Post-T group produced by M1 macrophages targeting mainly MDSCs and monocytes (Figure 20). A summary of the stepwise signaling communications during a WD is show in Figure 21.

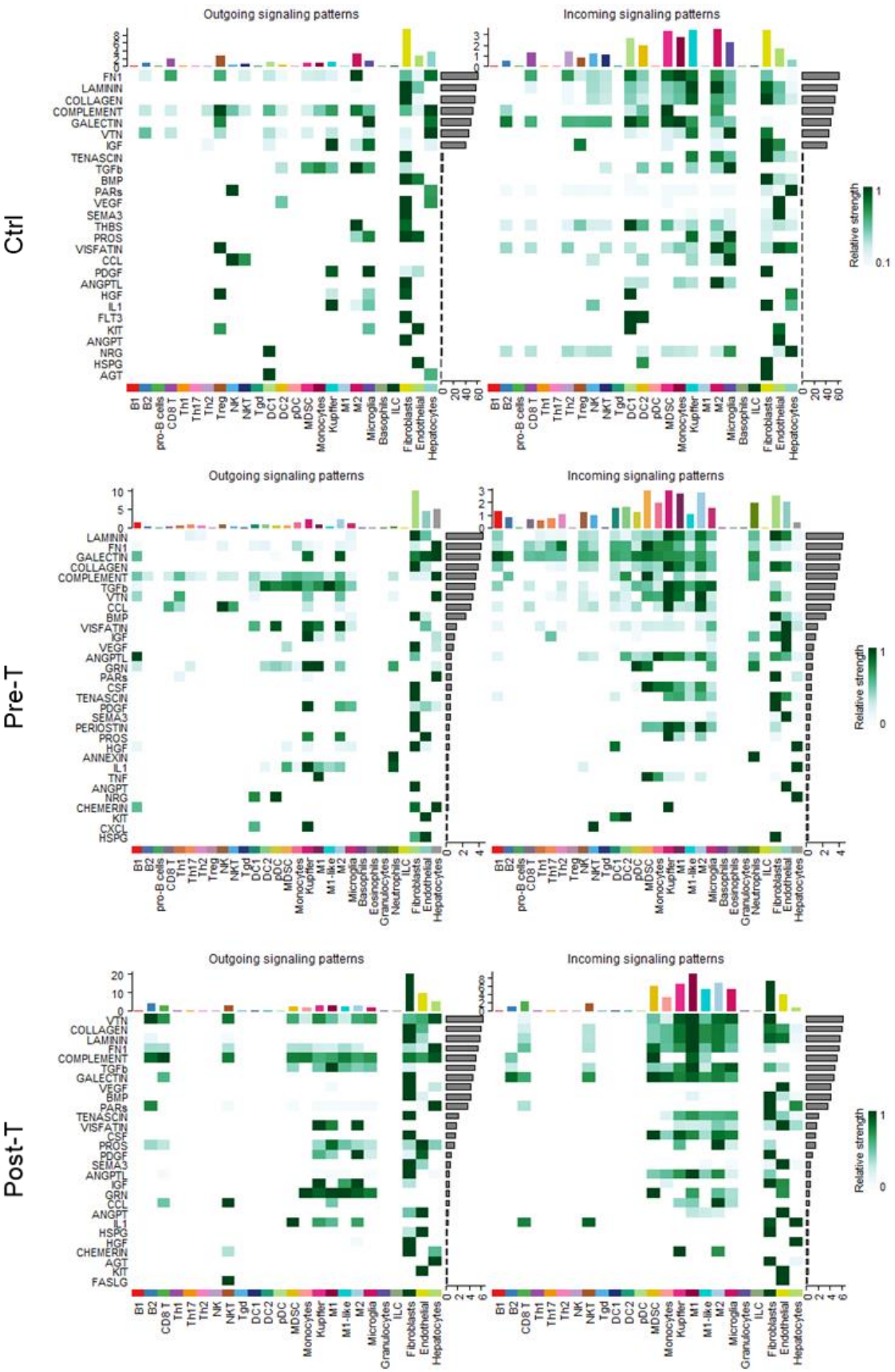


Figure 19. CellChat analysis of sorted subsets highlights the role of innate immune cells: Heatmaps portraying all identified significant signaling pathways by CellChat using default parameters (25% of cells expressing genes in each cell type. All heatmps were made through the use of CellChat version 1.5.0, along with the use of the dependent software ComplexHeatmap version 2.15.1 (https://github.com/jokergoo/ComplexHeatmap).

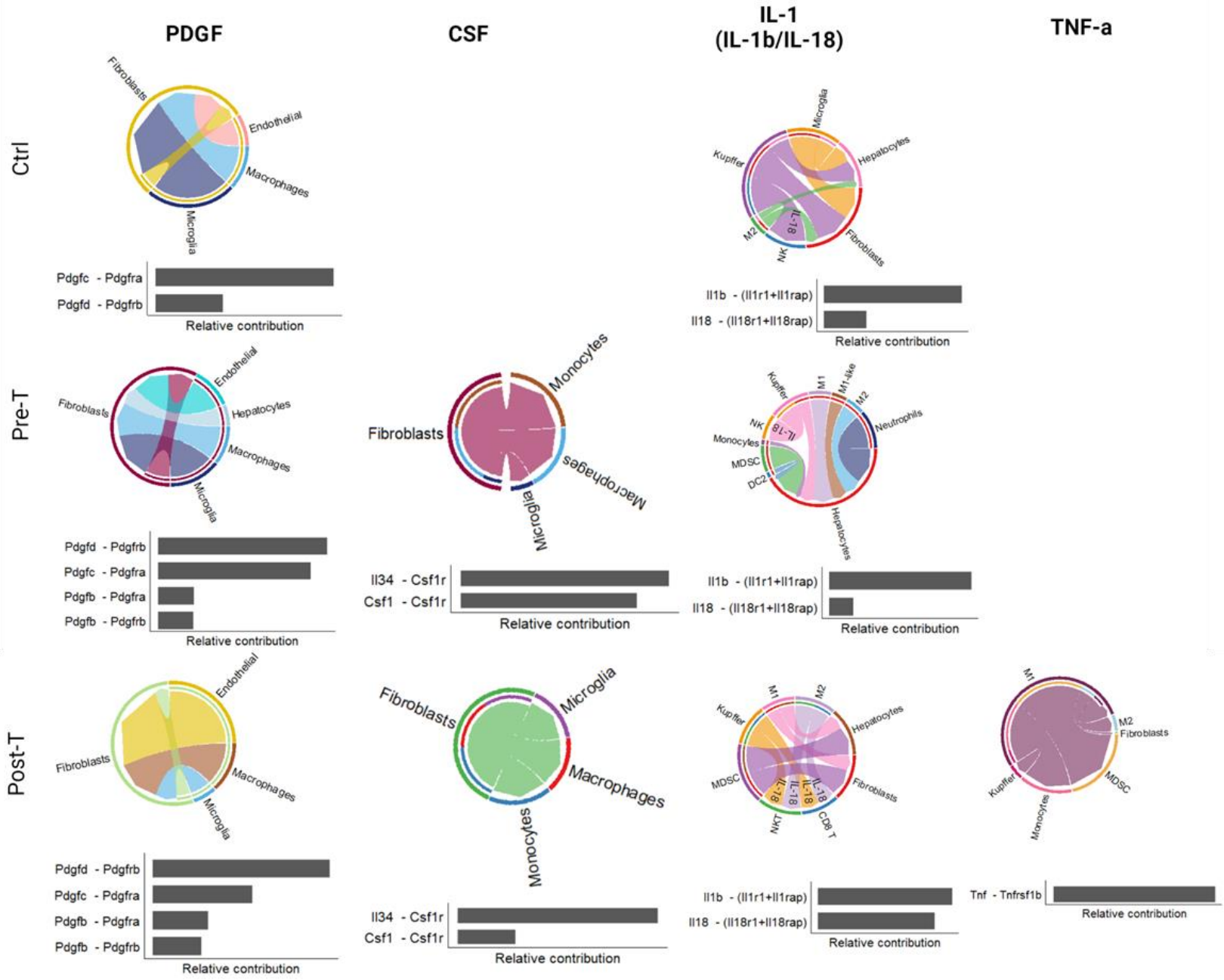


Figure 20. Signaling from innate immune cell subsets shows a central role in promoting HCC: Chord diagrams showing signaling pathways among hepatocytes, structural cells and innate immune cells in all groups. Blank spaces mean the pathway was not detected in the group (top row is the Ctrl, middle row for the Pre-T, and bottom row for Post-T). The chord diagrams displaying signaling events here were generated through the use CellChat version 1.5.0 and the dependent software circlize version 0.4.16 (https://github.com/jokergoo/circlize).

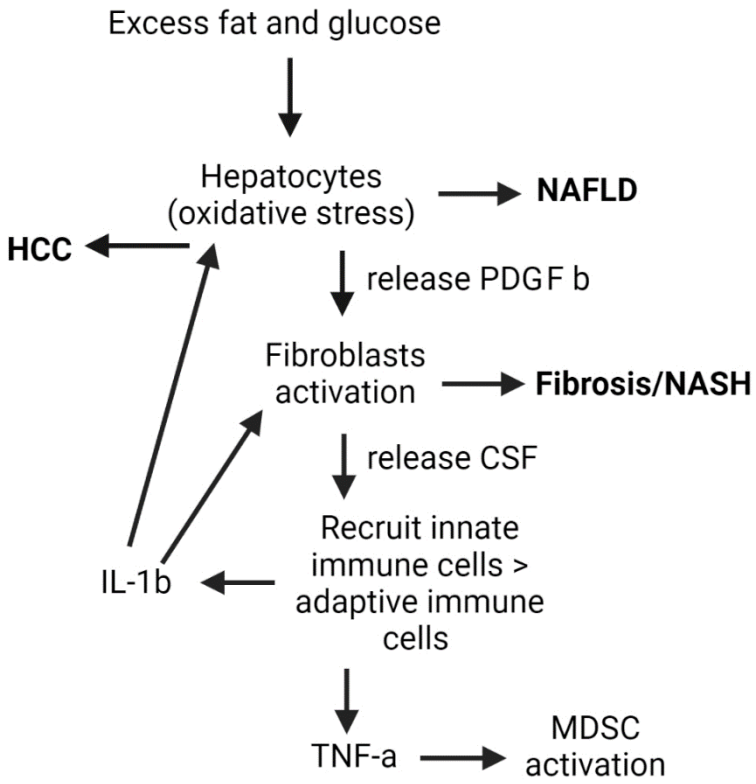


Figure 21. Overview of the functional understanding of NAFLD progression towards HCC development: A summary of the stepwise signaling communications in the liver during a WD.

Discussion

Immune responses to cancer functions as a system (immune system) in which immune cells are intimately connected through mutual interactions, thereby dynamically changing over time [137]. The outcome of the immune response in supporting or eliminating the tumor would depend on the internetwork immune cells interactions, rather than on a specific immune cell type. The strength of the reductionistic approach lies in a focused and targeted analysis of individual components alone, but it misses the mutual interconnection and feedback loops between the various cellular components comprising the dynamically changing immune system, as well as failing to understand the emergent properties of the immune response as a system. In fact, it misses the forest for the trees. Therefore, there is an urgent need to balance reductionism with a systems immunology approach for a comprehensive understanding of the immune function. A systems immunology approach suggests focusing on cellular interactions and analyzing feedback loops between the components, understanding the emergent properties or collective function of the immune responses, considering the immune cell interactions with the hepatic structural cells, and lastly adopting a holistic perspective that evaluates the immune responses as a collective function, rather than focusing solely on individual components. Although advances in methodology of research for collecting big data and data processing by means of different algorithms allows the implementation of systems immunology for a comprehensive understanding of the immunobiology of NAFLD and HCC, gaps in our methodological interpretation of big data results in an accumulation of descriptive knowledge without mechanistic insight. To address this gap, we devised a pattern discovery approach where collective function of the immune response is understood through dominant-subdominant interactions of the immune cell constituents [44,153]. Taking this systems immunology approach [141],

we discovered that i) innate immune cells recruited into the liver are the major player for orchestrating liver fibrosis and progression of HCC (Figure 21), ii) the hepatic immune system consisting of different immune cell types created a collective function beyond the function of its cellular constituents, which was not detectable by analyzing each cellular constituent separately; such collective function could be predicted from internetwork mutual interactions between all the various immune cell species, iii) each collective immune function is generated by dynamic ligand-receptor signaling interactions among immune cells, as well as with hepatic structural cells, resulting in dynamic changes in the function of each immune cell within different cellular patterns, and iv) analysis of dominant-subdominant relationships among the hepatic immune cells and structural cells can identify distinct immunological patterns that manifest their own collective functions, which can be understood through superior and inferior patterns of immune cells and their respective subsets during disease progression. Such pattern discovery approaches can be utilized for a comprehensive understanding of the immunobiology of NAFLD and HCC, and thereby offers immune modulatory interventions to induce a particular therapeutic immunological pattern, rather than inducing or suppressing specific immune cells types for the treatment of HCC.

The use of big data has led us to an era of information overload, but there is starting to become an appreciation for the limitations of reductionistic approaches for evaluating the role of the immune system and its network of interactions between immune cells and the tissue microenvironment as a complete system [145,181]. To this end, the net biological impact is highly complex and multifaceted, consisting of different cellular processes simultaneously and dynamically changing over time. Thus, we devised a method for studying the collective function of the hepatic immune pattern, similar to recent demonstrations that the proportion of immune

cell types interacting with one another could elucidate a mechanism by which the immune system in the liver functions as a network of interactions [44,58,153]. Assessing all of the immune cell species together identified the presence or absence of specific cytokine signals like IL1RN, TNFSF12, IFNL1, IFNB1, IL36A, EDN1, FASLG, IL37, CCL2, CXCL8, IL27, CCL20, IFN type I, CXCL3, CXCL2, SCGB1A1, C10orf99, IL7, TNFSF10, IL33, IL18, and IL20 (Figure 5A), all of which were only detected in the collective immune function. The hepatic immune system also displayed components of the immune response like increased phagocytosis and antigen presentation, as well as cell death of lymphocytes associated with decreased quantity of T and B cells during the development and presence of HCC (Figure 5B), further portraying contributions that are unique to the cumulative immune pattern. Alterations in the collective function of the hepatic immune response were also associated with significantly reduced cellular metabolism during the progression of NAFLD and HCC, which again, were solely detected in the collective immune function and not for each immune cell type. Pathways associated with tumor immunosurveillance such as oxidative phosphorylation and glycolysis [171], were only increased prior to HCC development when assessing the cumulative immune function. However, immune cell metabolism was upregulated in the Post-T compared to the Pre-T group in terms of the collective function of the hepatic immune system (Figure 5C), which may be indicative of the inferior immune cell patterns present such as M1 macrophage dominance, increasing proportion of mMDSCs compared to monocytes, and predominant CD8+ T cells (Figure 3D). It is easy to get bogged down in the details of which exact cytokine and cell type were identified by their expression of these signaling molecules and immune functions, but the main purpose is to highlight that the hepatic immune pattern has many components undetectable in the individual cell types comprising the pattern, which were only identified when the immune pattern is

assessed at a systems level, and thus likely attributable to all the interacting features between the cells within the pattern. Few other studies have taken similar approaches to understand the immune system as a whole, especially given the sheer number of cellular interactions between immune cells alone, but one study was able to stratify different HCC tumor immune microenvironments into infiltrating immune cell patterns corresponding to functionally different biological processes, reflecting both prognostic potential and immunotherapy responsiveness in patients [182]. This is a key finding, as this supports how the immune system as a whole exerts different biological functions depending on the disease context and pattern of immune cells, resulting in effective or inefficient immune responses. Lastly, by assessing how such pattern alterations may augment the collective immune function in regard to hepatotoxicity, liver-damaging, and tumor-promoting events during disease progression, we found the hepatic immune patterns during a WD showed increased hepatoxicity functions (Figure 6A), alongside upregulated liver-damaging and tumor-promoting processes (Figure 6B). These findings provide evidence that the collective function of the hepatic immune pattern could be utilized to predict risk of HCC development without having to focus on specific tumor markers. This is clinically significant because there is no specific marker or mechanism to predict the risk of carcinogenesis and HCC development in NAFLD patients [183]. To the best of our knowledge, this is the first report showing that detection of the collective immune function could be utilized to reliably predict risk of HCC progression during NAFLD. Together, these data demonstrate the collective immune function is not the summation of functions of its cellular constituents, rather it is beyond the function of each cellular constituent and manifested independently as a unique entity.

Internetwork analyses of ligand-receptor signaling interactions among immune cells, as well as their interactions with hepatic structural cells portrayed differential signaling dynamics

within each hepatic immune pattern that influenced the outcome and function of immune and non-immune cells. Mutual signaling interactions between cell types within the hepatic cellular patterns were assessed to understand how these functions may influence the collective function of the hepatic immune system, as well as how they may modulate cellular functions during disease progression. Shared signaling pathways like FN1 and PARs were active in all groups, but targeted different cell types during a WD, whereas other pathways were unique to the Ctrl, Pre-T, and Post-T groups, namely Tenascin, CCL, and BMP or CSF, respectively (Figure 10). These observations demonstrate the variability of mutual signaling interactions occurring during both homeostasis and HCC progression, with mutually interacting partners functioning in a signaling axis, but the interacting cell types dynamically change depending on disease status and the collective function of the cellular pattern they are found within. Analysis of the signaling molecules stemming from innate immune cells and structural cells due to their high contribution in the hepatic signaling network showed us mechanisms specifically targeting other immune cells in the pattern, which could serve as a method for understanding the role these cells play in liver disease pathogenesis, as well as how they modulate the immunological pattern and its collective function. For example, mutual interactions were detected such as macrophages supporting structural cells in the Ctrl and Pre-T groups by providing IGF signals to both endothelial cells and fibroblasts, but this signal was lost in the presence of HCC (Figure 10), in which these fibroblasts in the Post-T group distinctly elicited CSF signaling through the production of IL-34, promoting the recruitment of macrophages that can exacerbate disease progression [184]. Macrophages exhibited differential mutual signaling interactions by receiving 4 signals in the Ctrl group (Laminin/Collagen/FN1/Tenascin), 4 signals in the Pre-T group with a new interaction through NK cells (Laminin/FN1/Collagen/CCL), and 5 signals were received by

macrophages in the Post-T group (Collagen/VTN/Laminin/FN1/CSF) (Figure 9). These data clearly portray that macrophages mutually interact and receive signals from different cell types within the hepatic microenvironment during disease progression, while also exhibiting functional loss of signaling mechanisms like IGF present during homeostasis and the development of HCC. This exemplifies the need for new perspectives in understanding the collective function of the immune system during NAFLD and HCC, as the exact cell types interacting with one another and their signaling pathways themselves are dynamically changing across groups. Understanding how mutual interactions manifest the collective immune function and change cellular functions dynamically can also be seen in T cells by FASLG signaling (Figure 15), where T cells in the Ctrl group sent signals to 9 cell types (B and T cells/NKT cells/monocytes/macrophages/ microglia/fibroblasts/endothelial cells/and hepatocytes), the Pre-T group sent signals to the same 9 cell types with the addition of 3 new species (NK cells/DCs/and neutrophils) for a total of 12 different cellular targets, whereas the Post-T group T cells only signaled to 5 cell types (T cells/ NKT cells/monocytes/endothelial cells/and hepatocytes). This further portrays that T cells and their mutually interacting partners are also dynamically changing from homeostasis to the establishment of HCC, especially in the Post-T group where fibroblasts are no longer expressing the FAS receptor sufficiently to be predicted to engage with T cells, in which this mechanism promoting the homeostatic turnover of fibroblasts during wound repair [185] would not work and could lead to a persisting population of apoptosis-resistant fibroblasts [186]. Therefore, dynamic mutual interactions in the liver constitutively change, in which alterations in the mutually interacting cell types and their signaling pathways perturb cellular patterns and individual cell type functions. The accumulation of functional changes in signaling pathways and interacting partners during a WD lead up to the generation of carcinogenesis and development of HCC,

which we were also able to detect. For example, IL-1α promotes constitutive hepatic inflammation and exacerbates liver damage mainly through its actions on hepatocytes [187,188], yet the source of IL-1α in the liver is still under debate, with some reports implicating hepatocytes and Kupffer cells as major sources of this inflammatory molecule [187,189,190]. We identified endothelial cells as a source targeting hepatocytes, fibroblasts, and themselves in an autocrine fashion across all groups (Figure 18), possibly serving as one source of inflammation and hepatocarcinogenesis. Importantly, we see similar observations in these cells by IL-1α (Figure 18) being sent to 6 cell types in the Ctrl group (Hepatocytes/B cells/NK/NKT/ fibroblasts/and endothelial cells), 7 cell types in the Pre-T (Hepatocytes/monocytes/macrophages/microglia/ fibroblasts/neutrophils/and endothelial cells), and 9 cell types in the Post-T (Hepatocytes/DC/ microglia/T cells/ NKT/monocytes/macrophages/fibroblasts/and endothelial cells). However, due to the lack of hepatic stellate cell markers in our reference database for cell type annotation, these cells could be endothelial cell-like hepatocytes, thereby warranting subsequent analyses incorporating annotation for this cell type in particular. On the other hand, IL-1β also exemplified carcinogenic potential by it being produced by macrophages in all groups, but shifting from targeting fibroblasts, microglia, and hepatocytes in the Ctrl group, to only targeting hepatocytes in the Pre-T group or hepatocytes and fibroblasts in the Post-T group (Figure 12). This could increase the rate of carcinogenic events due to the pro-inflammatory functions of IL-1β [191] during a WD, and once the tumor has been established like in the Post-T group, it may serve to alter the function of structural cells by targeting both hepatocytes and fibroblasts, which can induce gene expression programming in fibroblasts that promote tumor cell survival [192]. Further, the use of longitudinal studies instead of snapshot studies has even identified unique cytokines influencing the outcome of signaling during HCC [137], which could certainly be

engaging with vastly different cell types throughout disease progression. Therefore, we postulate that the collective immune function emerges from mutual ligand-receptor interactions between immune cells and structural cells, resulting in dynamic changes in cellular function. We assessed ligand-receptor interactions in this manner to be able to make such a claim, but the interactions could also be beyond cognate ligand-receptor pairs, such as exosome signaling and the effects of the hepatic microbiome. Analyzing exosomes could provide additional insight to signaling mechanisms we could not detect, as exosome signaling cannot be assessed without optimized techniques such as droplet microfluidics [193] or biosensors [194,195], as well as the resident microbial species, which can exert homeostatic effects or induce inflammation during dysbiosis [196], both warranting subsequent studies to paint the most comprehensive picture of the immunobiology of NAFLD and HCC.

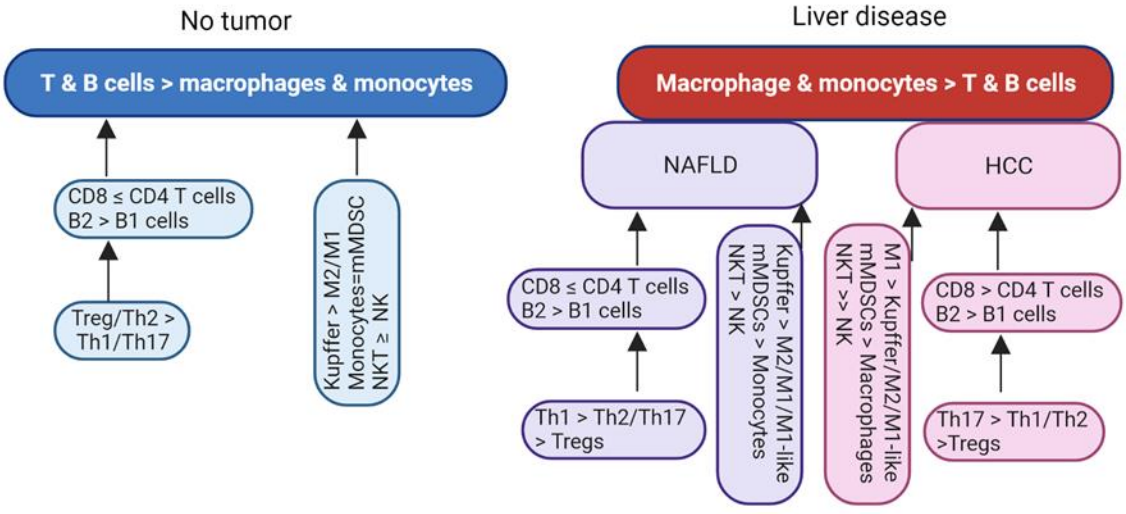


Figure 22. Multilayered immunological patterns during health and diseases: Super-patterns and inferior patterns were quantitatively analyzed by focusing on the ratios/proportion of immune cells interacting with each other in a network.

Despite dynamic changes in the hepatic signaling network and mutual interactions, the collective function can be understood by the discovery of dominant-subdominant patterns. We identified multilayered immunological patterns, in which the super-pattern of adaptive immune cell (B and T cell) dominance transitioned to innate immune cells (macrophages and monocytes) dominance during HCC progression (Figure 22). In addition, inferior patterns of immune cell subsets such as CD4+/CD8+ T cells, Th1/Th2/Th17/Tregs, and Kupffer cells/M1/M2 macrophages dynamically changed throughout disease progression (Figure 3D). This is consistent with reports that innate immune cells such as macrophages and monocytes increase and play a role in generating hepatic inflammation during liver disease [197,198]. However, other reports suggest CD8+ T cells and NKT cells, but not myeloid cells promote the development of HCC [54], in which this claim may be due to reductionistic perspectives that do not appreciate the hepatic immune pattern and thereby mask the role of innate immune cells, as this same report identified increased levels of monocytes and macrophages during high-fat diet feeding, but did not further interrogate these cells. MDSCs and Tregs are implicated in generating suppressive microenvironments during HCC [199], although we found increasing levels of mMDSCs as well, we found continual reductions in Tregs during HCC development (Figure 3D), suggesting this cell type may not be as important in NAFLD and HCC as implicated by other reports [200–202]. To this end, CD4+ T cells typically become Th1 and Th17 effector subtypes during chronic hepatic inflammation [203,204], which we detected as Th1 dominance in the Pre-T and Th17 dominance in the Post-T groups (Figure 3D). On the other hand, the proportion of T cells in the hepatic immune pattern constitutively decreased during HCC development, concordant with one report that immunotherapy led to tumor growth and loss of CD4+ T cells [97], while targeted approaches attempting to comprehend NAFLD and HCC suggest CD4+ T cells can inhibit HCC [56,57], another

study highlighted that restoration of CD4+ T cells could not prevent HCC development [58]. Although the status of the literature is convoluted with contradictory reports like these, utilization of pattern discovery methods could offer a strategy to generate a more uniform understanding of NAFLD and HCC, especially by evaluating the hepatic immune system as a pattern of different immune cells. The cell populations contributing the most to the hepatic signaling networks within our internetwork analyses suggested innate immune cells (macrophage and monocytes) and structural cells (fibroblasts and endothelial cells) play the most influential roles in intercellular signaling during HCC progression. This agrees with the hepatic cellular patterns we identified, as these innate immune cell species dominated the immune pattern during a WD, while the predominance of structural cell species fluctuated across groups (Figure 3). For example, restoration of endothelial cell predominance in HCC elicited different functionality compared to those in the Ctrl group, seen by the loss of FLT3 and IL-33 signals (Figure 18), suggesting although quantitative proportions of the non-immune cell patterns were restored to similar levels of the Ctrl, their function was altered within the new cellular pattern during HCC. Fibroblasts shared signaling of TGF-β, FLT3, and various IL-1 family cytokines across groups, but the qualitative pattern of signaling functions changed, with the loss of IL-7 expression while gaining IL-2 signaling in the Post-T group (Figure 18), perhaps serving as one axis mediating the decrease in T cells during NAFLD and HCC. In addition, fibroblasts acquired TSLP expression during both the progression and presence of HCC, which promotes Th2 programming of CD4+ T cells and can be targeted during HCC to improve the immunosuppressive environment created by Th2 cells [205,206]. However, the lack of TSLP expression in the Ctrl may not support this differentiation mechanism, as we found the highest levels of Th2 cells in the Ctrl group and similar proportions during a WD (Figure 3D), as well as T cells only being targeted by this

signaling in the Post-T group (Figure 18). These observations showed us although fibroblasts returned to a similar pattern as the Ctrl group, the hepatic cellular pattern during the presence of HCC altered the functional quality of fibroblasts and their mutually interacting partners, likely due to the signaling interactions within hepatic immune patterns dominated by innate immune cells. Evaluation of inferior immune cell subset patterns revealed similar observations, where pattern shifts not only were quantitatively diverse during HCC progression, but also qualitatively distinct in their new functions and contributions to the cumulative function of the hepatic immune system. TNF-α signaling was detected in all groups and exemplified this (Figure 16), where the shift from dominant T and B cells to macrophages and monocytes during a WD resulted in more TNF-α production by macrophages that could induce autocrine pro-survival signaling via their high expression of TNFR2 [178,207] (Figure 17A). This may be a potential mechanism facilitating their persistence and dominance within the hepatic immune pattern, but it also reflects the increasing prevalence of M1 macrophages replacing Kupffer cells in the inferior immune patterns (Figure 3D), consistent with reports that shifts toward predominant innate immunity results in epigenetic changes during high-fat diet feeding where even after weight loss and metabolic normalization the inflammatory gene expression signature persists [208]. Further, in relation to our previous studies utilizing flow cytometry [44,58], the patterns we identified such as M1 macrophage dominance over M2 cells during NAFLD and HCC are in agreement with the results from these studies looking at the expression of markers at the protein level. In addition, other observed pattern shifts in our data were concordant with previous findings, such as fluctuating dominance of CD4+ Th1 and Th17 cells during NAFLD and HCC [44], as well as increasing ratios of NKT to NK cells and CD8 to CD4 cells in the presence of HCC [44,58]. Even during adaptive immune cell dominance in the Ctrl group, their participation in the ligand-

receptor signaling network was less than that of innate immune cells, which further decreased with subdominant adaptive immune cell patterns during a WD. This could be due to only 20% of T cells being T effector phenotype in the Ctrl group, whereas the effector phenotypes increased to more than 80% during a WD [44]. Thus, analysis of pattern shifts and their signaling networks clearly correspond with functional signaling differences within distinct hepatic immune patterns, which can be further understood through the gain and loss of signaling pathways manifested within each pattern-specific gene expression programming. Unique signals in the Ctrl group included IL-6 and GITRL, in the Pre-T we detected OSM, IL-4, and IFN-γ, and the Post-T group showed RANKL (Figure 17B). We detected macrophages and DCs as the major senders of IL-6, in which macrophages are a typical source of this cytokine for exerting hepatoprotective effects [209], as well as homeostatic processes like initiating immune responses, liver regeneration, and metabolism [210]. The other unique signal in the Ctrl group, GITRL, has been shown to stimulate T cell functionality in HCC [211], along with modulating immune responses by acting as a stimulatory signal to enhance T cell activation, survival, and differentiation [212–214]. As for the Pre-T group, oncostatin M (OSM) signaling promotes fibrosis via inducing phenotypic changes in macrophages and hepatic stellate cells [215], as well as facilitating epithelial-mesenchymal transition and angiogenesis in HCC [216], IL-4 induces immunosuppressive functions [217,218], and IFN-γ is characteristic of liver dysfunction [219], stemming from NKT, NK, and T cells for immune surveillance [220]. Finally, RANKL signaling in the Post-T group is more complex, with signaling outcomes capable of stimulating or inhibiting the immune response [221–223]. Once more, we do not want to be distracted by the multitude of signaling pathways, but by solely focusing on those signals uniquely found within each distinct hepatic immune pattern, we see characteristics reflecting observed pattern shifts like the progressive loss of homeostatic functions in the Ctrl

group, such as IL-6 and GITRL, while during disease progression we discovered new signaling pathways within the immune pattern promoting pattern disruption and liver dysfunction, such as OSM, IL-4, and IFN-γ, further supporting our systems immunology approach coupled to pattern discovery as a means to provide a more in-depth understanding of the immunobiology of NAFLD and HCC. To this end, understanding the collective hepatic immune pattern and its signaling networks during NAFLD and HCC pathogenesis provides a foundation for immune modulation based on the patterns and mutual signaling interactions, which generate a collective function that could be used to achieve a pattern that results in the inhibition of tumor development.

Lastly, by focusing on the signaling pathways among hepatocytes, structural cells, and innate immune cells (Figure 19), we discovered that high fat/sugar diet induces hepatocytes to express the PDGF b isoform (Figure 20), which is typically produced by infiltrating macrophages during inflammation for the activation of fibroblasts by signaling through the PDGFRβ receptor, followed by its subsequent activation of PI3K/AKT pathways that prompt proliferation, fibrinogenesis, and collagen deposition [224–226]. Importantly, crosstalk between these cell types are implicated in hepatic inflammation, fibrosis, and carcinogenesis [227,228], while the activation of hepatic stellate cells and fibroblasts in this manner facilitates expression of CSF for the recruitment of innate immune cells in the liver [229–231]. Consequently, the innate immune cells, M1 macrophages in particular, predominated over Kupffer cells and expressed IL-1β to further activate fibroblasts and target hepatocytes, as well as TNF-α to recruit and activate MDSCs. IL-1β is canonically produced by Kupffer cells and infiltrating macrophages during inflammation and can activate these structural cell species [227,232,233], as well as converting fibroblasts into tumor-associated fibroblasts recruiting innate immune cells [231]. IL-1β was reported to increase

carcinogenic events [191] on hepatocytes during a WD, but it has also been shown to facilitate steatosis by its effects on hepatocyte fat accumulation [234]. TNF-α signaling through TNFR2 on MDSCs has been identified as a prominent mechanism in the accumulation, persistence, and survival of MDSCs in tumor sites [235,236], and also for their ability to exert suppressive and tumor promoting functions [237,238]. Therefore, this provides numerous pieces of evidence implicating innate immune cells play a major role in promoting HCC development and hepatic fibrosis through their interactions with fibroblasts and hepatocytes, as well as other innate immune cells. This is a key finding representing yet another component of the hepatic microenvironment that could be targeted to employ therapeutic interventions for immune pattern modulation.

Together, these data give multiple novel insights on the impact of immune-inflammatory and carcinogenic pathways, which may vary depending on the biological context and immunological pattern within which they are activated. Further, they promote the use of future integrated approaches via pattern discovery to identify a hepatic immune pattern that can elicit the best anti-tumor responses, based on the active mechanisms detected in the collective function of the immune system present, which may not be evident with studies that only assess one specific immune cell type and its subsets alone. Pattern discovery methods like ours implicate that progressive NAFLD or NASH and HCC cannot be characterized by tumor immune tolerance or suppression, as these mechanistic perspectives focus on each cell compartment and not the collective immune function. Other groups that have employed pattern recognition techniques through computational algorithms have identified distinct immune cell infiltration patterns in HCC patients, with different patterns corresponding to different prognosis [239], as well as survival outcomes and predicted responsiveness to immunotherapy [240]. Although immunotherapies have been useful for a subset of HCC patients, these treatments only alleviate

symptomologies associated with disease progression, and in fact perpetuate the cause, by facilitating further disruption of immune patterns by only targeting a single cell type. Of course, the dominant-subdominant patterns are only a single factor influencing the collective function of the hepatic immune system, in which feedback loops between immune cells, as well as those between immune cells and structural cells in the liver or resident microbial species could also be involved in shaping the immune patterns and the collective immune function. Therefore, we offer pattern discovery approaches to understand the dynamic network of immune cell interactions, instead of getting distracted with too many details, along with immune pattern modulation strategies as cancer therapies, rather than targeting a specific immune cell type to augment its individual immune response. Pattern modulation strategies are not well defined and an area under development, which requires novel approaches for pattern modulation such as targeting innate immune cells and structural cells given their high levels of contribution to the hepatic signaling networks and influence on the cellular patterns, in which structural cells are also known to be critical coordinators of immune responses but differ based on the anatomical organ site [179]. In addition, exosome signaling and the underlying hepatic microbiome can alter the status of inflammation and frequency of carcinogenesis. Resident microbial species have not only been implicated in facilitating NAFLD progression and displaying different microbiota composition compared to healthy individuals [241,242], but this is also seen in NASH patients with cancer by their abundance of Clostridium species and decreased amount of Bacteroidetes compared to patients with just steatosis [243,244]. Microbiome dysbiosis is often seen during NAFLD and HCC development, which facilitates chronic hepatic inflammation [245], along with compositional shifts in the resident microbial species during the development of HCC that induce suppressive T cell phenotypes [246]. Thus, the microbiome clearly is able to alter the hepatic

immunological pattern in both quantitative and qualitative manners, but the microbiome can even be modulated by rather non-invasive methods like dietary changes [247,248], hence this component of the microenvironment represents a promising avenue for immune modulation of the cellular patterns in the liver during NAFLD and HCC. Although this requires further elucidation of the exact patterns of microbial species and the effects of their byproducts needed to foster the most effective immune pattern, this could be attainable through subsequent studies employing both systems immunology and pattern discovery approaches, coupled with multilayered "omics" to provide the most comprehensive insight for devising effective immune pattern modulatory approaches for progressive NAFLD and NASH patients, prior to the inevitable development of HCC.

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VITA

Personal Summary

Nicholas James Koelsch was born on May 9th, 1999 in Virginia Beach, Virginia. After graduating high school in Chesapeake, Virginia in 2017, he attended Virginia Tech from 2017 to 2021 where he received his Bachelor of Science in Biology with minors in Chemistry and Spanish. He then continued on to pursue his graduate education at Virginia Commonwealth University in the Fall of 2021 as a master's student.

Publications

1. Isbell M, Mirshahi F, Aqbi HF, Guo C, Saneshaw M, **Koelsch N**, Idowu MO, Austin D, Gelber C, Wang XY, Sanyal AJ, Manjili MH. Restoration of CD4+ T Cells during NAFLD without Modulation of the Hepatic Immunological Pattern Is Not Sufficient to Prevent HCC. Cancers (Basel). 2022 Nov 9;14(22):5502. doi: 10.3390/cancers14225502. PMID: 36428596.
2. **Koelsch N**, Mirshahi F, Aqbi HF, Saneshaw M, Idowu MO, Olex AL, Sanyal AJ, Manjili MH. The crosstalking immune cells network creates a collective immune function beyond the function of each cellular constituent during the progression of hepatocellular carcinoma. Scientific Reports (under revision)

Upcoming Poster Presentation

1. **Koelsch N**, Mirshahi F, Aqbi HF, Saneshaw M, Idowu MO, Olex AL, Sanyal AJ, Manjili MH. A shift in the hepatic immunological pattern from predominant adaptive immunity to innate immunity compromises tissue-specific immunity and leads to HCC. Poster will be presented at: Immunology 2023 hosted by the American Association of Immunologists; May 12, 2023; Washington, D.C.
2. Isbell M, Mirshahi F, Aqbi HF, Guo C, Saneshaw M, **Koelsch N**, Idowu MO, Austin D, Gelber C, Wang XY, Sanyal AJ, Manjili MH. Restoration of CD4+ T Cells during NAFLD without Modulation of the Hepatic Immunological Pattern Is Not Sufficient to Prevent HCC. Cancers (Basel). 2022 Nov 9;14(22):5502. doi: 10.3390/cancers14225502. PMID: 36428596. Poster will be presented at: Immunology 2023 hosted by the American Association of Immunologists; May 14, 2023; Washington, D.C.
3. **Koelsch N**, Mirshahi F, Aqbi HF, Saneshaw M, Idowu MO, Olex AL, Sanyal AJ, Manjili MH. A shift in the hepatic immunological pattern from predominant adaptive immunity to innate immunity compromises tissue-specific immunity and leads to HCC. Poster will be presented at: Walter Lawrence Research Retreat hosted by the Massey Cancer Center; June 30, 2023; Richmond, VA