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Targeting gut microbiota to overcome immunotherapy resistance in hepatocellular carcinoma: from mechanisms to clinical practice

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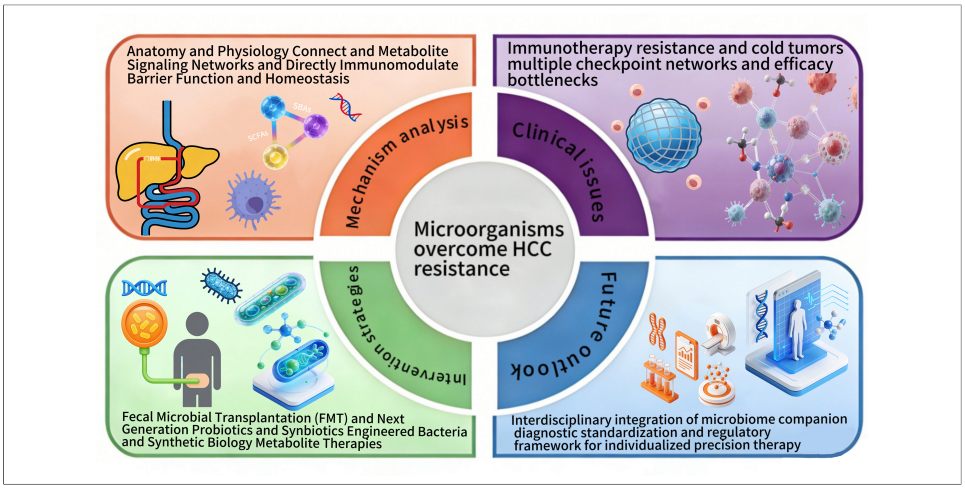

Abstract

Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer-related mortality worldwide. Despite significant improvements in the treatment landscape for advanced HCC in recent years through combination therapies centered on immune checkpoint inhibitors (ICIs), only approximately 20%-30% of patients achieve durable clinical responses. Primary and acquired resistance remain critical bottlenecks limiting broader efficacy. Traditionally, investigations into resistance mechanisms have predominantly focused on the tumor microenvironment (TME), emphasizing the formation of an immune-"cold" niche, the compensatory upregulation of alternative immune checkpoints [e.g., lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT)], and dynamic metabolic reprogramming. However, this tumor-centric perspective fails to fully account for the observed inter-patient heterogeneity in treatment efficacy, suggesting the critical involvement of systemic regulatory factors. Grounded in the unique anatomical and physiological connectivity of the "gut-microbiota-liver axis", the gut microbiota, as a critical extrahepatic regulatory system, is increasingly recognized for its

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role in influencing hepatic immune homeostasis and anti-tumor responses. This provides a novel breakthrough for systemically understanding and overcoming immunotherapy resistance in HCC. The gut microbiota systemically regulates immune surveillance in the liver and the immune status of the tumor microenvironment through various mechanisms, including metabolites (such as short-chain fatty acids, secondary bile acids, and tryptophan metabolites), the activation of pattern recognition receptors, and antigen cross-reactivity. Clinical evidence indicates significant differences in gut microbial community structure between responders and non-responders, while antibiotic use may impair the efficacy of ICIs by disrupting microbial homeostasis. Consequently, microbiota-targeted interventions have emerged as promising strategies to reverse immunosuppression and re-sensitize tumors to immunotherapy. Modalities such as fecal microbiota transplantation (FMT), next-generation probiotics/synbiotics, metabolite-based therapies, and engineered bacteria have progressed from concept to early-stage clinical practice, demonstrating initial safety and feasibility. This article systematically reviews the mechanisms and clinical evidence regarding the role of the gut microbiota in immunotherapy resistance for HCC, and discusses its translational prospects as a personalized combination therapeutic strategy, aiming to provide new insights for overcoming bottlenecks in HCC immunotherapy.

INTRODUCTION

From therapeutic challenges to the new hope of microbiome therapeutics

Hepatocellular carcinoma (HCC) remains a leading cause of cancer-related mortality worldwide, with an evolving etiological shift towards metabolic dysfunction-associated fatty liver disease (MAFLD)[1-4]. Its treatment landscape has transformed significantly from localized therapies to systemic options. The advent of tyrosine kinase inhibitors (TKIs), such as sorafenib and lenvatinib, marked the first breakthrough in targeted therapy for advanced disease[5-7]. Subsequently, a profound paradigm shift was ushered in by immune checkpoint inhibitors (ICIs). While initial monotherapy results were modest, combination strategies - such as atezolizumab plus bevacizumab ("T+A") and durvalumab plus tremelimumab ("STRIDE") - have significantly improved patient outcomes and established new first-line standards [Table 1][8-14]. Despite this progress, a substantial clinical bottleneck persists.

Currently, only 20%-30% of patients achieve durable objective responses to ICI-based regimens, leaving the vast majority to face primary or secondary resistance, which severely limits overall survival gains[10,15,16]. The mechanisms driving this resistance are primarily rooted in the tumor microenvironment (TME). Primary resistance is often associated with an immune-"cold" TME, characterized by a lack of cytotoxic T-cell infiltration. This is frequently driven by constitutive Wnt/β-catenin signaling that impairs chemokine-mediated immune cell recruitment[17-20]. Conversely, secondary resistance develops in initially responsive tumors through adaptive immune evasion, such as the compensatory upregulation of alternative inhibitory checkpoints [e.g., lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT)] leading to T-cell exhaustion, and the expansion of immunosuppressive cells [e.g., regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs)][21-24]. This results in the dynamic remodeling of the TME from an "inflamed" to an "excluded" state[25,26]. The multifaceted nature of these resistance mechanisms is illustrated in Figure 1 and further detailed in Table 2[27-34].

While landmark trials such as CheckMate-040[13] and KEYNOTE-224[8] established the role of ICI monotherapy in HCC, the subsequent failure of KEYNOTE-240[35] highlighted the persistent challenge of primary resistance. Even with current front-line combinations (e.g., IMbrave150[10] and HIMALAYA)[12], the objective response rate (ORR) has plateaued at approximately 30% - a clinical ceiling increasingly attributed to the gut-liver axis. The most compelling evidence for this link comes from the "antibiotic penalty". Recent multicenter cohorts reveal that patients receiving broad-spectrum antibiotics shortly before or after initiating ICI therapy experience significantly worse clinical outcomes [ORR, progression-free survival (PFS), and overall survival (OS)]. This disruption of microbial diversity underscores that an intact, diverse gut microbiota is a prerequisite for optimal systemic anti-tumor immune responses[36].

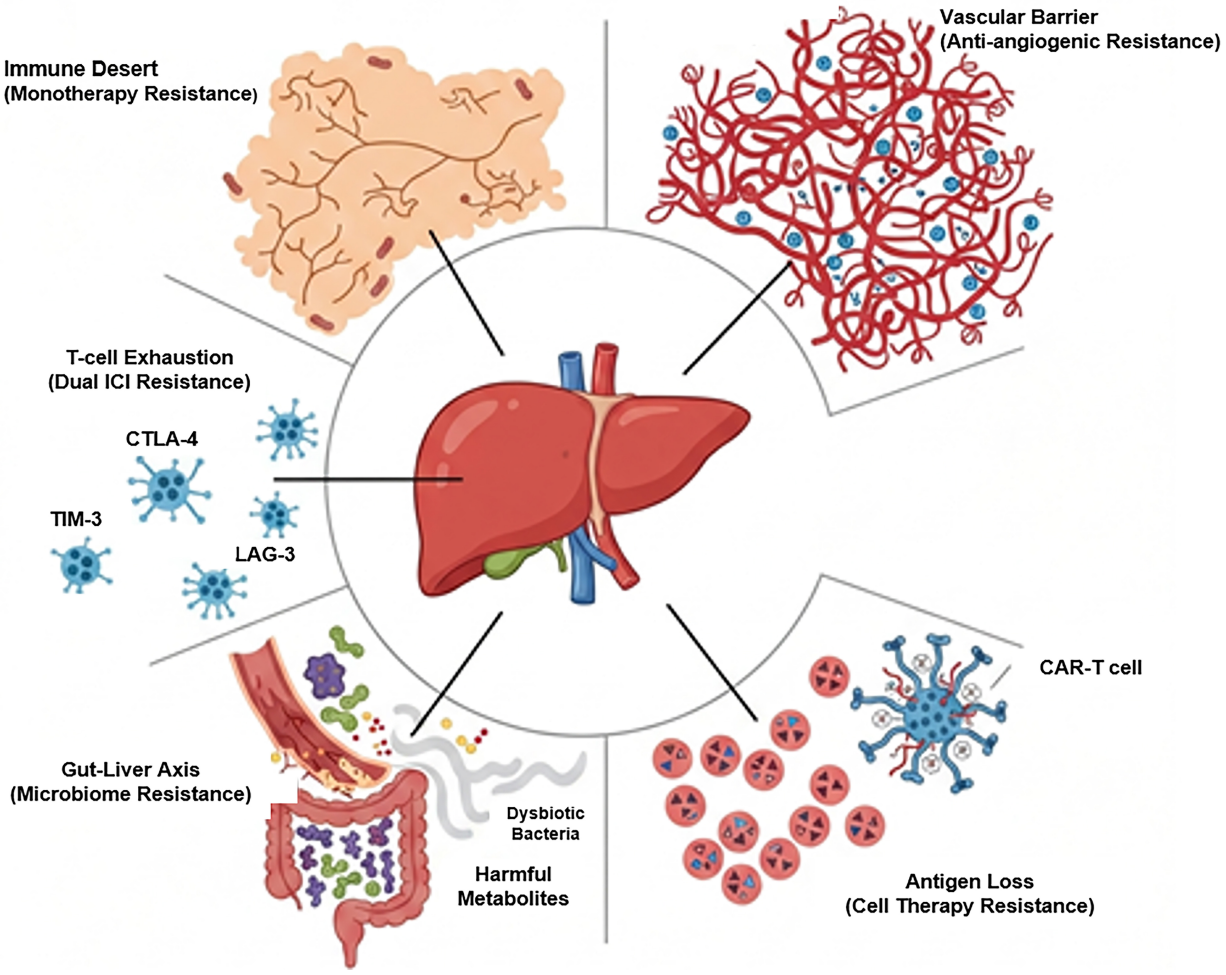

Figure 1. Resistance mechanisms in the HCC tumor microenvironment. The schematic illustrates five dimensions of resistance linked to the central liver. Black connecting lines indicate a correlation with specific therapeutic resistance, while icons and bold labels within each sector represent key biological drivers and resistance types. HCC: Hepatocellular carcinoma; ICI: immune checkpoint inhibitor; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; LAG-3: lymphocyte-activation gene 3; CAR-T: chimeric antigen receptor T-cell.

Table 1. Approved ICIs and combination regimens for HCC treatment

Treatment regimen	Drug class	Approved setting	Key study findings
Nivolumab	PD-1 inhibitor	Second-line (post-sorafenib)	CheckMate 040: Demonstrated a median OS of 15.6 months with favorable tolerability and durable clinical activity[9,13]
Pembrolizumab	PD-1 inhibitor	Second-line (post-sorafenib)	KEYNOTE-224: Yielded an ORR of 17% and a DCR of 62%, establishing notable survival benefits[8]
Atezolizumab + Bevacizumab ("T+A")	PD-L1 inhibitor + VEGF inhibitor	First-line	IMbrave150: Reported a median OS of 19.2 months and an ORR of 30%, showing efficacy significantly superior to sorafenib[10]
Durvalumab + Tremelimumab (STRIDE)	PD-L1 inhibitor + CTLA-4 inhibitor	First-line	HIMALAYA: Showed a median OS of 16.4 months and an unprecedented 5-year OS rate of 25.2%, highlighting the durable survival tail of dual checkpoint blockade[11,12]
Sintilimab + Bevacizumab biosimilar	PD-1 inhibitor + VEGF inhibitor	First-line (China)	ORIENT-32: Demonstrated an ORR of 29.8% with significant OS and PFS benefits, establishing a new standard for Chinese patient populations[14]

HCC: Hepatocellular carcinoma; ICIs: immune checkpoint inhibitors; ORR: objective response rate; PD-1: programmed cell death protein 1; OS: overall survival; DCR: disease control rate; PD-L1: programmed death-ligand 1; STRIDE: Single Tremelimumab Regular Interval Durvalumab; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; VEGF: vascular endothelial growth factor; PFS: progression-free survival.

Table 2. Resistance landscape of major immunotherapeutic strategies in hepatocellular carcinoma and potential links to the gut microbiota

Treatment strategy	Representative regimens	Clinical setting	Core resistance mechanism	Gut microbiota-related associations and interventions	Level of evidence and study type
ICI monotherapy (PD-1/PD-L1)	Nivolumab, Pembrolizumab	Second- or later-line advanced HCC	"Immune-cold" TME; lacking $CD8^+$ T-cell infiltration; Wnt/β-catenin activation impairing DC recruitment; defective antigen presentation (MHC-I loss)	Low diversity and depletion of beneficial taxa (e.g., Akkermansia) correlate with primary resistance; dysbiosis sustains TLR4-driven immunosuppression; FMT may convert "cold" to "inflamed" tumors	High: Human clinical cohorts and preclinical murine models[27]
ICI + Anti-angiogenic therapy	Atezolizumab + Bevacizumab; PD-1 + VEGFR-TKI	First-line unresectable HCC	Incomplete vascular normalization limiting T-cell trafficking; VEGF-driven enrichment of Tregs/MDSCs; adaptive resistance via alternative angiogenic pathways	Gut-derived PAMPs (e.g., LPS) activate the gut-microbiota-liver axis to enhance VEGF; microbiota-regulated bile acids and SCFAs affect vascular-immune homeostasis	Moderate: Human clinical cohorts and preclinical murine models[28]
Dual immune checkpoint blockade	STRIDE (Durvalumab + Tremelimumab); Nivolumab + Ipilimumab	First- or second-line immune-intensified therapy	Deep $CD8^+$ T-cell exhaustion; compensatory upregulation of TIM-3, LAG-3, TIGIT; TGF-β/IL-10-rich suppressive TME	Microbial metabolites (SCFAs, tryptophan derivatives) regulate effector T-cell *vs.* Treg balance; favorable microbiota linked to higher TCR diversity and delayed secondary resistance	Moderate: Early human cohorts and preclinical murine models[29]
Emerging checkpoint inhibitors	LAG-3, TIGIT, TIM-3 inhibitors (in development)	Multi-line ICI-refractory or combination settings	Redundant and compensatory checkpoint networks after PD-1/CTLA-4 blockade; immune editing and reduced neoantigen load	Chronic microbiota-driven inflammation promotes co-expression of multiple inhibitory receptors; restoring microbial homeostasis may improve therapeutic specificity	Low: Predominantly preclinical murine models and *in vitro* studies[30]
Gut microbiota-targeted strategies	FMT, next-generation probiotics, synbiotics, postbiotics	Primary or acquired ICI resistance; priming or sensitization	Dysbiosis with loss of immunostimulatory taxa and enrichment of pathobionts; barrier disruption and microbial translocation driving MDSC/Treg-dominant TME	Donor-selected FMT, targeted probiotics, and modulation of SCFAs/bile acids may reprogram the gut-liver-tumor axis and reverse ICI resistance	Moderate to High: Phase I/II human clinical trials and preclinical murine models[31]
Adoptive cell therapy	CAR-T, TCR-T, TILs (investigational)	Heavily pretreated, antigen-selected HCC	Antigen heterogeneity or loss; metabolically hostile TME (hypoxia, lactate); poor persistence and early exhaustion of infused cells	Microbiota shapes systemic inflammation and T-cell metabolism; microbiota-based conditioning may enhance infused cell persistence and reduce toxicity	Low: Preclinical murine models (extrapolated from other solid tumors)[32]
ICI + Locoregional therapy	ICI + TACE, radiotherapy, or ablation	Intermediate-stage or oligofocal disease	Limited/transient abscopal effects; therapy-induced inflammation recruiting MDSCs/TAMs	Locoregional therapies disrupt the gut barrier, increasing endotoxin translocation; peri-procedural microbiota-protective interventions may enhance immune synergy	Moderate: Observational human studies and preclinical murine models[33]
Engineered microbes/Synthetic biology	Engineered bacteria, OMVs, synthetic consortia	Experimental, early-phase developmen	Limited tumor-specific colonization; rapid innate immune clearance; biosafety and dose-control challenges	Microbiota-informed design of engineered microbes may enable precise tumor targeting with reduced systemic toxicity	Low: Preclinical murine models and *in vitro* studies[34]

HCC: Hepatocellular carcinoma; ICI: immune checkpoint inhibitor; TME: tumor microenvironment; TLR4: Toll-like receptor 4; FMT: fecal microbiota transplantation; MDSCs: myeloid-derived suppressor cells; LPS: lipopolysaccharide; SCFAs: short-chain fatty acids; TCR: T-cell receptor; TACE: transarterial chemoembolization; TAMs: tumor-associated macrophages; OMVs: outer membrane vesicles; Tregs: regulatory T cells; VEGFR-TKI: vascular endothelial growth factor receptor tyrosine kinase inhibitor; PD-1: programmed cell death protein 1; PD-L1: programmed death-ligand 1; $CD8^+$: cluster of differentiation 8 positive; DC: dendritic cell; MHC-I: major histocompatibility complex class I; PAMPs: pathogen-associated molecular patterns; VEGF: vascular endothelial growth factor; STRIDE: Single Tremelimumab Regular Interval Durvalumab; TIM-3: T-cell

immunoglobulin and mucin-domain containing-3; LAG-3: lymphocyte-activation gene 3; TIGIT: T-cell immunoreceptor with Ig and ITIM domains; TGF-β: transforming growth factor beta; IL-10: interleukin-10; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; CAR-T: chimeric antigen receptor T-cell; TCR-T: T-cell receptor-engineered T-cell; TILs: tumor-infiltrating lymphocytes.

The clinical challenge is profound, as the overarching efficacy of immunotherapy is fundamentally constrained by these resistance patterns[37]. However, the traditional tumor-centric perspective cannot fully explain the observed inter-patient heterogeneity in treatment response, pointing to the involvement of systemic host factors[38,39]. Recent evidence highlights the gut microbiota, operating through the "gut-microbiota-liver axis", as a key systemic regulator of hepatic anti-tumor immunity and a decisive factor in ICI efficacy[40,27]. To overcome the resistance observed in major trials such as IMbrave150, future efforts must focus on translating microbiome research into clinical practice. This includes developing microbiome-based companion diagnostics for patient stratification and integrating microbial "preconditioning" [e.g., fecal microbiota transplantation (FMT) or next-generation probiotics] with established ICI regimens. Such strategies, currently being explored in early-phase trials [e.g., Fecal Microbiota Transplant combined with atezolizumab/bevacizumab in patients with hepatocellular carcinoma (FAB-HCC)[41]], aim to transform the gut microbiota from a driver of resistance into a therapeutic ally, potentially breaking the current efficacy bottleneck in HCC immunotherapy. This review systematically explores the mechanisms by which the gut microbiota influences immunotherapy resistance in HCC and evaluates the translational potential of microbiota-targeting strategies to overcome this challenge.

THE GUT-MICROBIOTA-LIVER AXIS: A BRIDGE CONNECTING GUT MICROBES AND HEPATIC IMMUNITY

The anatomical and physiological basis of the "gut microbiota-liver axis"

The gut microbiota is a vast and dynamic microbial community residing within the human intestinal tract, primarily composed of bacteria, archaea, fungi, and viruses. The collective sum of their genomes is formally defined as the "microbiome". The functional repertoire encoded by this microbiome vastly exceeds that of the human genome, earning it the designation of the human "second genome"[42]. Beyond its fundamental roles in nutrient digestion and metabolic homeostasis, the gut microbiota plays a central role in educating host immunity and maintaining intestinal barrier integrity. In the context of HCC, the gut-microbiota-liver axis serves as a crucial bidirectional communication hub. Its unique functionality is rooted in distinctive anatomical connectivity and intricate physiological cross-talk.

The portal venous system constitutes the anatomical cornerstone of this axis. Venous blood from the intestines drains directly into the liver via the portal vein. This vascular hardwiring allows bioactive products derived from the gut microbiota - such as microbiota-associated molecular patterns (MAMPs), metabolic byproducts, and trace amounts of translocated bacteria - to bypass the systemic circulation and undergo efficient "first-pass" exposure within the hepatic sinusoids [Figure 2][43]. Consequently, the liver physiologically becomes the "first-line" organ for sensing and processing gut-derived signals. The liver is rich in innate immune cells, particularly resident macrophages known as Kupffer cells. These cells, equipped with an abundance of pattern recognition receptors (PRRs) [e.g., Toll-like receptor 4 (TLR4)], continuously monitor these gut-derived signals, thereby coupling the dynamic state of the gut microbiota in real-time with the liver's immune milieu[44].

Intestinal barrier integrity acts as the critical gatekeeper maintaining this homeostasis. An intact epithelial barrier effectively restricts the pathological translocation of bacteria and their structural components.

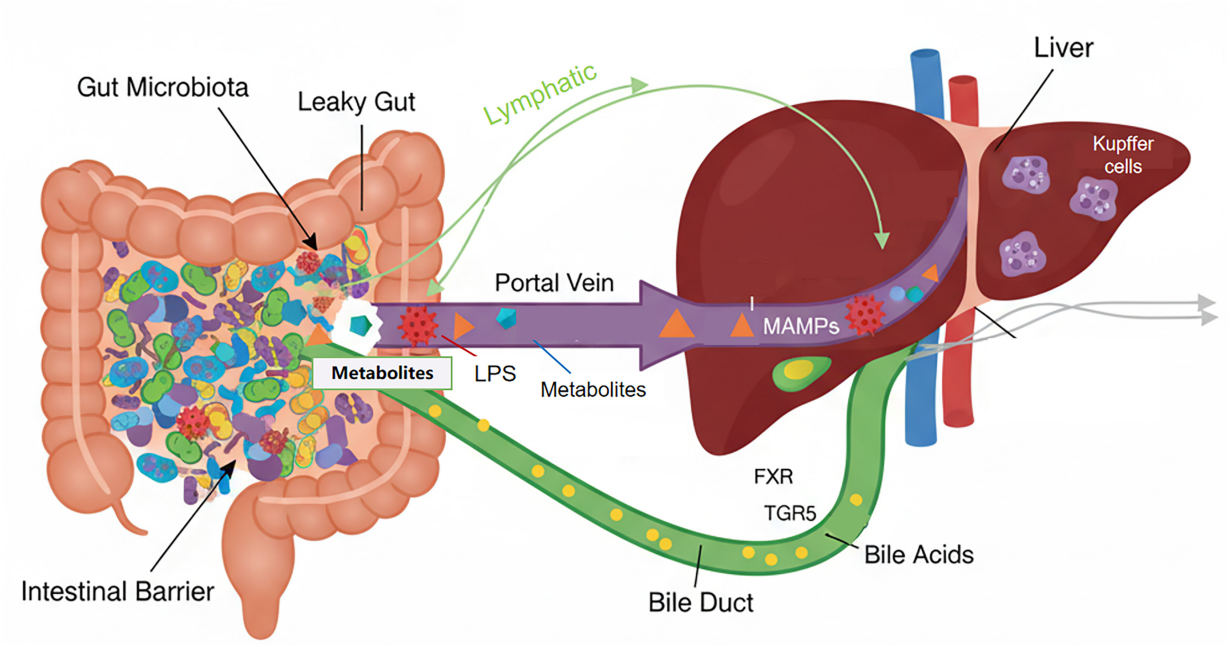

Figure 2. Anatomical and functional bidirectional gut-microbiota-liver axis in HCC. Schematic diagram illustrating key communication pathways with simplified components. Solid purple tube/arrows indicate the portal vein, transporting gut-derived signals; thin green arrows indicate lymphatic pathways; and solid green tube/arrows indicate the biliary tract pathway. Character definitions: Red spiky spheres (LPS); Orange triangles (MAMPs); Blue/green polygons (Gut-derived metabolites); Yellow spheres (Bile acids). Specific physiological mechanisms and cellular signaling details are provided in the corresponding text. HCC: Hepatocellular carcinoma; LPS: lipopolysaccharide; MAMPs: microbiota-associated molecular patterns; FXR: farnesoid X receptor; TGR5: Takeda G protein-coupled receptor 5.

Conversely, barrier dysfunction (often termed "leaky gut") precipitates the influx of highly immunogenic substances, such as lipopolysaccharide (LPS), into the portal circulation. This endotoxemia triggers persistent hepatic inflammatory responses and fosters a profoundly immunosuppressive microenvironment conducive to tumorigenesis[45-47]. Beyond MAMPs, enterohepatic bile acid circulation establishes another sophisticated bidirectional regulatory loop. Primary bile acids synthesized by the liver enter the intestine, where they are biotransformed by the microbiota into secondary bile acids with distinct bioactivities. These secondary metabolites can activate critical nuclear receptors [e.g., farnesoid X receptor (FXR)][48] and membrane-bound receptors [e.g., Takeda G protein-coupled receptor 5 (TGR5)][49] within hepatocytes to modulate both metabolic pathways and innate immunity. Concurrently, these bile acids exert antimicrobial effects, forming a feedback loop that shapes the composition and spatial organization of the gut microbiota itself, culminating in a dynamically balanced regulatory system[50,51]. The complexity of this axis is further amplified by systemic network extensions, including lymphatic trafficking and neural connections between the gut and liver, which collectively constitute a multi-layered bidirectional communication network beyond the portal vein[52,53].

In summary, the gut-microbiota-liver axis is an integrated biological system encompassing anatomical hardwiring, continuous material exchange, and sophisticated signal transduction. It empowers the gut microbiota to exert systemic, remote control over the hepatic immune microenvironment, thereby providing a robust physiological rationale for targeting the microbiota to overcome immunotherapy resistance in HCC.

Targeting the gut microbiota is a promising strategy to overcome drug resistance in HCC

The efficacy of immunotherapy for HCC exhibits significant individual variability, rooted not only in the tumor itself but also intimately linked to the systemic immune state of the host. In recent years, the gut microbiota has been established as a key systemic modulator and a core factor driving differential responses to ICI therapy, demonstrating dual potential as both a predictive biomarker and a therapeutic target.

Table 3. Summary of differentiated microbiota characteristics between HCC immunotherapy responders and non-responders

Species/Taxa	Enriched population	Clinical outcome	Relevant metabolites or functions	Potential immunomodulatory mechanisms	Research methods	Level of evidence and study type
Lachnoclostridium	Responders	Prolonged OS[54]	Secondary bile acids (e.g., UDCA)	Regulates bile acid metabolism and produces immunomodulatory metabolites	Metagenomics	Moderate: Human clinical cohort
Veillonella	Responders	Prolonged OS[54]	Propionate, acetate	Maintain microbial community stability and influence systemic inflammatory status	16S rRNA Sequencing	Moderate: Human clinical cohort
Coprococcus comes	Responders	Positively correlated with ORR[27]	Butyrate	Digests dietary fiber and produces immunomodulatory SCFAs	Metagenomics	High: Human clinical cohort and murine FMT validation
Ruminococcus gnavus	Non-responders	Decreased OS[55]	Mucus degradation	Disrupts the intestinal mucus layer, increases intestinal permeability, and promotes systemic inflammation	16S rRNA Sequencing	Moderate: Human clinical cohort
Enterococcaceae (family)	Non-responders	Decreased OS[56]	Associated with barrier damage	Disrupts the intestinal epithelial barrier, increases microbial translocation, and drives systemic inflammation	16S rRNA Sequencing	Moderate: Human clinical cohort
Bacteroides thetaiotaomicron	Responders (context-dependent)	Reduced risk of tumor recurrence[57]	Acetate	Produces acetate, promotes M1 macrophage polarization, and enhances $CD8^+$ T-cell effector function	Metagenomics, metabolomics	High: Human clinical cohort and *in vivo* models
Subdoligranulum	Responders	Improved immune response[27]	Butyrate （Putative）	Regulates intestinal immune homeostasis and produces anti-inflammatory metabolites	Metagenomics	Moderate: Human clinical cohort
Roseburia	Responders	Prolonged PFS[58]	Butyrate	Produces butyrate, enhances intestinal barrier function, and regulates T-cell differentiation	16S rRNA Sequencing	Moderate: Human clinical cohort

HCC: Hepatocellular carcinoma; UDCA: ursodeoxycholic acid; ORR: objective response rate; SCFAs: short-chain fatty acids; FMT: fecal microbiota transplantation; OS: overall survival; $CD8^+$: cluster of differentiation 8 positive; PFS: progression-free survival.

Through metagenomic, 16S rRNA sequencing, and metabolomic analyses of HCC patients undergoing ICI treatment, studies consistently reveal systematic differences in the gut microbiota between responders and non-responders in terms of species composition, ecological networks[27], and functional output [Table 3][54-58]. For instance, early clinical observations from specific cohorts suggest that responders' intestines are often enriched with beneficial genera such as *Akkermansia*, Lachnospiraceae, and *Faecalibacterium prausnitzii*, whose metabolic output is associated with enhanced anti-tumor immunity[28]. However, it is critical to interpret these associations with caution. Microbiome studies in oncology are notoriously heterogeneous, and the identification of universal predictive taxa has proven challenging. Microbial signatures predictive of ICI response in one cohort often fail to reproduce in independent cohorts across different geographic regions or hospital centers[59]. Conversely, non-responders frequently exhibit a dysbiotic pattern characterized by genera such as *Ruminococcus* and Enterococcaceae, often accompanied by the formation of an immunosuppressive microenvironment[60]. These differential profiles hold promise as potential biomarkers, but their broad clinical utility demands validation in large-scale, multi-center prospective trials.

While ICI-based combination therapies have significantly improved the treatment landscape for advanced HCC, primary and acquired resistance mediated by an immune-"cold" tumor microenvironment remains a central bottleneck. Traditional localized sensitization strategies are often limited by profound tumor heterogeneity; thus, targeting the gut microbiota offers a novel "outside-in" systemic intervention paradigm. The feasibility of this strategy is firmly rooted in the unique pathophysiological wiring of the gut-microbiota-liver axis in HCC. Against the backdrop of chronic liver disease, impaired intestinal barrier function drives the continuous translocation of microbial products (e.g., lipopolysaccharide, LPS), which act directly on the liver via the portal vein. By engaging PRRs (e.g., TLR4) on hepatic innate immune cells (e.g., Kupffer cells), these translocated products trigger chronic local inflammation and actively sculpt an inhibitory immune niche[61,62]. Consequently, intervening at the level of the gut microbiota effectively intercepts erroneous immune signaling at its source, achieving a "remote reprogramming" of the TME.

In-depth mechanistic studies elucidate that the gut microbiota precisely regulates therapeutic resistance through a complex, multi-pathway network[63]. Specific microbial taxa can directly orchestrate a resistant microenvironment. For example, specific pathobionts such as *Fusobacterium nucleatum* and *Escherichia coli* (*E. coli*) have been shown to upregulate host multidrug resistance efflux pumps - most notably ATP binding cassette subfamily B member 1 (ABCB1) (P-glycoprotein) and ATP binding cassette subfamily C member 1 (ABCC1) - on the surface of malignant cells, thereby actively pumping out chemotherapeutic agents and targeted drugs. Concurrently, these microbial signals can activate the Janus kinase 1/protein kinase B/signal transducer and activator of transcription 3 (JAK1/AKT/STAT3) signaling cascade to inhibit tumor cell apoptosis, or induce protective autophagy via the TLR4/myeloid differentiation primary response 88 (MyD88) axis, collectively dampening the efficacy of systemic therapies[64-66]. Furthermore, preclinical studies demonstrate that FMT from clinically resistant patients is sufficient to transfer the ICI-resistant phenotype to recipient mice[67]. Simultaneously, specific microbiota-derived metabolites, such as indole-3-acetic acid (IAA) and butyrate, have been shown under certain contexts to directly impair anti-tumor immunity by enhancing the suppressive function of regulatory T cells or by exacerbating the pre-cancerous inflammatory environment via interleukin-35 (IL-35)[68,69]. Conversely, beneficial dominant bacteria and their metabolites can play crucial sensitizing roles. For instance, specific Clostridium species can reverse the Wnt/β-catenin signal-dominated "immune desert" state by modulating secondary bile acid metabolism, promoting cytotoxic T cell infiltration[70]. Moreover, the tryptophan metabolite indole-3-aldehyde, derived from *Bifidobacterium*, acts as a competitive ligand for the aryl hydrocarbon receptor (AhR), antagonizing the potent immunosuppressive signals of tumor-derived kynurenine, restoring cluster of differentiation 8 positive ($CD8^+$) T-cell function, and downregulating inhibitory checkpoints such as TIM-3[71,72] [Figure 3].

Despite these compelling mechanistic insights, gut microbiome research is characterized by significant heterogeneity, with seemingly contradictory associations reported for certain taxa across different studies, underscoring the profound complexity of microbe-host interactions. For example, while the genus *Veillonella* was found to be enriched in responders in a study by Lee *et al.*[54], it is frequently associated with a pro-inflammatory state in contexts such as inflammatory bowel disease. These discrepancies may stem from several key factors: First, population and etiological heterogeneity - driven by differences in genetic backgrounds, underlying liver disease etiology [e.g., hepatitis B virus (HBV), hepatitis C virus (HCV), metabolic dysfunction-associated steatotic liver disease (MASLD)], dietary habits, and medication history among different cohorts collectively shape unique microbiota profiles[73]. Second, technical and methodological inconsistencies - ranging from sample collection and DNA extraction protocols to sequencing platforms (16S rRNA *vs.* shotgun metagenomics) and bioinformatics pipelines - drastically reduce cross-study reproducibility. Third, functional divergence at the strain level is critical; different strains within the same genus can harbor vastly different genomic and metabolic capacities. Metagenomic analyses increasingly suggest that strain-specific features, rather than species-level abundance, are the true determinants of biological function[58]. Additionally, methodological differences in research may also lead to inconsistent results[74].

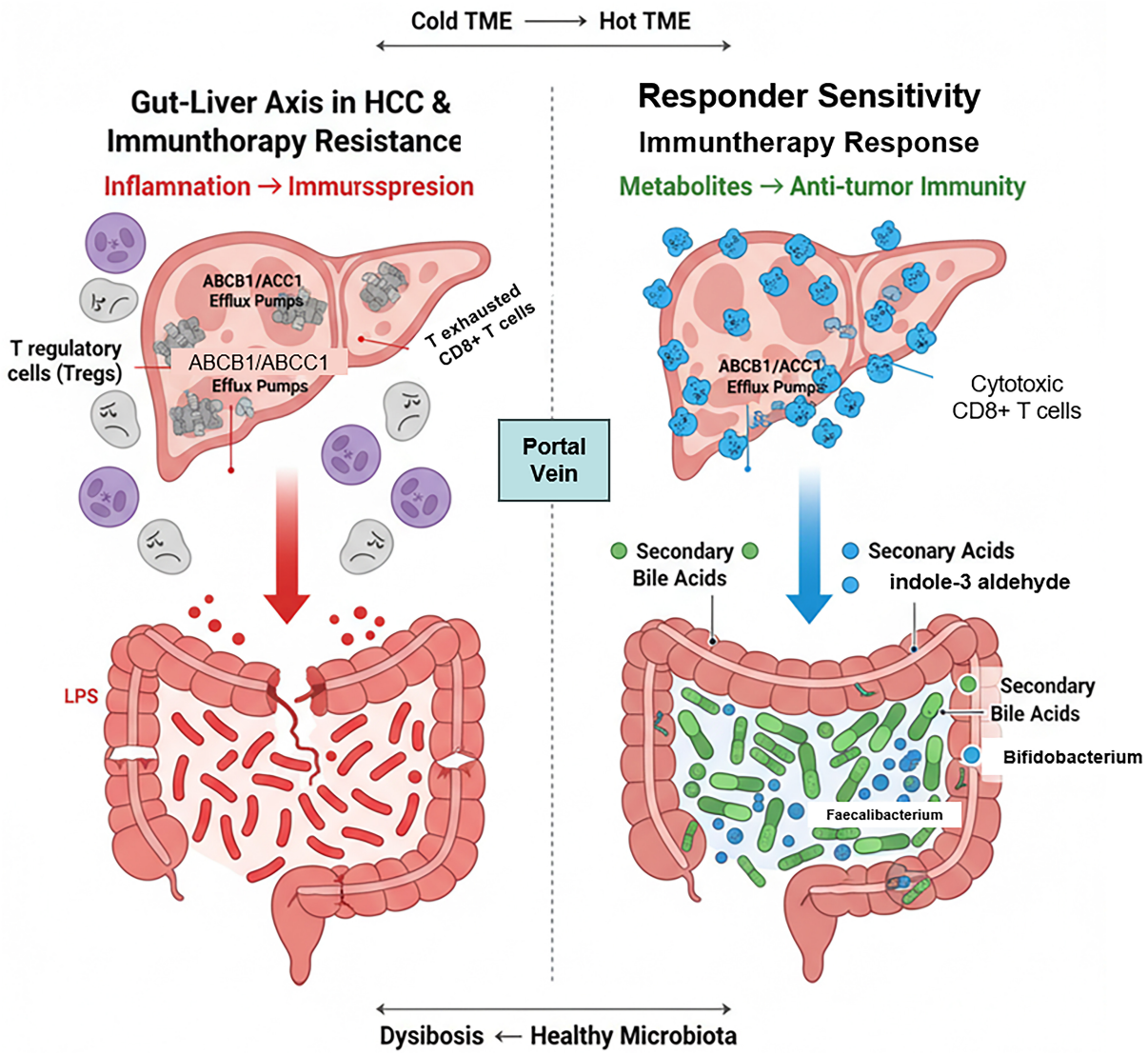

Figure 3. Mechanistic paradigm of the gut-microbiota-liver axis modulating ICI therapeutic response in HCC. Schematic illustrating the systemic immune and TME distinctions between non-responders (left) and responders (right). Arrows indicate flow directions (downward: harmful translocation; upward: beneficial metabolite transfer). Icons defined: red broken walls (leaky intestinal barrier); green intact walls (intact barrier). HCC: Hepatocellular carcinoma; ICI: immune checkpoint inhibitor; TME: tumor microenvironment; Tregs: regulatory T cells; ABCB1: ATP binding cassette subfamily B member 1; ACC1: acetyl-CoA carboxylase 1; ABCC1: ATP binding cassette subfamily C member 1; $CD8^+$: cluster of differentiation 8 positive; LPS: lipopolysaccharide.

Therefore, future predictive modeling must transcend reliance on single bacterial species and adopt a multi-dimensional, integrative strategy. This necessitates the combined analysis of specific strains with clear functional annotations, microbiota functional outputs (e.g., metabolomic profiles), and the ecological network structure of the microbial community. Combinatorial biomarkers - such as a structural ratio of "high *Akkermansia* + high *Faecalibacterium* + low *Ruminococcus*", the secondary-to-primary bile acid ratio, and the abundance of microbial butyrate synthesis genes - hold immense potential for accurately predicting ICI efficacy. Recently, machine learning frameworks have been deployed to navigate this complexity. For instance, one study utilized a random forest algorithm to integrate gut microbiota features (such as the relative abundances of *Akkermansia* and *Ruminococcus*) with clinical variables, constructing a robust predictive model that significantly outperformed any single clinical indicator in distinguishing treatment responses[75].

In summary, the gut microbiota represents a critical node for understanding and therapeutically intervening in the HCC immunotherapy response. Its composition and functional output directly dictate treatment outcomes by modulating intestinal barrier integrity, systemic inflammation, and the TME landscape. While current preclinical models and early clinical cohorts provide a robust conceptual foundation for developing microbiome-based companion diagnostics and precision intervention strategies, these approaches are still in their infancy. Moving forward, the field must transition from associative small-cohort studies to standardized, adequately powered, multi-center randomized clinical trials to critically evaluate their true clinical utility. Future translational research must dissect the microbe-host interaction network at a granular level, propelling HCC immunotherapy into a new era of systemic remodeling and precise regulation.

MECHANISM EXPLORATION: THE MOLECULAR PATHWAYS BY WHICH THE GUT MICROBIOTA RESHAPES THE IMMUNE MICROENVIRONMENT OF HCC

Microbiota composition and functional dynamics: the biological basis for shaping immune tone

The composition of the gut microbiota, at both the species and strain levels, is not a passive bystander but an active, preconfigured determinant that shapes hepatic immune surveillance. A healthy, diverse, and stable microbial community supports systemic immune homeostasis by maintaining intestinal barrier integrity and generating beneficial metabolites. Specific beneficial microbiota fortify host defenses through both direct and indirect mechanisms[76].

Akkermansia muciniphila (A. muciniphila) represents a prototypical species in this context. Its outer membrane protein, Amuc_1100, can specifically interact with Toll-like receptor 2 (TLR2) on intestinal epithelial cells. This interaction triggers downstream signaling cascades that promote the synthesis and secretion of mucin 2 (MUC2) by goblet cells, thereby thickening and fortifying the protective mucus layer[77]. Furthermore, short-chain fatty acids (SCFAs, e.g., acetate) produced by *A. muciniphila* metabolism can activate G protein-coupled receptors (e.g., GPR43) on epithelial cells, subsequently upregulating the expression of tight junction proteins and directly enhancing epithelial barrier function[78]. Human evidence robustly supports these preclinical findings: a randomized controlled trial demonstrated that supplementation with pasteurized *A. muciniphila* significantly reduced plasma levels of gut permeability markers (e.g., LPS-binding protein) and systemically improved host insulin sensitivity and inflammatory status. This provides crucial support for its translational value in remodeling the HCC immune microenvironment[79].

Beyond *A. muciniphila*, classical probiotics such as *Bifidobacterium* and *Lactobacillus* also reinforce the barrier through distinct functional pathways. For instance, *Bifidobacterium longum* subsp. *infantis* produces specific glycosyl hydrolases that degrade dietary fibers to generate functional oligosaccharides. These molecules serve as essential precursors to promote mucin synthesis[80]. Certain *Lactobacillus* strains, via the binding of their cell wall component lipoteichoic acid to TLR2 on dendritic cells, can strongly induce the production of IL-10 and transforming growth factor beta (TGF-β). This cytokine milieu, in turn, promotes Treg differentiation. These cells can secrete tissue repair factors, such as keratinocyte growth factor, directly stimulating epithelial cell proliferation and tight junction protein synthesis, thereby achieving an immune-mediated barrier repair[81].

Other beneficial bacteria, such as *Limosilactobacillus reuteri* (*L. reuteri*), can induce the expression of heat shock proteins in intestinal epithelial cells, enhancing cellular resistance to oxidative stress and maintaining epithelial integrity. A pilot study in cirrhotic patients found that *L. reuteri* supplementation successfully

reduced serum endotoxin levels and improved liver function scores, suggesting its therapeutic potential to alleviate liver disease via the gut-microbiota-liver axis[82]. In a study of patients with unresectable HCC, fecal samples from immunotherapy responders showed significant enrichment of Lachnospiraceae, *Lachnoclostridium*, and *Veillonella*. Notably, the abundance of *Lachnoclostridium* was highly positively correlated with systemic levels of secondary bile acids, such as ursodeoxycholic acid and ursocholic acid. Furthermore, the coexistence of *Lachnoclostridium* enrichment and *Prevotella* 9 depletion emerged as a significant predictor of superior overall survival[54]. *Coprococcus*, particularly *Coprococcus comes*, is another consistently observed critical genus in responders. This bacterium is closely associated with dietary fiber digestion and the robust production of butyrate; its abundance strictly correlates with a favorable treatment response. Microbial interaction network analyses have revealed denser positive correlations among species enriched in responders, indicating a more synergistic, resilient, and stable community structure[27].

Conversely, the enrichment of pathogenic bacteria driven by gut dysbiosis serves as a major risk factor promoting HCC progression and ICI resistance. The Fap2 protein of *Fusobacterium nucleatum* has been shown to specifically bind and activate the inhibitory receptor TIGIT on natural killer (NK) cells and T cells, directly suppressing the cytotoxic function of NK cells and $CD8^+$ T cells, thereby fostering a profoundly immunosuppressive niche at the tumor site[83]. A recent study combining metagenomics and liver tissue bacterial culture revealed a significant enrichment of *Klebsiella pneumoniae* in both the intestines and livers of HCC patients and their corresponding transplanted mouse models. Mechanistic models demonstrated that monocolonization with this specific bacterium was sufficient to drive HCC development by disrupting the gut barrier and inducing severe systemic and local hepatic inflammation. The core mechanism involves the direct interaction of the bacterial surface protein penicillin-binding protein 1B (PBP1B) with the TLR4 receptor on hepatocytes, thereby hyperactivating downstream oncogenic signaling pathways[84]. This crucial finding not only elucidates the pathogenic role of specific gut pathobionts in HCC but also provides novel targets for developing precision therapies targeting the "pathogen-host" interaction interface. Similarly, *Ruminococcus* was consistently found enriched in non-responders. In studies related to chronic hepatitis B progression, *Ruminococcus* enrichment was associated with delayed viral clearance and the maintenance of an immune-tolerant state. It may indirectly foster an inhibitory hepatic immune environment by encoding bile salt hydrolase (BSH), which severely alters the host's bile acid metabolic balance[55]. Additionally, Enterococcaceae exhibited a clear trend of enrichment during HCC progression, with its relative abundance significantly increasing from early to advanced disease stages. This dynamic change was heavily correlated with elevated levels of the gut injury marker regenerating islet-derived protein 3 alpha (REG3α) and the microbial translocation marker soluble cluster of differentiation 14 (sCD14), underscoring its potential role in disrupting intestinal barrier integrity and driving relentless systemic inflammation[56].

In summary, the homeostasis of intestinal barrier function serves as a crucial and actionable target within the HCC immune landscape [Figure 4]. Future microbiota-targeted intervention strategies must delicately balance "reinforcing the beneficial" and "eliminating the pathogenic". This dual strategy entails supplementing with beneficial consortia that possess barrier-repair functions, while simultaneously employing targeted eradication of specific pro-carcinogenic pathobionts or utilizing precise receptor (e.g., TLR4) signal modulation. Through such a comprehensive approach, it becomes possible to drastically reduce the antigenic and inflammatory burden on the liver at its source, laying the essential foundation for reversing the "cold" tumor microenvironment and maximizing the efficacy of immunotherapy.

Systemic messenger roles of metabolites

The double-edged sword effect of SCFAs

SCFAs, primarily generated by the gut microbiota through the fermentation of dietary fibers, play an indispensable role in maintaining intestinal immune homeostasis. However, within the tumor immune microenvironment of HCC, SCFAs (particularly butyrate, propionate, and acetate) exhibit a pronounced

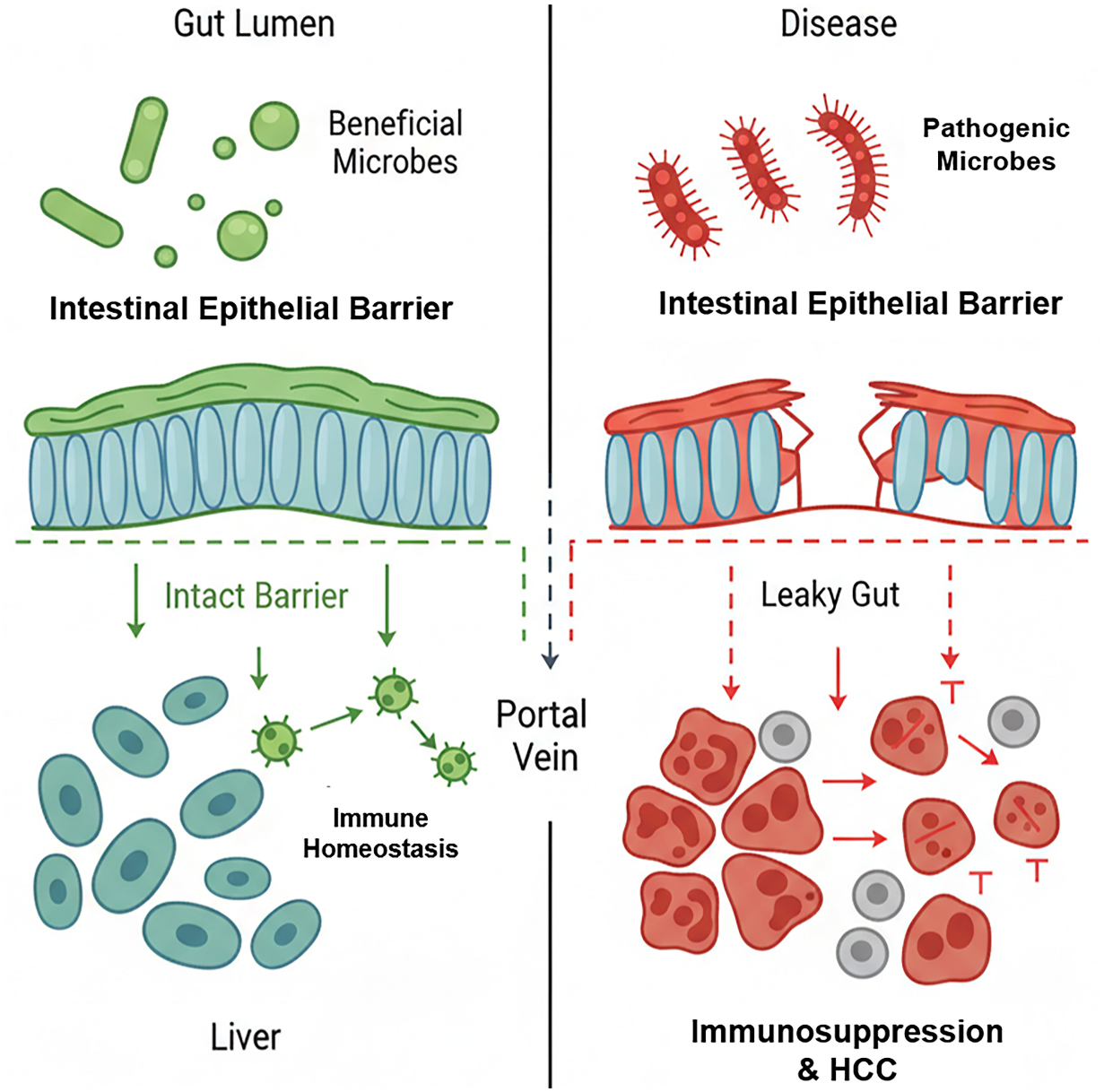

Figure 4. Bidirectional role of the gut microbiota-liver axis in shaping the immune microenvironment of HCC. The diagram illustrates the dual mechanisms by which microbiota composition dictates hepatic immune tone. Solid green arrows indicate direct beneficial interactions or activation; solid red arrows indicate pathogenic activation or oncogenic signaling; dashed arrows represent translocation from the gut to the liver via the portal vein; red T-bars represent direct suppression or inhibition of immune cells. Detailed cellular and molecular mechanisms, including specific microbial strains, are discussed in the corresponding text. HCC: Hepatocellular carcinoma.

"double-edged sword" characteristic [Figure 5]. On one hand, they actively sculpt an immunosuppressive microenvironment via multiple parallel pathways, such as driving Treg differentiation and inducing T-cell exhaustion via histone deacetylase (HDAC) inhibition. On the other hand, butyrate can also stimulate potent anti-tumor immunity. Specifically, butyrate can induce chromatin remodeling in HCC cells, leading to the upregulation of the chemokine C-X-C motif chemokine ligand 11 (CXCL11). This effectively recruits NK cells to infiltrate the tumor site and exert anti-tumor cytotoxic effects[85]. Thus, the net immunological outcome of SCFAs demonstrates a profound dependence on local concentration gradients and the specific cellular context.

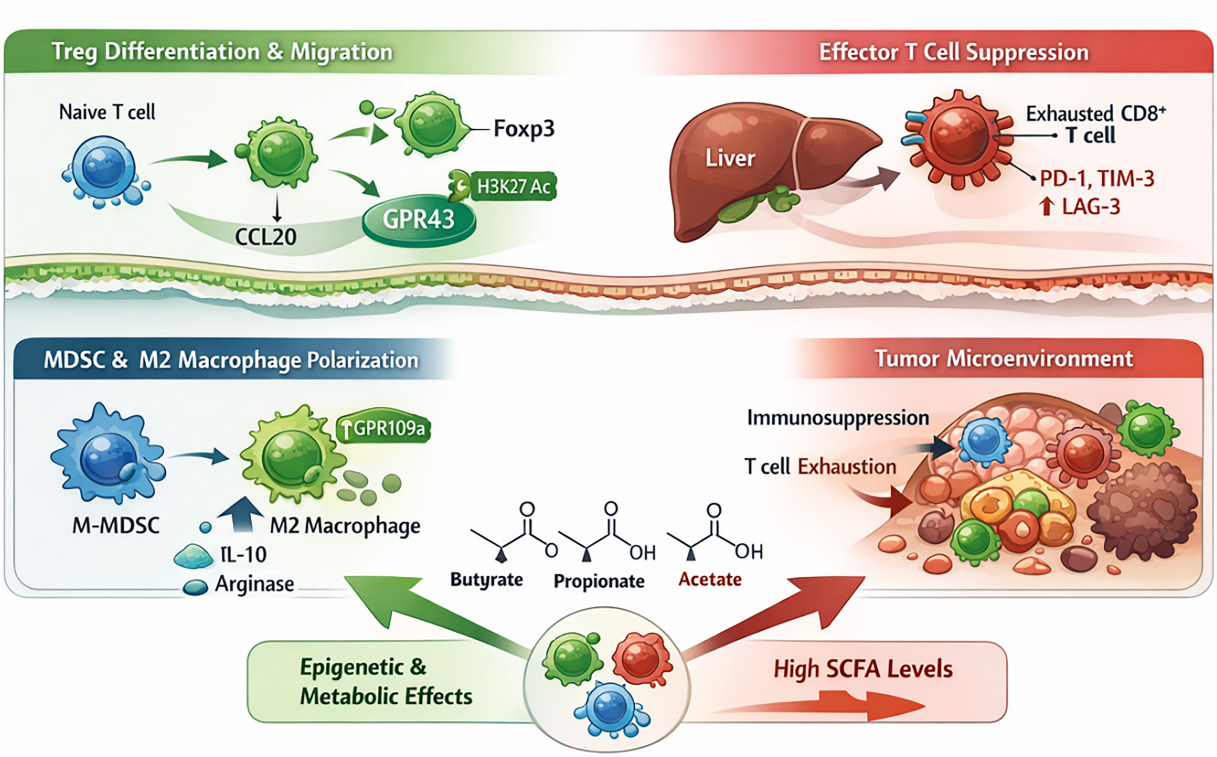

Figure 5. The immunosuppressive "edge" of the SCFA double-edged sword in the HCC microenvironment. While SCFAs exhibit context-dependent anti-tumor properties (discussed in the text), this schematic illustrates their primary pathways driving immune tolerance. Green arrows indicate differentiation towards suppressive phenotypes (Tregs and M2 macrophages); red arrows indicate pathways leading to effector $CD8^+$ T cell exhaustion; red up-arrows (↑) denote the upregulation of specific exhaustion markers (PD-1, TIM-3, LAG-3). Detailed receptor-mediated signaling (GPR43, GPR109a) and epigenetic regulations are explicitly discussed in the corresponding text. HCC: Hepatocellular carcinoma; SCFA: short-chain fatty acid; Tregs: regulatory T cells; $CD8^+$: cluster of differentiation 8 positive; PD-1: programmed cell death protein 1; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; LAG-3: lymphocyte-activation gene 3; CCL20: C-C motif chemokine ligand 20; GPR43: G protein-coupled receptor 43; H3K27 Ac: histone H3 lysine 27 acetylation; MDSC: myeloid-derived suppressor cell; GPR109a: G protein-coupled receptor 109A.

A primary immunosuppressive mechanism of SCFAs is the enhancement of Treg differentiation. Butyrate potently inhibits histone deacetylases, thereby epigenetically activating forkhead box P3 (Foxp3) expression, a master transcription factor critical for Treg lineage commitment[86]. Preclinical studies in HCC mouse models have demonstrated that systemic butyrate supplementation significantly increases intratumoral Treg accumulation and impairs $CD8^+$ T-cell effector functions, ultimately accelerating tumor progression[87,88]. Furthermore, SCFAs can induce Treg differentiation within gut-draining lymph nodes and promote their liver migration via chemokine axes such as C-C motif chemokine ligand 20 (CCL20)[89]. In addition to expanding suppressive populations, SCFAs reinforce immunosuppression by inducing metabolic reprogramming and profound exhaustion in $CD8^+$ T cells. Butyrate-mediated HDAC inhibition severely impairs mitochondrial function and upregulates the expression of co-inhibitory receptors, such as programmed cell death protein 1 (PD-1), TIM-3, and LAG-3, actively driving effector T cells into a terminally exhausted state[90]. Propionate, via a distinct mechanism involving the activation of mechanistic target of rapamycin complex 1 (mTORC1) signaling, promotes oxidative phosphorylation within Tregs. This metabolic rewiring enhances Treg survival and suppressive function under mitochondrial stress, allowing them to metabolically outcompete effector T cells in the nutrient-deprived tumor niche.

The regulatory role of SCFAs on MDSCs also warrants significant attention. Emerging research indicates that butyrate can promote the rapid expansion of monocytic MDSCs and potentiate their immunosuppressive functionality via the G protein-coupled receptor 109A (GPR109a) signaling pathway[89].

Meanwhile, SCFAs (particularly butyrate and acetate) have been shown to induce macrophage polarization toward an anti-inflammatory M2 phenotype, a process intricately associated with HDAC inhibition and cellular metabolic reprogramming[91]. Although this mechanistic axis was initially elucidated in intestinal resident macrophages, the underlying principles strongly suggest that within the HCC tumor microenvironment, SCFAs similarly propel the polarization of tumor-associated macrophages (TAMs) toward an M2-like phenotype, characterized by the secretion of IL-10 and arginase, thereby consolidating the immunosuppressive state.

In the clinical context of HCC, the correlation between aberrant SCFA profiles and immunotherapy resistance has been robustly confirmed[28]. A landmark study revealed that the gut microbiome of patients resistant to PD-1 inhibitors was significantly enriched with genes encoding key enzymes for butyrate synthesis. Correspondingly, tumor tissues from these patients exhibited a diminished $CD8^+$/Treg ratio[92], strongly suggesting that systemically elevated SCFA levels may serve as a negative predictive biomarker for immunotherapy response. These concentration- and microenvironment-dependent immunomodulatory effects perfectly encapsulate their dual nature[71,72,93].

From a signal transduction perspective, SCFA-mediated signaling via specific G protein-coupled receptors (e.g., GPR41, GPR43, GPR109a) also actively contributes to immune regulation. This response exhibits profound cell-type specificity, adding another layer of complexity to their regulatory network[94]. In summary, through epigenetic regulation, metabolic reprogramming, receptor-mediated signaling, and the multidimensional modulation of various immune populations, SCFAs play an extremely complex role in the HCC tumor microenvironment [Figure 5], with the net effect often tipping toward the promotion of immune tolerance. Future microbiota-targeted intervention strategies, such as the selective modulation of butyrate-producing consortia, the development of tissue-specific delivery systems, or the application of specific receptor modulators, hold significant translational potential for overcoming immunotherapy resistance in HCC.

"Biphasic regulation" of secondary bile acids (SBAs)

The gut microbiota exerts a far more complex role in the HCC immune microenvironment than traditionally recognized, primarily through its profound regulation of bile acid metabolism. Central to this role is the "biphasic regulatory" nature exhibited by SBAs - where the same overarching class of molecules can elicit diametrically opposing immunomodulatory effects under different metabolic contexts. The molecular basis of this phenomenon lies in the differential microbial biotransformation of primary bile acids by the intestinal microbiota: specific anaerobic bacteria convert primary bile acids into hydrophobic molecules, such as lithocholic acid (LCA) and deoxycholic acid (DCA)[95], via 7α-dehydroxylation, which generally drive immunosuppression and tumor evasion. Conversely, distinct bacterial metabolic pathways yield structurally and functionally divergent hydrophilic metabolites, including ursodeoxycholic acid (UDCA) and 3β-hydroxy-cholic acid (3β-HCA), which predominantly exert anti-tumor, immune-activating effects[51]. This striking dichotomy in metabolic pathways not only reflects individual heterogeneity in microbial community composition but also directly sets the systemic tone of hepatic immune surveillance [Figure 6].

On the immunosuppressive axis, hydrophobic SBAs actively promote HCC progression and immune evasion. In non-alcoholic steatohepatitis (NASH)-associated HCC models, the aberrant accumulation of DCA drives tumor development[96]. Mechanistically, DCA activates mechanistic target of rapamycin (mTOR) signaling within hepatocytes[97] and downregulates the expression of CXCL16 in liver sinusoidal endothelial cells, thereby severely impairing natural killer T (NKT) cell recruitment and actively contributing to the formation of a "cold" tumor microenvironment [Figure 6][95].

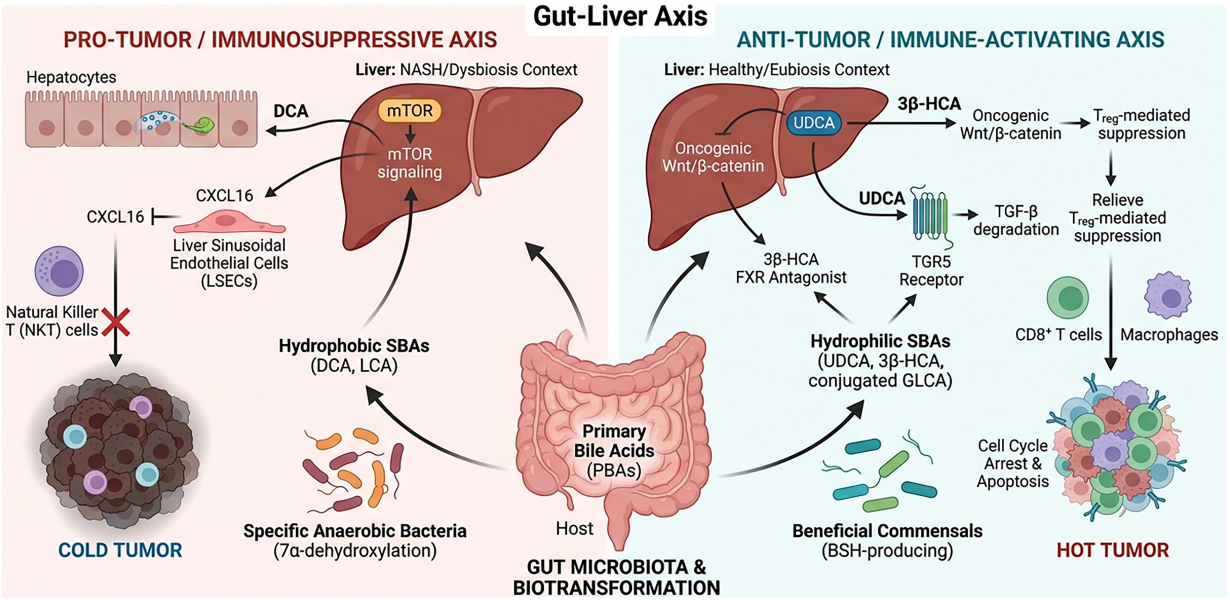

Figure 6. Biphasic regulation of SBAs in the HCC gut-liver axis. The schematic contrasts the immunosuppressive pathways driven by hydrophobic SBAs (left) with the immune-activating pathways driven by hydrophilic SBAs (right). Black arrows indicate pathway activation, metabolite conversion, or cellular recruitment; Red cross (X) indicates the blockade of immune cell infiltration. Detailed specific microbial conversions (e.g., 7α-dehydroxylation) and receptor-mediated signaling (mTOR, FXR, TGR5) are comprehensively discussed in the corresponding text. HCC: Hepatocellular carcinoma; SBAs: secondary bile acids; DCA: deoxycholic acid; FXR: farnesoid X receptor; NKT: natural killer T; LCA: lithocholic acid; UDCA: ursodeoxycholic acid; 3β-HCA: 3β-hydroxy-cholic acid; GLCA: glycolithocholic acid; BSH: bile salt hydrolase; CXCL15: C-X-C motif chemokine ligand 15; LSECs: liver sinusoidal endothelial cells; mTOR: mechanistic target of rapamycin; TGR5: Takeda G protein-coupled receptor 5; TGF-β: transforming growth factor beta; PBAs: primary bile acids; $CD8^+$: cluster of differentiation 8 positive.

Human epidemiological studies robustly corroborate this immunosuppressive axis. A prospective cohort study of viral hepatitis patients demonstrated a significant positive correlation between pre-diagnostic circulating bile acid concentrations and the subsequent risk of HCC[98]. The Singapore Chinese Health Study further substantiated this relationship by profiling serum bile acids using ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS). The study revealed that HCC patients exhibited significantly elevated levels of conjugated primary bile acids in their serum, alongside a markedly lower ratio of secondary to primary bile acids compared to healthy controls. This strongly suggests that an imbalance in the microbial conversion of primary to secondary bile acids serves as a critical predictor of HCC risk[99].

Conversely, specific hydrophilic SBAs generated by beneficial commensals can actively reverse hepatic immune tolerance. For instance, 3β-HCA acts as a naturally occurring FXR antagonist, effectively inhibiting oncogenic Wnt/β-catenin signaling and relieving the repression of T-cell-recruiting chemokines[100]. Similarly, UDCA activates the TGR5 receptor, promoting the degradation of TGF-β and directly relieving Treg-mediated suppression of effector T cells[101]. Clinical transcriptomic profiling has further revealed that HCC patients exhibiting a high "bile acid metabolism profile" - quantitatively defined by the coordinated transcriptomic upregulation of a specific signature of host genes governing bile acid biosynthesis, active transport, and biotransformation [e.g., cytochrome P450 family 7 subfamily A member 1 (CYP7A1), solute carrier family 10 member 1 (SLC10A1)] - demonstrate superior survival outcomes. This favorable metabolic phenotype is characterized by significantly enhanced intra-tumoral infiltration of $CD8^+$ T cells and macrophages, functioning in tandem with distinct beneficial gut microbiota and metabolic signatures[102].

Intervention studies further illuminate the intricate complexity of this biphasic regulation. One comprehensive study found that the proportion of serum SBAs, particularly conjugated DCA, was significantly reduced in both HCC patients and murine models. Concurrently, the fecal abundance of

BSH-producing bacteria was markedly depleted[103]. While vancomycin treatment further eradicated these bacteria and promoted liver tumor growth, exogenous supplementation with conjugated secondary bile acids - specifically glycolithocholic acid (GLCA) - effectively inhibited HCC proliferation and invasion both *in vivo* and *in vitro*. The underlying mechanism involves the induction of cell cycle arrest and mitochondrial apoptosis[104]. This critical finding underscores that the specific molecular conjugation status of certain SBAs (e.g., conjugated *vs.* free forms) profoundly dictates their biological functionality.

Ultimately, the biphasic regulation by SBAs reflects a highly dynamic equilibrium between pro- and anti-tumor signals. In the context of HCC, the vicious cycle of chronic inflammation, intestinal barrier damage, and severe gut dysbiosis fundamentally disrupts this balance, allowing hydrophobic SBAs to dominate the metabolic pool and systemically suppress anti-tumor immunity[105].

Other key metabolites

Beyond SCFAs and secondary bile acids, the gut microbiota orchestrates host immunity through the biotransformation of other key molecules and the shedding of its own structural components, forming a profound regulatory network that influences immunotherapy outcomes in HCC [Figure 7].

In mammalian cells, approximately 95% of tryptophan is metabolized via the kynurenine pathway, primarily driven by indoleamine 2,3-dioxygenase 1 (IDO1). Conversely, approximately 5% of unabsorbed tryptophan in the gastrointestinal tract can be converted by specific microbial species (such as Bifidobacterium) into indole derivatives[106]. Tryptophan catabolism plays a pivotal role in shaping the immune phenotype of HCC. Within tumor cells and tumor-infiltrating myeloid cells, the enzyme IDO1 oxidatively cleaves tryptophan into kynurenine. Kynurenine subsequently activates the AhR, driving a potent immunosuppressive cascade by promoting Treg differentiation, inducing effector T-cell exhaustion, and polarizing TAMs toward an anti-inflammatory M2 phenotype[107]. Notably, the kynurenine-AhR signaling axis has been identified as a critical upstream driver for upregulating the expression of the immune checkpoint TIM-3 on T cells, thereby directly exacerbating the immune-"cold" tumor phenotype of HCC.

The gut microbiota provides a crucial counteracting pathway by converting unabsorbed tryptophan into various indole derivatives, which function as competitive AhR ligands that elicit distinct, context-dependent immunological outcomes. In cohorts of HCC patients resistant to PD-1 inhibitors, a diminished abundance of Bifidobacterium strongly correlates with high kynurenine-to-tryptophan ratios and an elevated proportion of TIM-3$^+$ CD8$^+$ T cells[108]. Preclinical models demonstrate that intestinal colonization with Bifidobacterium actively shifts tryptophan metabolism toward the production of indole-3-aldehyde. This microbially derived metabolite effectively antagonizes kynurenine-mediated AhR signaling, downregulates the T-cell exhaustion transcriptional program, and ultimately reverses PD-1 inhibitor resistance[109].

Transitioning from soluble metabolites to microbial structural components, Polysaccharide A (PSA) - a capsular polymer derived from *Bacteroides fragilis* (*B. fragilis*) - also acts as a potent immunomodulator. PSA directly engages TLR2 on dendritic cells, promoting robust IL-10 production and subsequent Treg differentiation[110]. More importantly, this immunomodulatory effect has profound systemic implications. In HCC models, pre-treatment via oral supplementation of PSA-producing *B. fragilis* significantly suppressed hepatic inflammatory responses and reduced the incidence of chemically induced HCC[111]. The underlying mechanism involves a PSA-induced state of systemic immune tolerance, which helps control pathological, non-specific inflammation but may concurrently suppress anti-tumor immune surveillance, perfectly reflecting its dual nature in cancer biology[112]. However, when strategically combined with ICIs, the immunomodulatory role of PSA may present a new therapeutic dimension; emerging studies suggest it can enhance the antigen-presenting capacity of dendritic cells and promote the initiation of robust T helper 1 (Th1)-type immune responses[113].

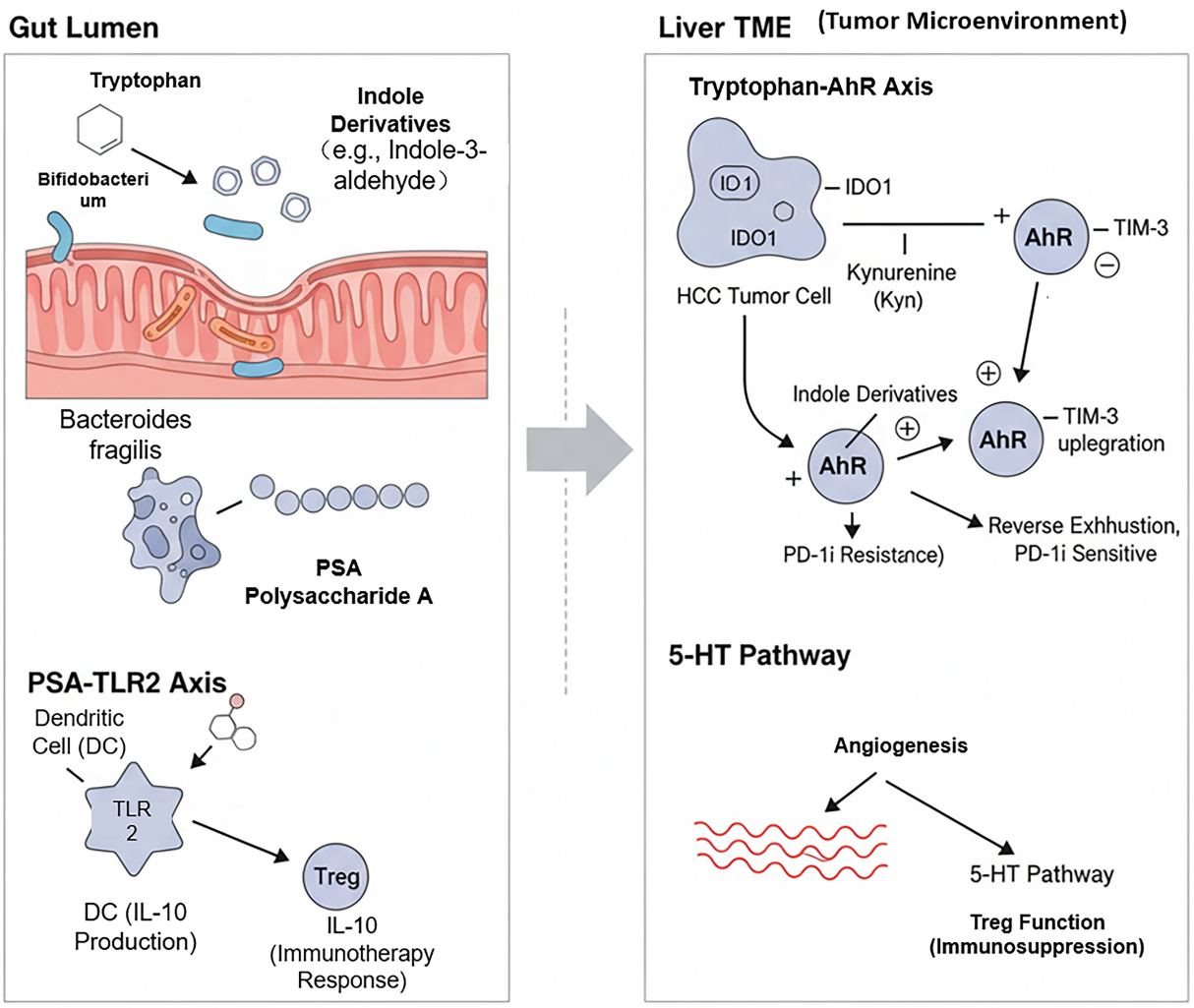

Figure 7. Immunomodulatory mechanisms of gut microbiota-derived tryptophan metabolites, structural components (PSA), and 5-HT. The schematic depicts the production of key bacterial derivatives in the gut lumen (left) and their subsequent signaling axes within the liver tumor microenvironment (right), primarily focusing on the AhR, TLR2, and 5-HT pathways. Solid arrows indicate promotion, activation, or metabolic conversion; blunt-ended lines indicate inhibition or receptor antagonism; dashed arrows indicate translocation from the gut to the liver. Detailed cellular interactions and clinical implications for ICI response are comprehensively discussed in the corresponding text. PSA: Polysaccharide A; AhR: aryl hydrocarbon receptor; TLR2: Toll-like receptor 2; 5-HT: 5-hydroxytryptamine; IDO1: indoleamine 2,3-dioxygenase 1; HCC: hepatocellular carcinoma; Treg: regulatory T cell; DC: dendritic cell; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; PD-1i: programmed cell death protein 1 inhibitor.

Furthermore, the gut microbiota is deeply implicated in the peripheral synthesis of neuroactive monoamines, notably 5-hydroxytryptamine (5-HT, serotonin). Over 90% of host 5-HT is synthesized within the gastrointestinal tract, a process highly dependent on the regulatory cues from specific microbial taxa[114]. In the context of HCC, elevated systemic 5-HT levels can directly promote the suppressive functionality of Tregs and indirectly support an immunosuppressive microenvironment by driving tumor angiogenesis[115].

In summary, gut microbiota-derived metabolites - encompassing SCFAs, secondary bile acids, and tryptophan catabolites - constitute a vast "soluble signaling network" that intimately regulates the hepatic immune microenvironment. Through concentration-dependent, receptor-mediated, and epigenetic programming mechanisms, these molecules finely tune the function and fate of critical immune populations,

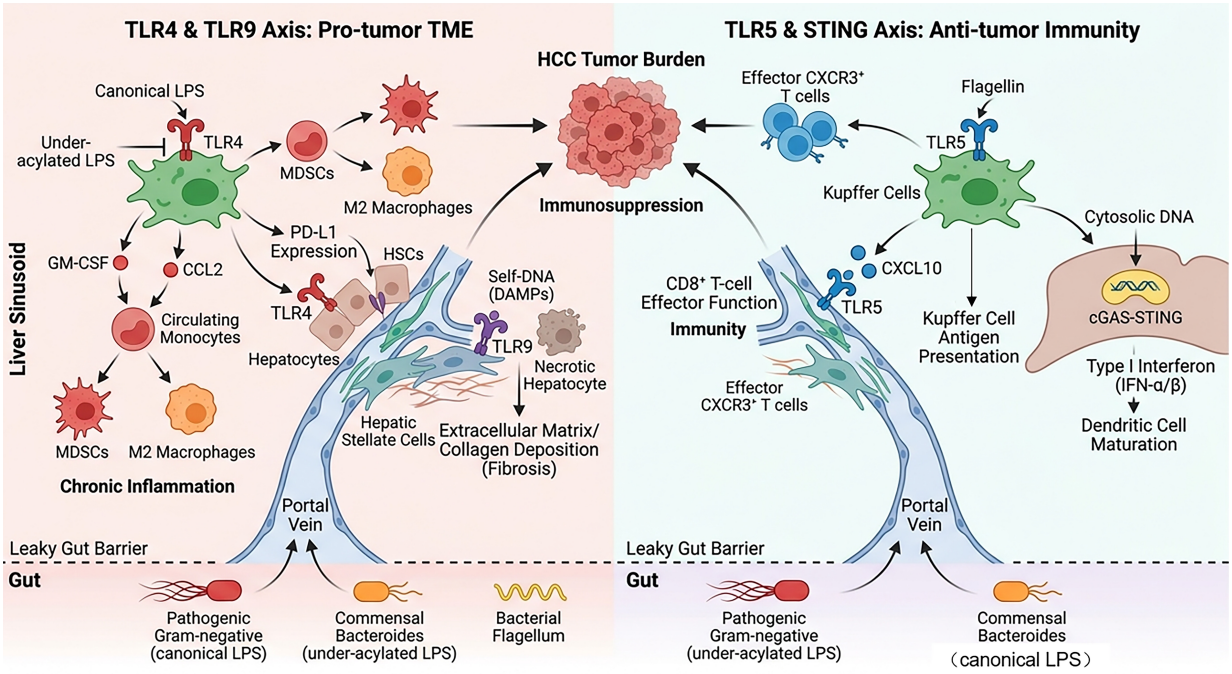

Figure 8. Direct immunomodulatory mechanisms of microbial pattern molecules via hepatic PRRs. The schematic contrasts the pro-tumorigenic TLR4/TLR9 axes (left) with the anti-tumorigenic TLR5/STING axes (right) within the liver microenvironment. Arrows indicate pathway activation, cellular recruitment, or immune cell maturation; T-bars denote receptor antagonism or inhibition (e.g., under-acylated LPS antagonizing TLR4). Detailed ligand-receptor interactions, including the distinct roles of canonical *vs.* under-acylated LPS, flagellin-mediated CXCR3$^+$ T cell recruitment, and cGAS-STING signaling, are comprehensively discussed in the corresponding text. PRRs: Pattern recognition receptors; STING: stimulator of interferon genes; LPS: lipopolysaccharide; TLR4: Toll-like receptor 4; MDSCs: myeloid-derived suppressor cells; GM-CSF: granulocyte-macrophage colony-stimulating factor; DAMPs: damage-associated molecular patterns; TLR5: Toll-like receptor 5; CXCR3$^+$: C-X-C motif chemokine receptor 3 positive; cGAS-STING: cyclic GMP-AMP synthase-stimulator of interferon genes; TLR9: Toll-like receptor 9; PD-L1: programmed death-ligand 1; CCL2: C-C motif chemokine ligand 2; HSCs: hepatic stellate cells; CD8$^+$: cluster of differentiation 8 positive; CXCR3: C-X-C motif chemokine receptor 3; INF-α/β: interferon-α/β.

including T cells, Tregs, MDSCs, and macrophages, thereby systemically setting the immunological tone for HCC's response to ICI therapy. However, the microbiota's regulation of the host immune system extends far beyond this indirect, metabolite-mediated influence. The gut microbiota and its inherent structural components can also rapidly and directly shape innate and adaptive immune responses by activating host PRRs or mediating antigen cross-reactivity.

Direct immunomodulatory mechanisms

Activation of PRRs

In the communication along the "gut-microbiota-liver axis", microbial structural components act as critical "pattern molecules" by directly activating PRRs widely expressed in the liver, constituting a rapid and potent channel for immunomodulation [Figure 8][116]. As an organ continuously exposed to gut-derived antigens, the liver harbors Kupffer cells, hepatocytes, and hepatic stellate cells that constitutively express a rich repertoire of PRRs[117]. These receptors function as an indispensable "sentry system" within the hepatic sinusoids, serving not only as the first line of defense against invading pathogens but also as a crucial bridge connecting innate and adaptive anti-tumor immune responses.

The LPS-TLR4 axis represents the most extensively studied pathway in this context. LPS is a major structural component of the outer membrane of Gram-negative bacteria. However, it is critical to recognize that LPS represents a highly heterogeneous group of molecules. The immunogenic activity of LPS is heavily

dependent on its structural composition, particularly the specific acylation pattern of its Lipid A moiety, which varies significantly across different bacterial species. Therefore, not all Gram-negative bacteria are inherently deleterious to HCC patients. For instance, canonical, highly acylated LPS (e.g., from certain pathogenic Enterobacteriaceae) acts as a potent TLR4 agonist. When these highly immunogenic LPS molecules translocate into the bloodstream during "leaky gut" and reach the liver via the portal vein at high concentrations, their recognition by TLR4 on Kupffer cells initiates robust downstream signaling. This cascade leads to the massive release of pro-inflammatory cytokines and chemokines, thereby triggering and sustaining chronic hepatitis[116]. Conversely, LPS derived from certain commensal bacteria, such as *Bacteroides* species (e.g., *Bacteroides thetaiotaomicron*, which is enriched in immunotherapy responders), often possesses an under-acylated Lipid A structure. These structural variants act as weak agonists or even competitive TLR4 antagonists, potentially mitigating excessive inflammation rather than exacerbating it[118].

This TLR4-driven chronic inflammation, primarily fueled by highly immunogenic LPS, serves as a classic "soil" for HCC development and directly participates in constructing an immunosuppressive tumor microenvironment. Mechanisms include driving the massive expansion of immunosuppressive cellular subsets: LPS-activated Kupffer cells and hepatic stellate cells produce granulocyte-macrophage colony-stimulating factor (GM-CSF) and CCL2, actively recruiting circulating monocytes to the liver, where they differentiate into MDSCs and M2-type TAMs[119]. Concurrently, chronic LPS stimulation can powerfully induce programmed death-ligand 1 (PD-L1) expression on various cellular compartments, including hepatocytes. A pivotal preclinical study confirmed that in a chemically induced HCC model, the targeted depletion of Gram-negative bacteria or pharmacological TLR4 antagonism significantly reduced tumor burden. This was accompanied by downregulated intratumoral PD-L1 expression and restored $CD8^+$ T-cell effector function, firmly establishing a causal relationship for this signaling pathway[120]. Translational clinical evidence further corroborates this: in advanced HCC patients treated with the "T+A" regimen, elevated pre-treatment plasma levels of soluble cluster of differentiation 14 (CD14) (a robust surrogate marker of systemic LPS exposure) are significantly associated with shorter overall survival[27]. Recent research also reveals that the immunological outcome of TLR4 signaling exhibits a highly complex "dose and time" effect. This includes the induction of endotoxin tolerance and even "trained immunity" - a paradigm where innate immune cells undergo persistent epigenetic and metabolic reprogramming following initial exposure to microbial antigens, potentially locking cells, such as Kupffer cells, into a "pro-tumor trained" state[121].

Notably, bacterial flagellin activates Kupffer cells via TLR5, exhibiting immunomodulatory properties distinctly different from those of TLR4. Unlike TLR4, which primarily drives aggressive pro-inflammatory and often pro-tumorigenic responses, TLR5 activation induces Kupffer cells to produce a unique cytokine profile. This includes the robust secretion of CXCL10, a key chemokine that specifically facilitates the recruitment of effector C-X-C motif chemokine receptor 3 positive ($CXCR3^+$) T cells to the hepatic niche[122]. More importantly, TLR5 signaling can intrinsically enhance the antigen-presenting capacity of Kupffer cells. One pivotal study directly elucidated the role of TLR5 activation in remodeling the immunosuppressive properties of the liver: treating mice with recombinant flagellin significantly alleviated the tolerogenic state mediated by primary liver immune cells, thereby markedly enhancing the activation and cytotoxic function of $CD8^+$ T cells *in vitro*. Crucially, this effect was exclusively mediated through the TLR5 [not NLR family CARD domain containing 4 (NLRC4)] signaling pathway, providing strong experimental rationale for developing flagellin or synthetic TLR5 agonists as a combination strategy to reprogram the HCC immune microenvironment[123]. The potent "adjuvant-like" signal provided by this flagellin-TLR5 axis offers a promising intervention strategy for converting immune-"cold" tumors into "hot" ones.

Beyond TLR4 and TLR5, other PRRs play highly significant roles. In non-alcoholic fatty liver disease (NAFLD)-associated HCC, signaling through TLR2 and TLR9 has been shown to be aberrantly hyperactivated. Self-DNA released from necrotic hepatocytes acts as a damage-associated molecular pattern

(DAMP), activating hepatic stellate cells via TLR9 and directly driving the excessive fibrotic deposition of extracellular matrix components such as collagen[124]. On the other hand, the activation of the cytosolic DNA sensor cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway typically induces robust type I interferon production and promotes dendritic cell maturation, establishing it as a highly attractive target for novel immunotherapies. Although functional impairment and silencing of this pathway have been observed in numerous human HCC samples[125], the intratumoral administration of specific STING agonists has shown promising signs of triggering systemic anti-tumor immunity in early-phase clinical studies[126].

In summary, gut microbial structural components constitute the fundamental core of direct immune regulation within the "gut-microbiota-liver axis" by systematically activating a complex network of PRRs in the liver. The impact of this direct pathway is profound, ranging from driving the chronic inflammatory basis of HCC initiation to actively maintaining an immunosuppressive TME through multi-layered mechanisms - such as inducing MDSC expansion, driving compensatory PD-L1 upregulation, and epigenetically shaping "trained immunity". Future precision intervention strategies must focus on delicately modulating the dynamic balance of this complex PRR network, thereby opening new, rational avenues for combination therapies aimed at decisively overcoming resistance to HCC immunotherapy.

Antigen cross-reactivity and molecular mimicry

In recent years, research elucidating the mechanisms linking the gut microbiota and systemic anti-tumor immunity has deepened significantly, with antigen cross-reactivity and molecular mimicry emerging as critical bridges connecting intestinal microbes to systemic immune responses. The core mechanism lies in the striking structural homology between certain gut microbial antigens and antigenic epitopes expressed on the surface of tumor cells. This phenomenon enables host-derived T cells, originally primed by exposure to gut microbiota, to "cross-react" by recognizing and attacking tumor cells, thereby achieving a profound form of "outside-in" adaptive immune activation. This mechanism not only enriches the immunological dimensions of the "gut-microbiota-liver axis" but also provides a novel perspective for understanding and overcoming HCC immunotherapy resistance [Figure 9][127].

The biological foundation for antigen cross-reactivity stems from the inherent flexibility - or degeneracy - of T-cell receptor (TCR) recognition of peptide-major histocompatibility complex (pMHC) structures. When microbial antigens and tumor antigens share homologous or identical epitopes, specific T-cell clones originally activated to target the gut microbiota can break their initial specificity to recognize and eradicate tumor cells expressing the analogous antigen[128]. This mechanism is particularly significant in the context of HCC. As the primary organ receiving intestinal venous drainage, the liver is continuously exposed to a massive influx of gut-derived antigens and primed immune cells, providing an optimal physiological site for the local accumulation and functional execution of these cross-reactive T cells.

Multiple landmark studies corroborate this paradigm. In melanoma and colorectal cancer research, T-cell clones capable of simultaneously recognizing antigens from gut commensals (such as *Bacteroides* species) and tumor neoantigens have been robustly identified. These T cells are typically primed in gut-associated lymphoid tissues, subsequently enter the systemic circulation, and home to tumor sites where they exert potent cytotoxic effects[129,130]. A pioneering study further elucidated a related dynamic: the therapeutic efficacy of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitors is highly dependent on the presence of specific *Bacteroides* species in the gut. The checkpoint blockade induces a Th1-skewed immune response against these commensal bacteria; the activated immune cells then migrate to the tumor site, where they remodel the TME via interferon-γ production, thereby indirectly amplifying the anti-tumor attack. In patients with metastatic melanoma, this treatment-induced anti-*Bacteroides* immune response strictly correlated with superior clinical outcomes. This reveals a novel immunological paradigm: adaptive immunity originally targeting commensal bacteria can be "co-opted" and redirected against the tumor[131].

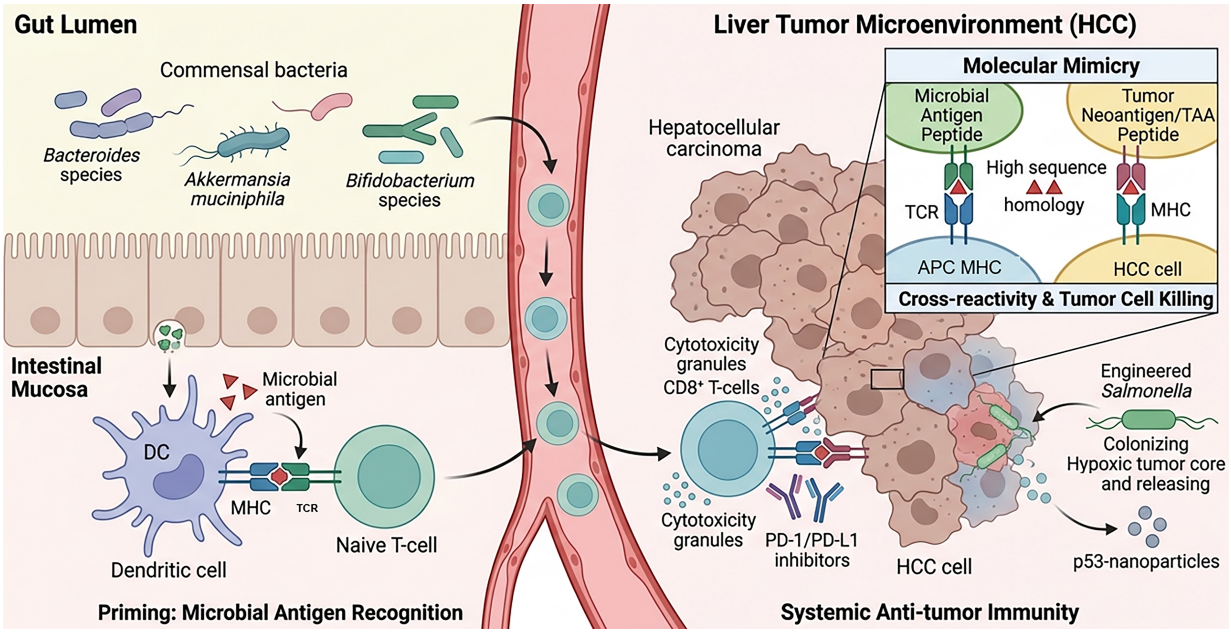

Figure 9. Mechanisms of microbiota-mediated antigen cross-reactivity and synergistic engineered immunotherapy. The schematic illustrates two critical pathways. (Left and Top Right) Molecular Mimicry: Commensal bacteria (e.g., Akkermansia, Bacteroides) provide microbial peptides with high sequence homology to tumor neoantigens/TAAs. DCs present these peptides via MHC to naive T cells (priming), which then migrate to the liver TME to cross-react with and kill HCC cells. (Bottom Right) Engineered Synergy: Attenuated Salmonella colonizes the hypoxic tumor core to release p53-nanoparticles, functioning in tandem with PD-1/PD-L1 inhibitors to restore systemic anti-tumor immunity. Specific cellular interactions and molecular homologies are detailed in the corresponding text. TAA: Tumor-associated antigen; TME: tumor microenvironment; HCC: hepatocellular carcinoma; TCR: T-cell receptor; DC: dendritic cell; MHC: major histocompatibility complex; PD-1: programmed cell death protein 1; PD-L1: programmed death-ligand 1; APC: antigen-presenting cell; $CD8^+$: cluster of differentiation 8 positive.

Molecular mimicry is the crucial molecular engine driving this antigen cross-reactivity. In the context of HCC, the tumor antigens targeted through this mimicry can primarily be categorized into two classes: tumor-specific neoantigens (TSAs) and tumor-associated antigens (TAAs). TSAs are generated by somatic mutation processes during tumorigenesis, creating entirely novel peptide sequences that the immune system perceives as foreign. Conversely, TAAs (such as cancer-testis antigens or alpha-fetoprotein) are non-mutated, wild-type proteins aberrantly overexpressed in HCC cells. The molecular basis of this mimicry relies on shared immunogenic motifs - typically short peptide sequences of 8 to 11 amino acids - that are presented by the major histocompatibility complex (MHC). When specific peptides derived from gut microbial proteins exhibit high sequence or conformational homology to the epitopes of these HCC neoantigens or TAAs, molecular mimicry occurs. For instance, well-documented homologous motifs from commensal bacteria (e.g., *Bacteroides* or *Bifidobacterium* species) can perfectly mimic the structural conformation of tumor neoepitopes. Driven by this structural overlap, T cells originally primed by gut bacteria to recognize microbial motifs break immunological tolerance and cross-react with HCC cells expressing the mutated or overexpressed antigens. Consequently, the immune system mistakes the tumor cells for "foreign invaders" and launches a targeted cytotoxic attack, providing a precise molecular explanation for how the microbiota systemically pre-conditions anti-tumor immunity.

Beyond naturally occurring cross-reactivity, contemporary research is actively exploring the use of engineered microorganisms to deliberately induce anti-tumor immunity. For example, one innovative study designed an attenuated *Salmonella* Typhimurium strain capable of specifically colonizing the hypoxic

interior of HCC tumors. Engineered as a microbial "Trojan horse", this bacterium locally and continuously releases nanoparticles carrying the p53 gene directly within the tumor bed, thereby restoring the function of this critical tumor suppressor gene in malignant cells. In preclinical models, the oral administration of this engineered bacterium, combined with PD-1 inhibitor therapy, demonstrated powerful synergistic effects. It more effectively inhibited tumor growth and elicited robust tumor-specific T-cell immune responses, providing compelling proof-of-concept for leveraging synthetic biology to overcome ICI resistance[132,133].

Observational clinical studies provide robust translational evidence supporting these microbiota-immune interactions. A multi-cohort study revealed that HCC patients responding to PD-1/PD-L1 inhibitors harbored significantly higher gut microbial diversity, and the specific enrichment of *Akkermansia* was independently associated with prolonged progression-free survival[27]. In preclinical settings, mechanistic studies utilizing murine models showed that FMT from human responders to tumor-bearing mice could reshape the tumor immune microenvironment of the recipient mice, manifested by increased $CD8^+$ T cell infiltration and a decreased proportion of regulatory T cells, successfully transferring the treatment-responsive phenotype. Recognizing that murine microbiomes and immune systems differ from humans, a recent prospective study focusing on Asian HCC patients provided crucial clinical validation. It identified that a high baseline abundance of *Akkermansia muciniphila* and *Bifidobacterium longum* were independent positive predictors of durable clinical benefit from PD-1 inhibitors. Subsequent murine experiments confirmed that transplanting fecal microbiota enriched with these two taxa profoundly enhanced the antigen-presenting function of dendritic cells. This critical antigen-presenting step bridged the microbial signals to the robust activation and proliferation of intratumoral $CD8^+$ T cells, ultimately synergizing with anti-PD-1 therapy to overcome resistance[28].

Systemic effects on intestinal barrier function and immune homeostasis

The intestinal barrier is a critical structural defense line separating the luminal contents from the host's internal milieu. Its integrity is paramount for maintaining systemic immune homeostasis and preventing gut-derived inflammation and liver injury. During the development and progression of HCC, the gut microbiota profoundly influences the hepatic immune microenvironment through its bidirectional regulation of barrier function [Figure 10]. On one hand, specific beneficial bacteria consolidate the barrier structure through synergistic mechanisms; on the other hand, the overgrowth of certain pathobionts can directly compromise the barrier and activate hepatic oncogenic signaling, constituting a key environmental driver of HCC progression.

The most immediate therapeutic consequence of fortifying intestinal barrier function is the effective attenuation of the pathological translocation of gut microbes and their metabolites into the portal venous system. This process is not merely a physical isolation; rather, it fundamentally mitigates the chronic inflammatory burden on the liver and reverses the resulting systemic and local immunosuppression by short-circuiting a pathological amplification cascade.

Under physiological conditions, the host establishes a dual defense system to manage the continuous influx of intestinal antigens. The mesenteric lymph nodes (MLNs), acting as the "primary firewall", efficiently intercept bacteria or their products that have breached the epithelial barrier, confining the immune response locally to prevent systemic dissemination[134]. However, when the intestinal barrier is severely compromised and the antigen load exceeds the processing capacity of the MLNs, this primary defense is overwhelmed. Bacteria and highly immunogenic products such as LPS flood into the liver via the portal vein. The liver is then mobilized as the crucial "secondary firewall": Kupffer cells efficiently phagocytose and clear these invaders. This clearance is amplified by LPS-binding protein (LBP, encoded by the liver-specific gene LBP), which is synthesized by hepatocytes. Although LBP does not directly initiate intracellular signaling, it acts as

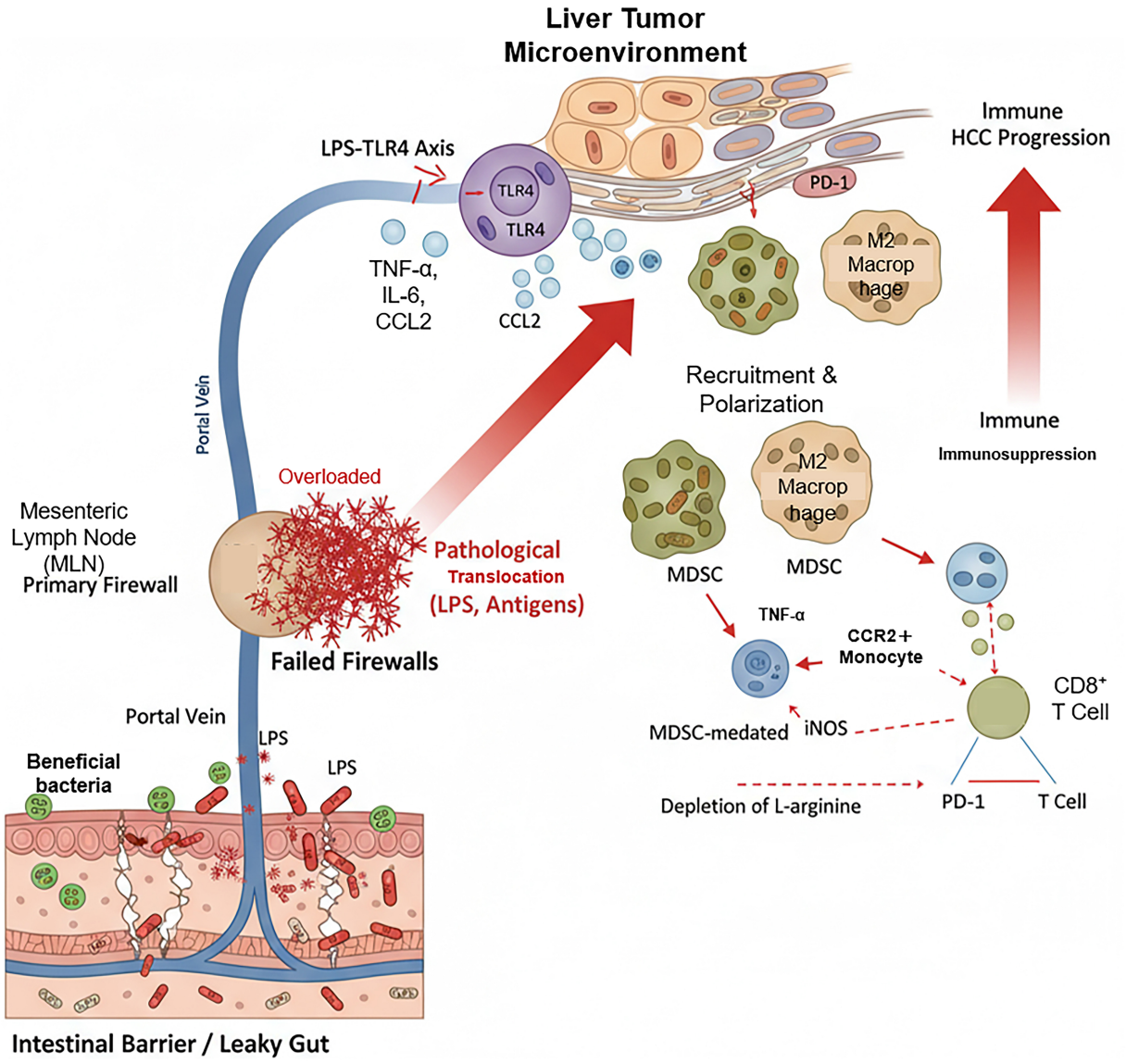

Figure 10. Mechanisms of intestinal barrier dysfunction and the "failed firewall" driving hepatic immunosuppression in HCC. The schematic illustrates the pathological translocation of LPS via the portal vein and the subsequent hyperactivation of the hepatic LPS-TLR4 axis. Solid red arrows indicate pro-inflammatory signaling, cellular recruitment, and polarization toward immunosuppressive phenotypes (e.g., MDSCs, M2 macrophages); dashed red arrows represent mechanisms of effector cell suppression (e.g., L-arginine depletion, iNOS); red T-bars denote direct functional inhibition of $CD8^+$ T cells (e.g., via the PD-1/PD-L1 axis). Detailed descriptions of the primary and secondary immunological firewalls are provided in the corresponding text. HCC: Hepatocellular carcinoma; LPS: lipopolysaccharide; MDSCs: myeloid-derived suppressor cells; iNOS: inducible nitric oxide synthase; MLN: mesenteric lymph node; LPS-TLR4: lipopolysaccharide-Toll-like receptor 4; PD-1: programmed cell death protein 1; PD-L1: programmed death-ligand 1; TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; CCL2: C-C motif chemokine ligand 2; $CCR2^+$: C-C motif chemokine receptor 2 positive; $CD8^+$: cluster of differentiation 8 positive.

an essential soluble lipid transfer protein that extracts LPS monomers and facilitates their delivery to CD14 and the TLR4-myeloid differentiation factor 2 (MD2) complex, thereby sensitizing the hepatic immune response to even trace levels of endotoxins. In a healthy state, this system maintains robust homeostasis. However, in the context of chronic liver disease and HCC, persistent pathological intestinal permeability (commonly termed "leaky gut") exposes the liver to chronic, high-dose antigenic assaults, ultimately causing the secondary defense to fail. Consequently, the liver transitions from a stringent immune surveillance organ into a continuous source of chronic inflammation and immune tolerance[135].

The collapse of this defense architecture directly triggers a cascade of immunosuppression within the hepatic niche. The influx of LPS binds with high affinity to TLR4 on the surface of hepatic resident cells, including Kupffer cells and hepatic stellate cells, driving a self-reinforcing inflammatory cycle. Activated Kupffer cells secrete vast quantities of TNF-α, IL-6, and the chemokine CCL2. The latter actively recruits C-C motif chemokine receptor 2 positive ($CCR2^+$) monocytes from the systemic circulation into the liver, where, heavily influenced by signals such as LPS, they polarize into highly immunosuppressive MDSCs and M2-type TAMs[120,136]. MDSCs potently suppress T-cell function by upregulating arginase-1 (ARG1) and inducible nitric oxide synthase (iNOS), thereby depleting the microenvironment of L-arginine - an amino acid absolutely essential for T-cell proliferation - and producing inhibitory mediators that directly induce T-cell anergy or apoptosis[137]. Furthermore, the LPS-TLR4 axis can directly upregulate the expression of immune checkpoint molecules, such as PD-L1, on both liver sinusoidal endothelial cells and infiltrating immune cells. Key preclinical studies have confirmed that in chemically induced murine HCC models, the administration of a TLR4 antagonist not only significantly reduced tumor burden but also downregulated intratumoral PD-L1 expression and restored $CD8^+$ T-cell effector function, definitively establishing the causality of this pathway[120].

Clinical cohort studies provide compelling corroborating evidence. A comprehensive meta-analysis published in 2025 systematically evaluated the prognostic value of the systemic immune-inflammation index (SII) in HCC, pooling data from 39 high-quality cohort studies. The results conclusively demonstrated that an elevated pre-treatment SII was significantly associated with shortened overall survival, recurrence-free survival, and progression-free survival[138]. This strongly suggests that a baseline state of systemic inflammation and immune imbalance - characterized by elevated neutrophil and platelet counts paired with decreased lymphocyte counts, as captured by the SII, and likely driven by upstream factors such as severe "leaky gut" - profoundly predisposes HCC patients to poor therapeutic outcomes. This hostile systemic inflammatory environment is fundamentally underpinned by the LPS-TLR4 axis-driven expansion and hyperactivation of immunosuppressive myeloid cells.

Therefore, the core translational value of intervention strategies targeting the intestinal barrier lies in consolidating the multi-layered immune defense network formed by the MLNs and the liver. By drastically reducing the translocation of products such as LPS, these strategies effectively prevent the "primary firewall" from being breached, thereby rescuing the liver from descending into an immunosuppressive incubator due to chronic antigen overload. For HCC immunotherapy, proactively maintaining the structural and functional homeostasis of the gut-microbiota-liver axis represents a foundational strategy for systemically reversing hepatic immunosuppression and maximizing the clinical efficacy of ICIs.

TRANSLATIONAL INTERVENTIONS: PATHWAYS FROM CONCEPT TO CLINIC PRACTICE

Microbiota-targeted intervention strategies: from FMT, and probiotics to engineered bacteria

Strategies for modulating the gut microbiota are rapidly advancing from conceptual frameworks toward early-phase clinical exploration, forming a diverse intervention armamentarium that ranges from whole-community transplantation to precision rational design, and from broad-spectrum to fine-tuned approaches. These translational strategies primarily encompass FMT, next-generation probiotics and synbiotics, and synthetic biology-driven engineered bacteria. They collectively aim to therapeutically reshape the "gut-microbiota-liver axis", reverse established immunosuppression, and thereby sensitize tumors or overcome resistance to immunotherapy in HCC. However, it is crucial to note that the translational readiness of these modalities varies significantly, spanning from experimental proof-of-concept models to ongoing early-phase clinical trials [Figure 11].

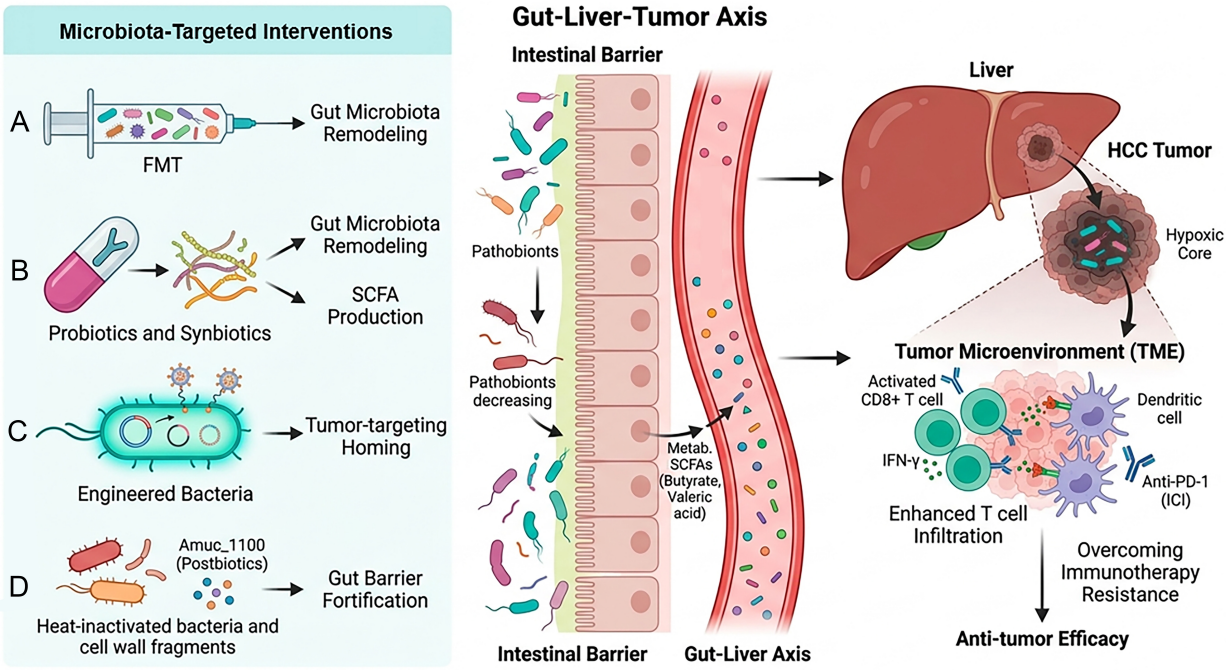

Figure 11. Translational microbiota-targeted interventions and their systemic mechanisms in HCC. The schematic outlines four primary clinical strategies: (A) FMT; (B) probiotics/synbiotics; (C) engineered bacteria; and (D) postbiotics. Black arrows indicate the sequential biological impact of these interventions, from remodeling the gut microbiota and fortifying the intestinal barrier (left) to the systemic delivery of metabolites (e.g., SCFAs) and tumor-homing bacteria via the gut-liver axis (middle). (Right) In the liver tumor microenvironment, these interventions synergistically reverse immunosuppression, enhancing dendritic cell activation, $CD8^+$ T cell infiltration, and IFN-γ production, thereby overcoming resistance to immune checkpoint inhibitors (e.g., anti-PD-1). Specific strain functions and clinical translation progress are detailed in the corresponding text. HCC: Hepatocellular carcinoma; FMT: fecal microbiota transplantation; SCFAs: short-chain fatty acids; ICI: immune checkpoint inhibitor; TME: tumor microenvironment; $CD8^+$: cluster of differentiation 8 positive; IFN-γ: interferon gamma; PD-1: programmed cell death protein 1.

FMT, serving as the most direct whole-microbiota intervention [Figure 11A], has successfully entered early clinical exploration. A prospective single-arm study demonstrated that among 13 patients with advanced gastrointestinal cancers (including 4 with HCC) who were refractory to nivolumab, the administration of FMT combined with continued nivolumab therapy enabled previously resistant HCC patients to achieve a partial response after receiving an FMT from a responder donor. Crucially, this clinical benefit was tightly associated with the successful engraftment of specific immune-activating bacterial strains[139]. Furthermore, interim analysis from a Phase II pilot study (FAB-HCC) evaluating patients resistant to the first-line "T+A" regimen reported highly encouraging results: following a single donor FMT and the continuation of the original systemic therapy, an ORR of 33% and a disease control rate of 83% were observed among six evaluable patients, preliminarily validating the safety and feasibility of this approach[140].

In-depth longitudinal analyses reveal that successful FMT is a highly dynamic process of microbial community reconstruction. In clinical responders, beneficial donor strains stably colonize and expand, while the relative abundance of potentially deleterious pathobionts correspondingly decreases[141]. This microbial shift translates into the detectable clonal expansion of tumor antigen-specific T cells and an improved TCR repertoire diversity in the host's peripheral blood, subsequently leading to enhanced infiltration and cytotoxic function of $CD8^+$ T cells within the tumor microenvironment[142,143]. Expanding beyond systemic ICIs, the microbiota's influence also dictates responses to locoregional interventions. For instance, in the context of radiotherapy, studies corroborate that gut dysbiosis can suppress tumor antigen presentation and effector T-cell function via the impairment of the cGAS-STING-type I interferon pathway. Conversely, microbiota-mediated activation of this pathway can synergistically enhance the anti-tumor efficacy of PD-1 blockade combined with radiation[144].

Despite these highly promising prospects, establishing FMT as a routine, standardized adjuvant therapy for HCC still faces multiple hurdles, including the strict standardization of donor screening, the mitigation of long-term safety risks (e.g., pathogen transfer), and the navigation of complex regulatory pathways[145,146]. Driven by the sheer complexity and undefined nature of FMT, intervention strategies are increasingly evolving towards precision medicine using next-generation probiotics and synbiotics [Figure 11B] based on specific, highly characterized functional strains. This paradigm shift is grounded in the recognition that the strain - rather than the species - is the core functional unit. Different strains within the same species can exhibit profound differences in their genomic architectures, metabolomic profiles, and immunomodulatory capacities. For example, not all *Akkermansia muciniphila* strains effectively fortify the intestinal barrier; their therapeutic efficacy strictly depends on the expression and structural conformation of the outer membrane protein Amuc_1100, which is highly strain-specific[147]. Similarly, the capability of *Bifidobacterium* to sensitize tumors to immunotherapy exhibits strict strain dependency.

Clinical and preclinical studies have firmly validated the potential of strain-specific interventions. One notable trial found that in patients receiving dual checkpoint blockade (nivolumab plus ipilimumab), concurrent supplementation with the live biotherapeutic *Clostridium butyricum* strain MIYAIRI 588 (CBM588) significantly extended median progression-free survival (12.7 months *vs.* 2.5 months)[29]. Additionally, *Lactobacillus rhamnosus* Probio-M9 has been shown to accelerate post-antibiotic microbiota reconstitution and subsequently enhance anti-PD-1 efficacy[148]. In the context of altering the pre-tumorigenic background, Lactobacillus acidophilus exerts potent preventive effects in NASH-HCC models through the production of its metabolite valeric acid. This SCFA activates GPR41/43 receptors and inhibits the Ras homolog family member A (RhoA) pathway [a canonical member of the Ras homolog-guanosine triphosphatase (Rho-GTPase) family], thereby suppressing inflammation-driven tumor progression[149]. Furthermore, a rationally constructed synthetic consortium comprising 11 defined bacterial strains was shown to robustly induce the generation of interferon gamma positive (IFN-γ^+) $CD8^+$ T cells in the intestine, enhancing host anti-infection immunity and synergistically improving the efficacy of ICIs[150].

However, the continuous administration of exogenous probiotics may occasionally delay the autochthonous recovery of the host's indigenous microbiota[151]. Therefore, synbiotics - rational combinations of specific probiotics with targeted prebiotics - are strategically designed to selectively enhance the *in vivo* colonization and metabolic output of the target strains. For instance, a synbiotic combination of bilberry anthocyanins and citrus pectin significantly increased fecal butyrate levels, synergistically enhancing the anti-tumor effect of PD-L1 blockade[152]. Modulating the underlying hepatic background is also a critical pre-conditioning strategy. In patients with NAFLD, meta-analyses of multiple randomized controlled trials indicate that interventions with probiotics, prebiotics, or synbiotics can significantly ameliorate liver steatosis, fibrosis markers, and elevated liver enzymes[153]. For example, the daily consumption of probiotic yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 for eight weeks significantly reduced serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, and low-density lipoprotein (LDL)-cholesterol levels[154]. Similarly, treatment with a tailored probiotic mixture of 6 strains for 12 weeks in obese patients with NAFLD [confirmed by magnetic resonance imaging-proton density fat fraction (MRI-PDFF)] significantly reduced intrahepatic fat content. This clinical improvement was accompanied by a marked expansion of beneficial endogenous taxa, such as *Agathobacter* and *Blautia*[155].

A more cutting-edge frontier involves leveraging synthetic biology to engineer bacteria [Figure 11C], developing them as programmable "living therapeutics" capable of actively homing to tumors, sensing the hostile tumor microenvironment, and intelligently releasing therapeutic payloads. For example, researchers

have successfully constructed engineered *E. coli* capable of specifically targeting and colonizing the hypoxic cores of HCC tumors. Drug-loaded nanoparticles anchored to their bacterial surface enable *in situ* antigen expression and the targeted co-delivery of chemotherapeutic agents and genetic payloads, demonstrating potent anti-tumor and anti-metastatic effects in murine models[156]. Another breakthrough study designed an intelligent, self-lysing delivery system based on attenuated *Salmonella*. This engineered bacterium efficiently invades malignant cells and lyses intracellularly to release therapeutic protein drugs, effectively inhibiting primary HCC and lung metastases[157]. Additionally, engineered *E. coli* designed to specifically sense the acidic tumor microenvironment and locally induce the expression of anti-PD-1 single-chain variable fragments have successfully activated robust anti-tumor immunity and suppressed post-surgical recurrence in HCC models[158].

Within this translational landscape, postbiotics [Figure 11D] - formally defined as preparations of inanimate microorganisms and/or their components that confer a health benefit on the host - have emerged as a highly promising intervention modality. Their distinct advantages include superior safety profiles, enhanced structural stability, and highly standardized manufacturing processes. Clinical studies have demonstrated that combined supplementation with heat-inactivated preparations of specific *Limosilactobacillus fermentum*, *Limosilactobacillus reuteri*, and *Lactiplantibacillus plantarum* for 60 days significantly improved liver function and modulated uric acid levels in patients with MAFLD, while concurrently optimizing their baseline gut microbiota architecture[159]. Basic research further corroborates this, showing that postbiotic compounds extracted from *Lactiplantibacillus plantarum* exert profound protective effects in preclinical acute liver injury models[160]. Remarkably, even pasteurized *Akkermansia muciniphila* retains the potent biological activity of its outer membrane protein Amuc_1100, fully maintaining its function in preserving the critical intestinal barrier in experimental models[79].

In summary, the seamless clinical translation of microbiota-targeted therapies must still overcome significant challenges related to long-term safety, standardized pharmaceutical-grade production, and individualized efficacy prediction. Integrating deep multi-omics data - such as high-resolution metagenomics and metabolomics - to construct AI-driven predictive models represents a paramount future perspective. This technological leap will eventually enable clinicians to precisely match individual patients with their optimal bacterial strains or synthetic formulations, firmly driving microbiota-targeted interventions away from the era of "broad-spectrum" empirical supplementation and into a transformative new epoch of precision microbial modulation.

Microbiota-derived metabolite therapy

A primary mechanism by which the gut microbiota dictates the efficacy of HCC immunotherapy is through its functional output: bioactive metabolites. Consequently, "metabolite therapy" - a strategy that directly targets these signaling molecules with defined biological activities - offers a highly controllable and translationally viable avenue for overcoming immune resistance. This therapeutic strategy primarily encompasses two distinct directions: first, the exogenous supplementation of beneficial immunomodulatory metabolites; and second, the precise pharmacological blockade of deleterious metabolic pathways. Both approaches aim to actively reshape the host systemic immune tone and remodel the TME [Figure 12].

The direct systemic or targeted supplementation of beneficial metabolites is a widely investigated strategy. Its core translational challenge lies in traversing the "double-edged sword" nature of metabolite function and achieving efficient, spatiotemporally targeted delivery. Butyrate, a prominent SCFA, serves as a prototypical example. Systemically high concentrations of butyrate may paradoxically promote regulatory T-cell (Treg) differentiation by inhibiting HDACs, which can inadvertently suppress anti-tumor immunity[161]. However, robust research has also revealed its potent capacity for localized immune activation: butyrate can stimulate

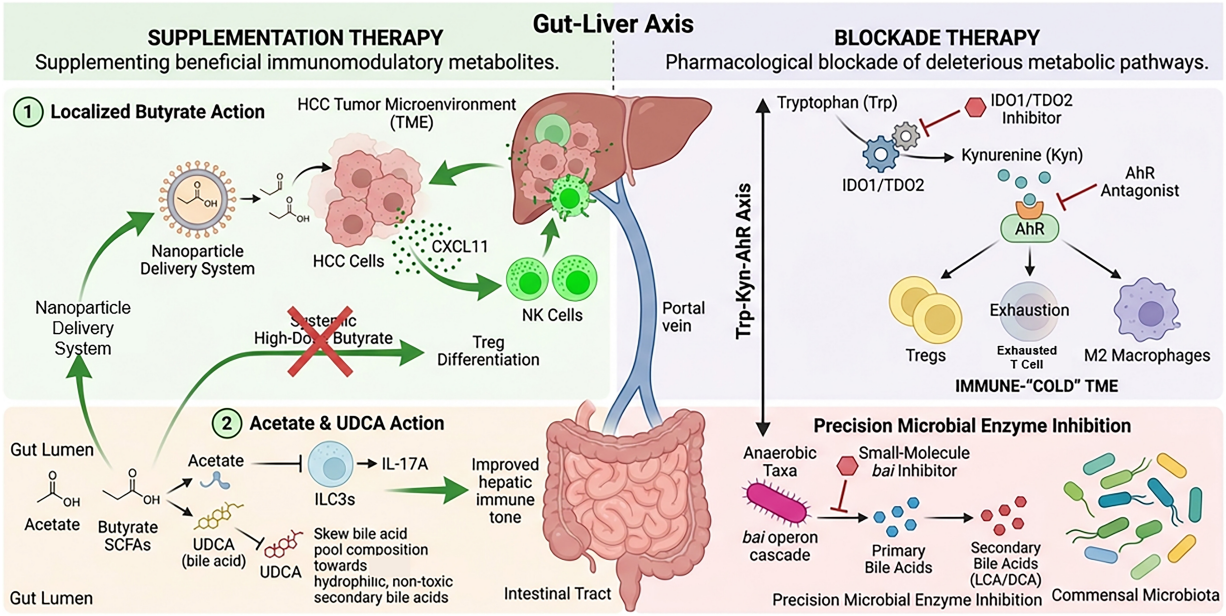

Figure 12. Microbiota-derived metabolite therapies for modulating the HCC immune microenvironment. The schematic illustrates two distinct pharmacological strategies. (Left) Supplementation Therapy: Localized delivery of butyrate (e.g., via nanoparticles) promotes CXCL11-mediated NK cell recruitment while avoiding systemic Treg induction. Acetate inhibits IL-17A production by ILC3s, and UDCA skews the bile acid pool toward non-toxic profiles. (Right) Blockade Therapy: Pharmacological interventions target the immunosuppressive Trp-Kyn-AhR axis using IDO1/TDO2 inhibitors or AhR antagonists. Furthermore, precision microbial enzyme inhibition targets specific bacterial operons (e.g., bai) to block the synthesis of pro-tumorigenic secondary bile acids without disrupting overall microbiota ecology. Detailed mechanisms are discussed in the corresponding text. HCC: Hepatocellular carcinoma; NK: natural killer; ILC3s: type 3 innate lymphoid cells; UDCA: ursodeoxycholic acid; IDO1: indoleamine 2,3-dioxygenase 1; AhR: aryl hydrocarbon receptor; TME: tumor microenvironment; SCFAs: short-chain fatty acids; LCA: lithocholic acid; DCA: deoxycholic acid; Tregs: regulatory T cells; CXCL11: C-X-C motif chemokine ligand 11; IL-17A: interleukin 17A; TDO2: tryptophan 2,3-dioxygenase.

HCC cells to upregulate the expression of the critical chemokine CXCL11 through epigenetic chromatin remodeling, effectively recruiting NK cells to infiltrate the tumor bed. Preclinical models confirm that oral butyrate supplementation significantly inhibits tumor growth, an effect that is strictly dependent on the presence of functional NK cells[85]. These nuanced findings suggest that localized administration (e.g., via specialized enemas) or the development of liver-targeting/TME-responsive nanodelivery systems may maximize the immunostimulatory effects of butyrate while firmly mitigating its systemic immunosuppressive risks. Furthermore, exogenous acetate supplementation has demonstrated significant therapeutic potential; it attenuates the production of the pro-inflammatory cytokine interleukin 17A (IL-17A) by type 3 innate lymphoid cells (ILC3s) in the liver through HDAC inhibition mechanisms, exhibiting potent synergistic anti-tumor effects when combined with PD-1 inhibitors in preclinical models[162].

Within the realm of bile acid metabolism, the direct supplementation of UDCA - a hydrophilic secondary bile acid already FDA-approved for specific liver diseases with a well-established safety profile - is considered a highly translatable strategy. Accumulating evidence suggests that while specific hydrophobic secondary bile acids (e.g., lithocholic acid[163]) possess profound immunosuppressive properties, UDCA actively counteracts these effects. Direct UDCA supplementation aims to rapidly improve the hepatic immune microenvironment by beneficially skewing the composition of the circulating bile acid pool, and preliminary clinical explorations combining it with ICIs are currently underway[164].

Compared to direct supplementation, the pharmacological blockade of deleterious metabolic pathways represents a highly targeted intervention. Among these, antagonizing the tryptophan-kynurenine-AhR axis remains a major research focus. Within the TME, the microbially modulated enzymes IDO1 and tryptophan

2,3-dioxygenase (TDO2) metabolize tryptophan into kynurenine. Kynurenine acts as an endogenous AhR ligand, causing its persistent activation, which subsequently drives Treg differentiation, induces T-cell exhaustion, and polarizes macrophages toward a suppressive M2 phenotype. This axis is a fundamental driver in the formation of an immune-"cold" microenvironment[164,165]. Therefore, developing IDO1/TDO2 inhibitors or direct AhR antagonists aims to sever this harmful signaling cascade upstream. Although early clinical trials of IDO1 inhibitors have yielded complex and sometimes underwhelming results in certain solid tumors, this strategy remains potentially highly valuable in specific HCC subgroups characterized by intense IDO1 overexpression[165].

An even more innovative frontier is "precision microbial enzyme inhibition". This paradigm involves utilizing small-molecule drugs to specifically inhibit key bacterial enzymes responsible for catalyzing harmful metabolites, entirely bypassing the need to exert broad-spectrum bactericidal pressure. For instance, the enzyme cascades encoded by the bile acid-inducible (bai) operon, expressed by specific anaerobic taxa, are responsible for converting primary bile acids into highly immunosuppressive secondary bile acids (e.g., lithocholic acid and deoxycholic acid), which actively promote HCC progression[166]. Designing highly specific, non-lethal inhibitors targeting these bacterial enzymes holds immense promise for reducing the generation of harmful secondary bile acids directly at their source. Crucially, this approach preserves the overall structural integrity and diversity of the gut microbiota while profoundly improving the hepatic immune landscape.

In summary, metabolite therapy - by directly and rationally manipulating the functional output of the gut microbiota - provides a multidimensional framework for precise intervention to overcome HCC immunotherapy resistance. Nevertheless, this strategy must navigate substantial challenges on its path toward routine clinical translation. For supplementation strategies, issues regarding concentration gradients, context-dependent immunological outcomes, delivery efficiency, and profound patient heterogeneity must be resolved. For blockade strategies, ensuring absolute target specificity, overcoming metabolic pathway redundancy, and accumulating robust long-term safety data are imperative. Moving forward, integrating advanced nanotechnology, synthetic biology, and multi-omics-guided personalized diagnostics will propel metabolite therapy from conceptual blueprints to clinical reality, firmly establishing it as a fundamental pillar within the comprehensive HCC treatment paradigm.

Other intervention strategies

Beyond FMT, precision probiotics/synbiotics, engineered live biotherapeutics, and metabolite therapies, interventions targeting the gut microbiota are rapidly expanding into highly diversified and precision-guided dimensions. Although many remain in the conceptual or preclinical stages, these strategies either macroscopically modulate the host-microbiota interaction landscape or employ novel biologics for targeted microbial editing and delivery. Together, they constitute a robust, multi-layered, and complementary experimental armamentarium.

Dietary modulation represents the most accessible and fundamental intervention. Its role extends far beyond basic nutritional provision, serving as a core modality to systemically sculpt the structure and functional output of the gut microbiota. A large-scale prospective cohort study involving 63,275 Singaporean Chinese individuals (with an average follow-up of 17.6 years) demonstrated that long-term adherence to a healthy, plant-based dietary pattern was associated with a significantly reduced risk of HCC[167]. However, given the observational nature of this study, this association is inevitably subject to confounding variables, as these cohorts plausibly adhere to overall healthier lifestyles (e.g., smoking cessation, reduced alcohol consumption). Therefore, while it is strongly hypothesized that shaping a specific, protective microbial-metabolic environment through such diets contributes to these cancer-preventive benefits, the

definitive mechanistic link remains speculative and strictly warrants validation through controlled clinical trials. Beyond chronic dietary patterns, chrono-nutritional interventions also exhibit profound translational potential. Compelling preclinical studies indicate that short-term starvation cycles or fasting-mimicking diets can radically remodel the tumor immune microenvironment. Mechanistically, this involves regulating the metabolic reprogramming of TAMs [via the fructose-1,6-bisphosphatase (FBP1)/AKT/Ras-related protein Rab-27A (Rab27a) signaling axis] to drastically reduce the secretion of immunosuppressive PD-L1-positive exosomes, thereby significantly enhancing the efficacy of anti-PD-1/PD-L1 therapy in multiple murine models of HCC[168]. This provides critical proof-of-concept for deploying specific fasting regimens as "metabolic adjuvants" to immunotherapy. Ultimately, "precision nutrition" paradigms - leveraging specific functional components like defined dietary fibers or polyphenols - are expected to enable the targeted enrichment and functional activation of beneficial commensal consortia.

For the highly targeted elimination of specific pathobionts, bacteriophage (phage) therapy exhibits irreplaceable advantages. Unlike broad-spectrum antibiotics, which inflict devastating collateral damage on the commensal ecosystem, phages can specifically lyse target bacterial strains. This achieves the precise clearance of pathogens while maximally preserving the integrity of the beneficial gut microbiota. For instance, customized phage "cocktails" could be rationally designed against key bacteria implicated in HCC progression and immunosuppression, such as *Fusobacterium nucleatum* or *Klebsiella pneumoniae*. This strategy not only eradicates the pathogenic source but also holds immense promise for decisively reversing the bacteria-driven immunosuppressive TME through the precise, surgical editing of the microbiota composition[83,84]. Although phage therapy for HCC remains strictly in the preclinical arena, its unparalleled specificity offers a highly attractive translational approach for overcoming pathogen-driven resistance phenotypes.

Furthermore, bacterial outer membrane vesicles (OMVs) are rapidly gaining traction as naturally derived, nanoscale immunomodulatory carriers. OMVs intrinsically harbor various highly immunogenic components from their parent bacteria, endowing them with potent built-in adjuvant properties. An innovative preclinical study leveraged synthetic biology to construct dual-targeting engineered OMVs [OMV-CD47/glypican-3 (GPC3)] that simultaneously display an anti-CD47 nanobody and an anti-GPC3 single-chain variable fragment on their surface. These highly specialized vesicles can precisely recognize HCC cells. Mechanistically, they dually enhance macrophage-mediated tumor phagocytosis by blocking the CD47 "don't eat me" signal, while simultaneously exploiting their natural immunostimulatory payload to activate dendritic cells and radically remodel the TME. This dual-action strategy effectively inhibited tumor growth and metastasis in an orthotopic HCC model[169].

It is imperative to emphasize that the strategic timing and synergistic sequencing of these interventions will dictate their ultimate translational success. Microbiota-targeted therapies will rarely be administered as isolated monotherapies. Instead, the precise temporal integration of these interventions with established standard-of-care modalities - including surgery, transarterial chemoembolization (TACE), radiotherapy, and targeted systemic therapies - will profoundly shape the final therapeutic trajectory. For example, as a strong translational hypothesis, the peri-procedural adjuvant use of mucoprotective probiotics during TACE could strategically mitigate treatment-induced intestinal barrier damage and systemic endotoxemia, thereby cultivating a significantly more favorable, immune-permissive baseline for subsequent systemic ICI therapy[33]. Looking ahead, the formulation of personalized intervention blueprints - rooted deeply in the patient's baseline microbiota profile (enterotype) and metabolomic biomarkers - represents the ultimate translational zenith, acknowledging that this is a future paradigm rather than an immediate clinical reality. Guided by AI-driven multi-omics analyses, individual patients could theoretically be seamlessly matched with their most optimal probiotic consortium, synthetic community, FMT donor, or chrono-nutritional regimen. This milestone will finally propel microbiota-targeted oncology from an empirical, "one-size-fits-all" approach into the definitive era of true precision medicine.

Paradigm shift and capability reconstruction in laboratory medicine in the microbiome era

The crucial role of the gut microbiota in orchestrating the response and resistance to immunotherapy for HCC not only provides new therapeutic targets but also poses novel demands and opportunities for clinical laboratory practice. Advances in this field clearly dictate that future laboratory medicine must evolve beyond the traditional paradigm focused primarily on single-pathogen identification or static biochemical marker analysis. It must actively shift towards a novel microbiome diagnostics system that integrates multi-omics data, emphasizes dynamic longitudinal monitoring, and focuses on actionable functional interpretation. This transformation aims to more precisely serve efficacy prediction, treatment monitoring, and the guidance of personalized microbiota-targeted interventions in cancer immunotherapy.

Regarding the scope of laboratory samples, the traditional concept of the tumor "liquid biopsy" urgently warrants expansion. Currently, tumor-derived markers such as circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) dominate the primary focus. However, gut microbiome research highlights the equal strategic importance of a systemic host "microbiome liquid biopsy". This paradigm shift demands that clinical laboratories fundamentally elevate the clinical value of fecal samples. Feces must no longer be viewed merely as crude specimens for routine occult blood or parasite screening; rather, they are the core biological materials for accurately assessing the functional status of the "gut-microbiota-liver axis". In the future, establishing standardized workflows for fecal metagenomic and metabolomic testing, alongside the development of rapid molecular assays targeting key predictive bacterial taxa (e.g., *Akkermansia*) or functional gene clusters, should become integral components of companion diagnostics for immunotherapy. Concurrently, the analytical scope of peripheral blood samples requires simultaneous expansion. Beyond traditional tumor markers, laboratories must incorporate systemic indicators reflecting microbiota-host interactions, such as microbial translocation markers (e.g., LPS-binding protein, soluble CD14) and key microbiota metabolite profiles (e.g., SCFAs, specific secondary bile acids, and the tryptophan/kynurenine ratio). These indicators provide indirect yet vital real-time information regarding the systemic immunological tone of the tumor microenvironment. Integrating these microbiota-derived markers with traditional tumor molecular markers to construct a multi-dimensional biomarker landscape will significantly enhance the predictive capacity for treatment response and resistance risk.

This profound change directly necessitates a rapid capability upgrade in laboratory technologies. Comprehensive microbiome analysis relies heavily on high-throughput sequencing and complex bioinformatics. Therefore, laboratory professionals must master the basic principles, operational standards, and quality control essentials of next-generation sequencing (NGS) technologies. They must also acquire foundational bioinformatics interpretation skills to translate massive datasets - such as microbial diversity metrics, compositional structures, and functional pathway predictions - into clinical significance. Simultaneously, to promote widespread clinical adoption, developing rapid, cost-effective testing methods suitable for routine clinical laboratories - independent of massive sequencing platforms - is crucial. Examples include specific quantitative polymerase chain reaction (qPCR) assays for particular beneficial or pathobiontic strains, or automated chemiluminescence immunoassays for key immunomodulatory metabolites. A more profound paradigm shift addresses the inherent limitations of isolated, single-analyte test results. Future laboratory diagnostics will inextricably rely on advanced data integration and artificial intelligence (AI) models. The role of the laboratory department will inevitably evolve from merely outputting discrete data points to utilizing machine learning algorithms to integrate multi-omics datasets - encompassing the patient's microbial features, metabolite profiles, and clinical parameters. This integration will generate highly intelligible reports, such as an "Immunotherapy Response Index" or "Microbiota Modulation Recommendations", directly supporting personalized clinical decision-making.

This technological shift consequently drives a fundamental transformation in the mode of report interpretation and clinical communication. The interpretation of a microbiome test report transcends the complexity of routine laboratory panels. It requires laboratory physicians to pivot from simple binary judgments of "normal *vs.* Abnormal" towards a comprehensive, biologically grounded interpretation of microbiota composition, functional potential, and clinical relevance. Reports should strive to elucidate the biological significance behind the data, for example: "Reduced gene abundance in the butyrate synthesis pathway suggests potentially impaired immunomodulatory function of the gut microbiota, which is associated with a heightened risk of T-cell functional suppression." Furthermore, because the gut microbiota is highly dynamic, laboratory practice must advocate for and implement a longitudinal monitoring concept. Analyzing serial samples collected before, during, and after treatment allows for the dynamic assessment of the biological effects of microbiota-targeted interventions (e.g., probiotics, FMT) and provides a quantitative basis for real-time regimen adjustments. This dynamic landscape demands that laboratory physicians proactively strengthen communication and collaboration with multidisciplinary tumor boards (MTBs) - including oncology, hepatology, and clinical nutrition departments - positioning the laboratory as the indispensable bridge connecting complex microbiome data to bedside clinical practice.

Finally, the cornerstone for the robust, sustainable development of this new field lies in rigorous quality control, standardization, and strict ethical norms. The clinical translation of microbiome testing currently faces immense standardization challenges. Strict industry consensus and standardized operating procedures (SOPs) are urgently needed for every pre-analytical and analytical step - from sample collection, stabilization, and DNA extraction to sequencing library preparation and bioinformatics pipelines - to guarantee the reproducibility and comparability of results across different institutions. Strengthening the architecture of internal quality control (IQC) and external quality assessment/proficiency testing (EQA/PT) programs is paramount. Additionally, because microbiome data inherently contains highly sensitive genomic and personal health information, laboratory departments must establish impregnable data security and privacy protection frameworks, strictly adhering to ethical guidelines while vigorously promoting technological advancement.

In conclusion, gut microbiome research has unequivocally opened new therapeutic avenues for HCC immunotherapy and holds the immense potential to guide laboratory medicine into a transformative new era characterized by a greater systemic perspective, longitudinal dynamism, and unprecedented precision. For the laboratory profession, this represents not merely a technological innovation, but a fundamental future upgrade in clinical mindset and disciplinary positioning. Clinical laboratories are poised to transform from traditional service departments providing discrete diagnostic data into core clinical hubs that integrate multi-dimensional biological intelligence to generate personalized diagnostic and therapeutic decision support. By proactively embracing this profound capability reconstruction, laboratory medicine will play an increasingly critical and irreplaceable role in overcoming cancer therapy resistance and realizing the ultimate goal of truly personalized precision medicine.

CLINICAL IMPLICATIONS AND TRANSLATIONAL PERSPECTIVES IN HCC IMMUNOTHERAPY

Gut microbiota as predictive biomarkers and the impact of antibiotic exposure

The gut microbiota has emerged as a promising predictive biomarker for patient stratification in ICI therapy. Early clinical observations suggest that responders' intestines are often enriched with beneficial taxa such as Akkermansia, Lachnospiraceae, and Faecalibacterium prausnitzii, whose metabolic output is strongly associated with enhanced systemic anti-tumor immunity. Conversely, non-responders frequently exhibit a dysbiotic pattern. However, microbiome studies in oncology are notoriously heterogeneous. Single microbial signatures predictive of ICI response in one cohort often fail to reproduce across different geographic regions or hospital centers due to underlying population and methodological differences. Therefore, future

companion diagnostics must shift towards a multi-dimensional, integrative strategy. Combining multi-omics data (e.g., metagenomics, metabolomics, immunomics) and leveraging advanced artificial intelligence, such as random forest algorithms, can generate highly robust predictive models. Combinatorial biomarkers - such as a structural ratio of "high *Akkermansia* + high *Faecalibacterium* + low *Ruminococcus*," systemic secondary-to-primary bile acid ratios, and the abundance of microbial butyrate synthesis genes - hold immense potential for the early stratification of patients' therapeutic response and resistance risk.

When considering real-world clinical implications, the profound impact of concurrent medications - particularly antibiotics - on established immunotherapies cannot be overlooked. The current treatment landscape for advanced HCC has been unequivocally revolutionized by several pivotal registration trials, notably IMbrave150 (atezolizumab plus bevacizumab)[10], HIMALAYA (tremelimumab plus durvalumab)[12], as well as foundational studies (e.g., CheckMate-040[13], KEYNOTE-224[8], and KEYNOTE-240[35]) assessing monotherapy with PD-1 inhibitors. While these landmark trials successfully established ICIs as the standard of care, it is a critical limitation of the current oncology literature that these original study designs did not incorporate prospective gut microbiome profiling as primary or secondary endpoints. Consequently, direct microbiome sub-analyses from these specific pivotal cohorts remain sparse.

However, emerging real-world evidence bridging these established regimens with microbiome-disrupting factors provides crucial clinical insights. Recent retrospective cohorts evaluating HCC patients treated with the IMbrave150 "T+A" regimen or anti-PD-1 monotherapies have consistently demonstrated that prior or concurrent exposure to broad-spectrum antibiotics - which violently disrupt the delicate microbial interaction network required for optimal immune priming - significantly diminishes ORRs and strictly correlates with poorer overall survival in HCC patients[170]. This strongly implies that the profound clinical efficacy observed in these major trials is inherently dependent on an intact, eubiotic baseline gut microbiome. Therefore, critically analyzing these standard-of-care regimens through the lens of microbial ecology underscores an urgent translational need: rigorous antibiotic stewardship must be implemented to purposefully preserve the patient's baseline microbial immune tone, and future iterations of global, large-scale HCC clinical trials must prospectively integrate microbiome stratification to fully optimize personalized immunotherapeutic strategies.

Dietary modulation and potential role of probiotics and synbiotics

Beyond pharmacological interventions, dietary modulation represents a safe, highly accessible, and foundational strategy to fundamentally remodel the intestinal microenvironment. Diet profoundly shapes both microbiota composition and functional output. For instance, diets rich in specific fermentable fibers and prebiotics actively promote the expansion of SCFA-producing taxa (such as Lachnospiraceae and Ruminococcaceae). The resulting increase in systemic butyrate and other beneficial metabolites can finely modulate mucosal T-cell responses, strictly preserve intestinal epithelial barrier integrity, and favorably condition the hepatic immune microenvironment. Integrating personalized dietary guidance into the standard oncological care of HCC patients may serve as a potent, synergistic "preconditioning" strategy to enhance subsequent ICI regimens.

Direct microbiota-targeted interventions, including next-generation probiotics and synbiotics, are rapidly emerging as powerful tools to reverse immunotherapy resistance. Basic research confirms that defined specific strains hold immense translational value. For example, postbiotic compounds extracted from *Lactiplantibacillus plantarum* exhibit potent protective effects in liver injury models, and even heat-inactivated *Akkermansia muciniphila* retains the robust activity of its outer membrane protein (Amuc_1100) to maintain intestinal barrier integrity. Moving forward, intervention strategies must definitively evolve from a "one-size-fits-all", broad-spectrum supplementation approach towards true

precision medicine. By comprehensively evaluating a patient's unique enterotype and metabolic profile, clinicians could theoretically supplement specifically defined immune-sensitizing strains, employ customized phages to target pathogenic bacteria, or utilize engineered bacteria strictly designed to deliver specific immunomodulatory molecules directly to the gut.

In addition to specific dietary components, metabolic interventions have shown immense potential. Preclinical studies indicate that short-term starvation cycles can profoundly reshape the gut microbiota architecture, reduce systemic inflammation, and synergistically potentiate the anti-tumor efficacy of ICIs[171].

Ongoing clinical trials and future directions

While early correlative small-cohort studies provide intriguing evidence, these microbiota-targeted strategies are still in their clinical infancy. Strategies integrating microbial preconditioning (e.g., FMT or rationally designed consortia) with established ICI regimens are currently being aggressively explored in ongoing clinical trials, such as the FAB-HCC trial[41]. The ultimate success of these interventions hinges entirely on establishing a rigorous clinical validation loop. The field must rapidly transition to standardized, adequately powered, multi-center phase II/III randomized controlled trials to critically evaluate the optimal combination, optimal timing, and true clinical utility of these novel therapies. Furthermore, extensive collaborative efforts with regulatory agencies are urgently needed to establish clear evaluation and approval pathways, ensuring the long-term safety and standardized, pharmaceutical-grade production of FMT and live biotherapeutic products. Ultimately, standardizing the entire translational process - from precise sample collection to AI-driven data analysis - will propel HCC immunotherapy into a transformative new era of systemic remodeling and precise regulation.

CONCLUSIONS

Resistance to immunotherapy in HCC represents a profoundly complex challenge involving the TME, the host immune system, and systemic regulatory networks. This review systematically elucidates the central role of the gut microbiota in orchestrating the response to immunotherapy and mediating resistance in HCC via the "gut-microbiota-liver axis". Mechanistically, the gut microbiota and its downstream metabolites directly shape the functional states of effector T cells, regulatory T cells, myeloid-derived suppressor cells, and macrophages within the TME. By modulating key signaling pathways - such as SCFAs, secondary bile acids, and tryptophan metabolism - the microbiota profoundly dictates the "cold" *vs.* "hot" immune phenotypes. Concurrently, microbial structural components systemically regulate antitumor immune responses through pathways including PRR activation and the mediation of antigen cross-reactivity. Clinical cohorts further confirm that immunotherapy responders possess a characteristic, beneficial microbial architecture, whereas exposure to broad-spectrum antibiotics violently disrupts this balance and drastically diminishes therapeutic efficacy. These findings not only establish the potential of the gut microbiota as a predictive biomarker but also drive the development of a series of microbiota-targeted intervention strategies - from FMT and precision probiotics to metabolite therapies and engineered bacteria design. These strategies aim to reshape the host immune status "from the outside in", offering novel avenues to overcome ICI resistance.

While optimistically envisioning its therapeutic prospects, we must examine the complex role of the gut microbiota in HCC development with a more comprehensive and dialectical perspective. The gut microbiota is by no means merely a "beneficial resource"; its relationship with the host exhibits a significant "double-edged sword" characteristic. Under specific conditions, gut dysbiosis itself is a key environmental driver of liver inflammation, fibrosis, and even carcinogenesis. For instance, the enrichment of certain pathogenic or conditionally pathogenic bacteria (such as *Fusobacterium nucleatum* and *Klebsiella pneumoniae*) can actively shape a microenvironment conducive to tumorigenesis and immune evasion by disrupting the intestinal barrier, promoting chronic inflammation, producing genotoxic metabolites, or

directly activating oncogenic pathways. Therefore, future translational research must not only focus on how to strategically "harness" beneficial microbiota but also comprehensively delve into identifying which microbial species or functional modules act as absolute "pathogenic drivers", exploring precise pharmacological strategies for their targeted elimination or neutralization.

However, the field faces multiple formidable challenges on the road to routine clinical translation. First, the mechanistic network is exceptionally complex. The microbiota, its metabolome, the host immune system, and the tumor ecosystem form a multi-layered, highly dynamic interactive network. Clarifying definitive causal relationships within this web still relies heavily on refined experimental platforms, such as germ-free (gnotobiotic) murine models and broad-spectrum antibiotic depletion protocols. Second, profound inter-patient heterogeneity remains a massive hurdle. The baseline composition of the microbiota is deeply influenced by a myriad of factors - including host genetics, diet, and geographic environment - resulting in high personalization that poses a severe test for the development of universal, "off-the-shelf" intervention strategies. Third, the current level of clinical evidence remains insufficient. Because most existing studies are small-scale observational cohorts or preclinical animal models, there is an urgent imperative for large-sample, multicenter, phase II/III randomized controlled trials to provide high-level, definitive evidence prior to widespread clinical application. Finally, regulatory and safety frameworks are not yet fully matured. The long-term safety profiles, standardized pharmaceutical-grade production protocols, and clear regulatory approval pathways for novel modalities (e.g., FMT and live biotherapeutic products) remain to be rigorously established.

Confronting these current challenges, future research must achieve a definitive paradigm shift from mere "correlational analysis" to "mechanistic causality and precision intervention". Constructing a comprehensive microbiome-based companion diagnostic system represents a crucial foundational step toward precision oncology. This necessitates the deep integration of multi-omics data (metagenomics, metabolomics, immunomics) and the leveraging of advanced artificial intelligence models to develop robust microbiome biomarker maps capable of accurately predicting ICI efficacy and individual resistance risk. For example, combinatorial biomarkers based on specific microbial structural ratios, metabolic functional gene clusters, and key serum metabolite ratios could enable the highly accurate, early stratification of patients' therapeutic response potential. To decisively advance clinical translation, it is absolutely essential to establish a standardized, highly reproducible analytical and management framework. Standardization is the critical cornerstone for bridging the prevailing gap between basic research and bedside clinical practice, necessitating unified protocols encompassing the entire pipeline - from meticulous sample collection and processing to bioinformatic data analysis and clinical interpretation. Simultaneously, robust collaborative efforts with global regulatory agencies are urgently needed to establish clear, navigable regulatory and evaluation pathways for utilizing the microbiota as a "live drug" or a complex diagnostic biomarker.

The core of future intervention strategies lies in the development of personalized, modular microbiota-targeted treatment regimens. As a future conceptual framework, this paradigm explicitly requires moving beyond an empirical, "one-size-fits-all" probiotic supplementation approach. Instead, it demands precision rational design based exclusively on the patient's unique enterotype, functional metabolic profile, and baseline immune status: employing customized bacteriophages or specific metabolic enzyme inhibitors for precise therapeutic modulation in cases of defined microbial functional dysregulation; supplementing next-generation probiotics or tailored synbiotics with well-defined immune-sensitizing functions for patients severely lacking beneficial commensals; and for instances of severe, refractory dysbiosis, utilizing rigorously screened FMT or rationally designed synthetic microbial communities, which still hold immense therapeutic value.

The ultimate success of personalized microbiota intervention hinges entirely on deepening our scientific understanding from "correlation" to "causality" and firmly establishing a rigorous clinical validation loop. It necessitates the utilization of cutting-edge experimental models and high-resolution technologies to elegantly elucidate the precise molecular mechanisms by which specific bacterial strains or discrete metabolites dictate therapeutic efficacy. Concurrently, exceptionally well-designed randomized controlled trials are imperative to clinically validate the optimal combinatorial approaches and the precise timing of these intervention measures alongside existing systemic immunotherapies, while continuously monitoring their long-term safety and stability for both the host and the delicate microbial ecosystem. Ultimately, achieving the grand vision of precision microbiome medicine relies on seamless interdisciplinary integration and highly innovative clinical practice models. This requires forging integrated teams encompassing medical oncology, hepatology, clinical nutrition, and microbiology to deeply incorporate active microbiota management into the entire longitudinal HCC diagnosis and treatment cycle. Furthermore, it is necessary to actively explore value-based innovative payment models and significantly strengthen patient education to enhance overall awareness and long-term adherence to this novel "host-microbe" co-management model.

In summary, advanced strategies targeting the gut microbiota must be temporally and synergistically integrated with existing oncological therapies. By performing strategic microbial "preconditioning" prior to immunotherapy to profoundly optimize the tumor immune microenvironment, or by applying microbiota-targeted agents following local locoregional treatments to strictly maintain barrier function, there is immense translational potential to definitively transform the gut microbiota from an unpredictable "driver of resistance" into a highly predictable, pharmacologically intervenable "therapeutic ally". Through rigorous, systematic, and interdisciplinary global efforts, we have the unprecedented opportunity to usher in a new era of systematic regulation and holistic management in HCC immunotherapy - the era of precision microbiome medicine - thereby achieving substantive, paradigm-shifting breakthroughs in overcoming therapeutic resistance and dramatically improving patient survival.

DECLARATIONS

Authors' contributions

Investigation, writing-original draft, editing: Wang J
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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI and AI-assisted tools statement

During the preparation of this manuscript, the AI tools Gemini and its built-in Gemini 3 Flash Image model (Google) were used solely for the creation of illustrations (Graphical Abstract and Figures 1-12). The tools did not influence the study design, data collection, analysis, interpretation, or the scientific content of the work. All authors take full responsibility for the accuracy, integrity, and final content of the manuscript.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

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Not applicable.

Consent for publication

Not applicable.

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