Gut-Microbiota-Mediated Immune Regulatory Mechanisms by Immunotherapy

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Cancer contains tumor-initiating stem-like cells (TICs) that are resistant to therapies. Experimental evidence indicates that hepatocellular carcinoma (HCC) and TIC development are influenced by permissive conditions in response to changes in gut microbiota.

cancer stem cell (CSC) tumor-initiating stem-like cells (TICs) hepatocellular carcinoma (HCC)

1. Introduction

Treatment options for HCC are limited. The 3- or 5-year survival rate of HCC is 13–21% and 5%, respectively, without any curative treatment in advanced countries such as the U.S. [1][2][3][4][5]. The incidence rate of extrahepatic metastasis is 13% at 5 years [6][7]. Currently liver resection is the only viable option for HCC combined with cirrhosis that is the terminal stage of fibrosis, leading to hyperplasia formation [8]. Currently, only 10–23% of HCC patients are candidates for surgery [9][10][11]. Thus, HCV-associated HCC remains an incurable malignancy and an urgent unmet medical need. As 40% of HCCs are derived from TICs, TIC-mediated HCC development must be considered as clinically important.

TICs are resistant to conventional chemotherapy and immunotherapy and persist as recurrent tumors or circulating tumor cells (CTC) [12]. TICs express a core pluripotency-associated transcription factor (TF) network [13][14]. Up to forty percent of HCCs have clonality and are considered to originate from progenitor/stem cells [15][16][17][18]. TICs express stemness genes that are also expressed in pluripotent stem cells, including CD133 (Prominin in mice), Wnt/β-catenin, Nanog [19], NOTCH, Hedgehog/SMO, BMI, OCT3/4 [20][21][22][23][24][25][26][27][28][29][30][31], CD44 (cell adhesion molecule), and CD34. CD133+/CD49f+ HCC TICs confer resistance to chemotherapy, which hampers efficacy of therapy in HCC [32]. TICs exhibit a loss of this intrinsic asymmetry, leading to subsequent unchecked expansion of the progenitor cell pool [33][34][35][36][37][38]. Cell-fate-determinant molecule NUMB, and p53-MDM2-associated proteins, are targeted by interacting protein TBC1D15 in TICs [39]. These stemness factors are commonly expressed in TICs and pluripotent stem cells. Stemness factors promote therapy resistance and self-renewal ability.

1.1. Challenge in Targeting of Actionable Mutations

There are no current targeted therapy options for the most prevalent mutations (most are not “actionable”). HCC has only 2.5% of actionable mutations that can be clinically targeted by FDA-approved drugs, while biliary cancer

has 45% actionable mutations based on Oncokb.org (Level 3A, Level 3B) and HCC tumor genetics in a TCGA cohort [40]. These indicate many HCC mutations do not have conventional therapeutic targets. Therefore, the role of immunotherapy for the treatment of these diseases is an area of intense investigation [40].

1.2. Molecular Tumor Board (MTB) Review and Actionable Mutations in Liver Cancer

The molecular tumor board (MTB) review can guide choices of therapy for actionable mutations, clarify diagnosis, and identify patients who require germline testing. Prospective clinical sequencing of 10,000 patients revealed the mutational landscape of metastatic cancer [41] Clinical actionability of somatic alterations revealed by MSK-IMPACT was the lowest in HCC mutations at 2.5% [41][42][43][44][45], while clinical actionability of somatic alterations revealed by MSK-IMPACT showed that 45% of biliary cancer mutations are clinically actionable. The molecular tumor board (MTB) for intrahepatic cholangiocarcinoma (iCCA) shows clinically targetable mutations [46][47][48]. iCCA is a heterogeneous disease with several identifiable genetic driver mutations (i.e., FGFR2-fusions IDH mutations, etc.) [40]. For iCCA, fluorescence in situ hybridization (FISH), DNA/RNA-seq, and immunohistochemistry (IHC) analyses can identify cancer-driver mutations, including IDH1/2, CDK4/67, PRKACA/B, and BRCA1/2. FGFR2 and NYRK fusions, BRAF and IDH1 mutations, and microsatellite instability high (MSI-H)/dMMR (defective mismatch DNA repair) predict responses to targeted/immune therapies [41]. Tumor next-generation sequencing (NGS) should be considered in selected HCC patients with atypical histology/diagnostic features or who may be eligible for clinical trials. HCC classification, cells of origin, genetic and epigenetic abnormalities, molecular alterations, biomarker discovery, and treatments of iCCA have been well characterized [49].

2. Gut-Microbiota-Mediated Immune Regulatory Mechanisms by Immunotherapy

2.1. Bacterial Enzyme Inhibitors Can Be Used for Treatment

Gut microbial produced metabolites can be recognized by host pathogen recognition sensors to promote HCC progression. Metabolism of dietary components by the gut microbiota produces short-chain fatty acids, including other metabolites. When combined with microorganism fragments, these can stimulate the meta-organismal endocrine axis to promote HCC onset and growth. For example, trimethylamine (TMA) produced in the gut promotes ALD [44]. Thus, pharmacological interventions at the level of the gut microbiome should reduce HCC risk.

Targeting of the gut microbiota has great potential as a therapeutic modality for many diseases. However, relatively little is known regarding the contribution of commensal bacteria to normal host physiological functions [45]. For example, it was reported that 11 bacterial strains in feces obtained from normal human donors induce CD8 T cells to produce IFN-γ in the intestine in the absence of a generalized inflammatory response dependent on CD103+ DC and MHC class Ia [45]. These 11 strains also improved the efficacy of immune checkpoint inhibitors and aided host suppression against *Listeria monocytogenes* infection [45]. Thus, these 11 identified strains, which represent low-abundance components of the human microbiome, are potential biotherapeutics [45].

2.2. TLR2 Signaling in DCs Promotes Treg Differentiation to Attenuate the Inflammation

TLR2 senses components from bacteria, mycoplasma, fungi, and viruses [47] to activate NF-κB to promote a Th17 cell response to enhance the inflammation response and anti-inflammation responses [48][50]. *Lactobacillus acidophilus* stimulates the TLR2 pathway of murine myeloid dendritic cells (mDC) to induce interferon-β (IFN-β), while IL-10 secretion in plasmacytoid DC (pDC) is TLR9 dependent (**Figure 1**). *Bifidobacterium infantis 35624* stimulates the TLR2/TLR6 pathway to increase IL-10 secretion from human DCs. Polysaccharide A of Gram negative bacilli can activate TLR2 and promote the secretion of anti-inflammatory cytokine IL-10 [51]. These diverse immune responses depend on the appropriate co-receptor and microenvironment [48].

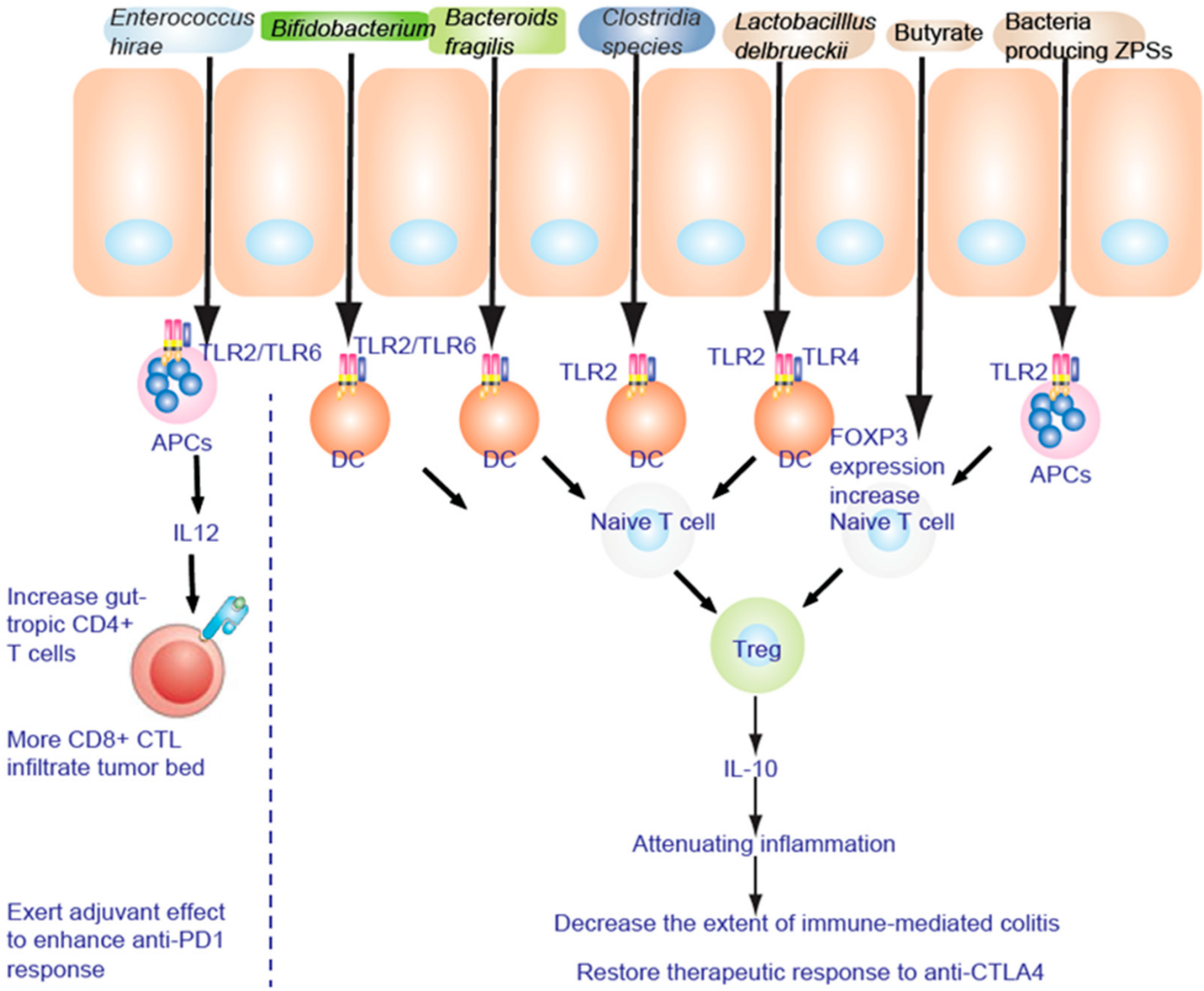

Figure 1. TLR2 is necessary to alleviate the inflammatory response. Fructo-oligosaccharide and inulin are considered as prebiotics, affecting IECs to be hyporesponsive to activation of NF-κB and MAPK induced by pathogens. NF-κB and MAPK reduce the inflammatory response to lipopolysaccharide (LPS).

2.3. Regulatory T Cells

Treg cells secrete the anti-inflammatory cytokine IL-10 to attenuate inflammation. IL-6, IL-21, and IL-2 dynamically regulate the balance between Th17 and Treg cell differentiation [52][53]. Intestinal bacteria act to stimulate and shape the T cell subsets. Short-chain fatty acid prime and induce Th17 cells undergo differentiation locally in the lamina propria. In addition, segmented filamentous bacteria antigen (SFB) adhesion to enterocytes stimulates serum amyloid A and ROS to induce Th17 cells [54]. MHCII-dependent antigen presentation of SFB occurs on DC [55] (**Figure 1**). Commensal bacteria (such as the *Lachnospiraceae* family, A4 bacteria) induce transforming growth factor β (TGF-β) production to inhibit Th2 cell development [56]. Clostridia colonization effects on T cell differentiation induce Treg cell expansion to suppress inflammation in mice [57][58]. In germ-free (GF) mice, colonization of gut bacteria and LPS-rich sterile diet induced T and B cell proliferation and differentiation in Peyer's patches (PP) and mesenteric lymph nodes (MLN), especially by CD4+ Foxp3+ T cells in MLN [59]. Polysaccharides do affect T cell differentiation. To reinforce its intestinal colonization, polysaccharide A (PSA) from *Bacteroides fragilis* promotes Treg cell secretion and suppresses Th17 activity [60]. The growth of bacteria encoding zwitterionic capsular polysaccharides (ZPS), as shown by genomic screen, results in stimulation of T cell differentiation of Treg cells and IL-10 production mediated by antigen presenting cells (APC) [61].

Zwitterionic polysaccharides bind the TLR2 complex on $CD11b^+$ DC to mobilize lamina propria $CD11b^+$ DC. This in turn stimulates Treg differentiation to promote anergy against immunity induced by CTLA-4 blockade [62] via interleukin-12 (IL-12)-dependent cognate TH1 immune responses against Bf capsular polysaccharides (**Figure 1**). CTLA4-mediated TH1 immune response is blocked by Treg to protect against experimental abscess formation [62] independent of TLR2/TLR4-mediated innate signaling [63][64]. A clustering of genus composition of stools [65][66] distinguished three clusters with *Alloprevotella or Prevotella* driving cluster A and distinct *Bacteroides* spp. driving clusters B and C. During anti-CTLA4 (ipilimumab) therapy, the proportions of MM patients falling into cluster C increased at the expense of those belonging to cluster B through the colonization of the immunogenic bacteria *Bf* and *Bt* [62][63][64][67][68][69].

2.4. Commensal Bacteria-Derived Products Stimulate DCs and Regulate Tregs

High-alcohol-producing *Klebsiella pneumoniae* causes fatty liver disease [70]. Intestinal microbiota in human stool contributes to susceptibility to ALD shown by the use of ALD-FMT in germ-free mice [71][72]. To edit gut microbiota, four distinct bacteriophages (podophages of the virulent *Picovirinae* group) were isolated from sewage water. Feeding of four podophages of the virulent *Picovirinae* group lyse the cytolytic *E. faecalis* strain [73]. Gavage of bacteriophages that target cytolytic *E. faecalis* attenuates alcoholic liver disease that promotes *E. faecalis* expansion (2700-fold increase) by reducing steatosis, inflammation, and liver injury of mice chronically fed ethanol [74]. Therefore, the gut microbiome is a potential therapeutic target in the pathogenesis (pro-inflammatory response) and treatment of chronic liver disease [75], since it is altered in liver cirrhosis [76]. Overgrowth by Clostridiales, Streptococcus, Lactobacillus, Bacteroides, and Enterobacteriaceae genera promotes gut injury and liver disease. In liver cirrhosis, Bacteroides increase while Firmicutes decrease. Rifaximin inhibits oral-originating

species and selectively decontaminates the gut. Further environmental factors mediating microbiota changes can promote excessive inflammatory signaling.

2.5. A Live Microbiome Co-Culture in a Gut-on-a-Chip Microfluidic Device

A live microbiome was co-cultured with micro-engineered human intestinal villi in a gut-on-a-chip microfluidic device [77]. The intestine–liver axis on-chip reveals the intestinal protective role on hepatic damage (**Figure 1**) by emulating ethanol first-pass metabolism [78][79][80][81]. Those who live longer customarily consume the following foods, including pasta (barley: fibers), soybean (flavone), seaweed (mineral), seafood (fish oil: DHA, BHA), and green tea (polyphenols, catechin epigallocatechin-3-gallate: EGCG). Prebiotics are nondigestible dietary supplements, including mucin or long-chain carbohydrates, which promote proliferation of beneficial commensal bacteria and improve the ecological balance of the gut. The effects of prebiotics can be tested in this system (**Figure 1**).

Synbiotic treatment normalizes gut microbiota and concomitantly reduces toxic gut microbiota to repair leaky gut syndrome [82]. These bacteria digest prebiotics to produce short-chain fatty acids which inhibit intestinal pathogen growth, provide enterocyte nutrition (butyrate), and promote mineral absorption. Bifidobacterium growth is enhanced with a prebiotic-containing formula (90% short-chain galacto-oligosaccharide, 10% long-chain fructo-oligosaccharide), fructo-oligosaccharides [83], and inulin [84].

Patients who responded to nivolumab (PD-1 antibody) were enriched with *Bacteroides caccae* [85] and *Fecalibacterium prausnitzii*, *Bacteroides thetaiotamicron*, and *Holdemania filiformis*, whereas patients who responded to pembrolizumab (another PD-1 antibody) showed that their gut microbiota was enriched with *Dorea formicogenerans*. This treatment increased bacterial diversity and abundance of bacteria from *Akkermansia muciniphila* [86], *Bifidobacterium* spp. (*B. longum*, *Collinsella aerofaciens*) [87], *Enterococcus faecium*, and the *Ruminococcaceae* family, to induce Treg accumulation by cooperating with DC in the colon (**Figure 1**).

2.6. TLR2 Is Necessary to Alleviate the Inflammatory Response

TLR2 senses components from bacteria, mycoplasma, fungi, and viruses [47]. TLR2 signaling induces both pro- and anti-inflammatory responses. *Bifidobacterium infantis 35624* treatment increases IL-10 secretion through the TLR2/TLR6 pathway in human myeloid dendritic cell (mDC) and monocyte-derived DC (MDDC), while IL-10 secretion in plasmacytoid DC (pDC) is TLR9 dependent. Tregs secrete the anti-inflammatory cytokine IL-10 to attenuate inflammation (**Figure 1**). Therefore, feeding of FMD + synbiotics preconditions gut microbiota and repairs the leaky gut to improve immunotherapy and chemotherapy (**Figure 1**).

2.7. Metabolism and Local Effects of SCFAs

Fermentation of dietary fiber in the colon generates short-chain (ranging from one to six carbon atoms) saturated fatty acids (SCFAs) [88]. Production of SCFA is dependent on dietary fiber and can result in gut production of approximately 500–600 mmol of SCFAs per day [89]. Acetate (C2) is the most abundant SCFA in the human body,

followed by propionate (C3) and butyrate (C4) (in a molar ratio of 60:20:20, dependent on microbiota composition) as the most abundant anions in the colon [90][91]. Bowel movements transfer gut contents from the terminal ileum to the proximal colon where SCFAs can reduce the pH. Lesser amounts of other SCFAs, including caproate, formate, and valerate, are also produced [90]. Monocarboxylate transporters (MCTs) allow SCFA absorption by colonocytes in a H^+-dependent, electroneutral manner, whereas the electrogenic, sodium-dependent monocarboxylate transporter 1 (SMCT1; known as SLC5A8) transports the SCFA anion [92].

Because of microbiota changes or intestinal microbiota transplantation in liver diseases and cirrhosis, use of pharmacotherapeutics must be cognizant of these issues when considering treatment options [93]. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome [94]. Allogenic fecal microbiota transplantation in patients with NAFLD improves abnormal small intestinal permeability, as shown in a randomized control trial [95]. Alkaline phosphatase can be used as a surrogate marker for liver–gut changes. *C. difficile* with cirrhosis is a deleterious combination with greater mortality via brain dysfunction due to SCFA downregulation. GF mice have altered microbial infection inflammatory markers (IL1β, MCP1, and IBA). Post-FMT GF mice recipients show improvement of neuro-inflammation [96]. Use of capsular fecal transplantation improves microbial function and supports better clinical outcomes in cirrhosis [97]. Oral capsule FMT (containing *Ruminococcaceae*) is currently under investigational new drug application (IND) guidance [98]. A randomized clinical trial of fecal microbiota transplant for alcohol use disorder is ongoing.

2.8. Exercise or Phage Therapy Retards Liver Diseases

Exercise reduces the incidence and progression of hepatocellular carcinoma in mouse models [99]. Personalized medicine approaches will stratify the HCC patient population into distinct subpopulations that may be responsive to HCC-type specific treatments [99]. As presented, there are several avenues of liver morbidities leading to HCC. For example, the microbiota is targeted for cytolysin+ alcoholic hepatitis patients. Future investigations will support a better understanding of antibiotic therapies for enteric pathogens, long-term effects of phage-based treatments, and use of precisely editing bacteria genomes by phage therapies (single phage or phage cocktail). These are all emerging areas of investigation, and these options reflect the original intent to reverse the triggering events leading to HCC.

2.9. Caveats for Fecal Microbiota Transplantation

FMT with multi-drug-resistant organisms (MDRO) can cause problems in donor recipient patients. Avoidance of *C. difficile* is important since it is responsible for the chronic liver diseases such as cirrhosis and alcoholic hepatitis. FMT trials for chronic liver diseases are currently in progress.

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