Universidade de Lisboa

Faculdade de Farmácia

Impact of microbiome in cancer nano-immunotherapy

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Monografia orientada pela Doutora Liane Isabel Ferreira Moura, Investigadora Júnior e coorientada pela Professora Doutora Helena Isabel Fialho Florindo Roque Ferreira, Professora Associada com Agregação

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Resumo

A microbiota humana (conjunto de microorganismos que vive no corpo humano) atrai muita atenção por parte dos investigadores devido ao seu impacto numa variedade de doenças, nomeadamente o cancro, uma das principais causas de mortalidade mundial. Nos últimos anos, vários estudos realçaram o duplo papel da microbiota intestinal na preservação da saúde. Certas bactérias têm a capacidade de ativar o sistema imunitário para combater o cancro, enquanto outras, por outro lado, medeiam uma imunossupressão, permitindo às células cancerígenas escaparem do sistema imunitário. As terapias convencionais no combate ao cancro incluem cirurgia, quimioterapia, radioterapia e imunoterapia. No entanto, limitações, tais como a falta de especificidade, citotoxicidade e multirresistência representam um desafio substancial para o tratamento do cancro. As abordagens tradicionais para a manipulação do microbioma, como antibióticos, dieta, prebióticos, probióticos e transplante de microbiota fecal – uma abordagem emergente -, demonstraram melhorar a eficácia das terapias tradicionais contra o cancro, como a quimioterapia e a imunoterapia. De forma a superar as limitações das terapias convencionais, uma melhor compreensão dos mecanismos relacionados com a progressão do cancro permitiu o desenvolvimento de sistemas de libertação de fármacos baseados em nanotecnologia que revolucionaram o tratamento do cancro. Destacam-se os avanços nestes sistemas que visam aumentar a eficiência da terapia através da modulação/manipulação da microbiota intestinal. Nesta dissertação, exploramos a importância da microbiota na tumorigénese e na terapia do cancro, a sua interação com o sistema imunitário e como estratégias direcionadas à microbiota podem melhorar a eficácia do tratamento do cancro, como estratégias tradicionais e sistemas de libertação de fármacos baseados em nanotecnologia.

Palavras-chave: cancro, microbioma, imunoterapia, nanotecnologia

Abstract

The human microbiota (a collection of microorganisms that live in the human body) is attracting a lot of attention from researchers due to its impact on a variety of diseases, including cancer, one of the major causes of mortality worldwide. In recent years, numerous studies have highlighted the dual role of the gut microbiota in preserving health. Some bacteria activate immunity to fight tumors, while others mediate immunosuppression to assist cancer cells evade the immune system. Conventional cancer therapies include surgery, chemotherapy, radiotherapy, and immunotherapy. However, limitations such as lack of specificity, cytotoxicity and multidrug resistance pose a substantial challenge for the treatment of cancer. Traditional approaches to microbiome manipulation, such as antibiotics, diet, prebiotics, probiotics, and fecal microbiota transplantation - an emerging approach -, have been shown to improve the efficacy of traditional cancer therapies, such as chemotherapy and immunotherapy. Nevertheless, in order to overcome the limitations of conventional therapies, a better understanding of the mechanisms related to cancer progression has led to the development of numerous nanotechnology-based drug delivery systems that have revolutionized cancer treatment. The advances in these systems that aim to increase the efficiency of the therapy through the modulation/ manipulation of the intestinal microbiota, are highlighted. In this dissertation, we explored the importance of microbiota in tumorigenesis and cancer therapy, its interaction with the immune system and how microbiota-targeted strategies may improve cancer treatment efficacy, such as traditional strategies and nanotechnology-based drug delivery systems.

Keywords: cancer, microbiota, immunotherapy, nanotechnology

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Acronyms

A. muciniphila - Akkermansia muciniphila

ACT - Adoptive cell therapy

AGS cells - Gastric epithelial cell membranes

AHSCT - Allogeneic hematopoietic stem cell transplantation

AMPs - Antimicrobial peptides

APCs - Antigen-presenting cells

B. fragilis - Bacteroides fragilis

BAs - Secondary bile acids

BC - Breast cancer

C. butyricum - Clostridium butyricum

C. jejuni - Campylobacter jejuni

C. pneumoniae - Chlamydophila pneumoniae

CDT - Cytolethal distending toxins

CpG ODN - Cytosine-phosphate-guanine Oligodeoxynucleotide

CRC - Colorectal cancer

CTL - Cytotoxic T lymphocyte

CTLA-4 - Cytotoxic T lymphocyte-associated antigen 4

DAMPs - Damage-associated molecular patterns

DCs - Dendritic cells

DDS - Drug delivery system

DNA - DeoxyriboNucleic Acid

E. coli - Escherichia coli

EcN - *E. coli Nissle*

F. nucleatum - Fusobacterium nucleatum

F. prausnitzii - Faecalibacterium prausnitzii

FMT - Fecal microbiota transplantation

GALT - Gut-associated lymphoid tissue

GF - Germ free

GI - Gastrointestinal

GVHD - Graft-Versus-Host Disease

H. Pylori - Helicobacter pylori

HPV - *Human papillomavirus*

HAPs - Hypoxia-activated prodrugs

ICB - Immune checkpoint blockade

ICI - Immune checkpoint inhibitors

IECs - Intestinal epithelial cells

IELs - Intraepithelial lymphocytes

IgA - Immunoglobulin A

IIS - Innate immune system

IL - Interleukin

ILCs - Innate lymphoid cells

IRT - Irinotecan

L. casei - Lactobacillus casei

LP - Lamina propria

LPS - Lipopolysaccharide

MDSCs - Myeloid-derived suppressor cells

MHC - Major histocompatibility complex

mLNs - Mesenteric lymph nodes

NCs - Nanoclusters

NK - Natural Killer

NO - Nitric oxide

NPs - Nanoparticles

NSCLC - Non-small-cell lung cancer

PAMPs - Pathogen-associated molecular patterns

PD1 - Programmed cell death protein 1

PD-L1 - Programmed cell death 1 ligand

PDAC - Pancreatic ductal adenocarcinoma

PLGA - Poly(lactic-co-glycolic acid)

PRRs - Pattern recognition receptors

PSA - Polysaccharide A

RCC - Renal Cell Carcinoma

RCDI - Refractory *Clostridioides difficile* infection

RES - Reticular endothelial system

ROS - Reactive oxygen species

SCFAs - Short-chain fatty acids

SFB - Segmented filamentous bacteria

SPF - Specific-pathogen-free

TAAs - Tumor-associated antigens

TCR - T cell receptor

T_H - T helper

TILs - Tumor-infiltrating lymphocytes

TLR - Toll-like receptor

TME - Tumor microenvironment

VEGFR2 - Vascular endothelial growth factor receptor 2

Index:

Figure Index:

Table Index :

1 Introduction

In the fourth century, researchers were already attempting to decipher the meaning and clinical implications of the microbiome. In fact, in the 1680s, by comparing oral and fecal samples from healthy and sick patients, Antonie van Leeuwenhoek was able to discover the vast diversity of the human microbiome (1).

The human body hosts trillions of microorganisms from diverse microbial taxa, the vast majority of which are symbiotic and non-pathogenic. Microbiota is usually defined as the assemblage of living microorganisms present in a defined environment. These microorganisms include Archaea, Yeast, Fungi, Viruses, Protozoa, and Bacteria (1). However, bacteria are the subject of most microbiological research (2).

The microbiome is defined as the set of genes and genomes of the microbiota, as well as the products of the microbiota and the host environment (3).

According to the Human Genome Project and the Human Microbiome Project, the human genome has a total number of 20.000 to 25.000 genes, whereas the number of microbial genes is believed to be around 2 million, approximately 100 times the number of human genes (4).

While each person's gut microbiota is distinct, the broad architecture is often similar, with the phyla *Firmicutes* and *Bacteroidetes* accounting for over 90% of the gut microbiota. The majority of bacteria belong to the *Clostridium* and *Bacteroides* genera, with *Lactobacillus spp*. and *Bifidobacterium spp*. being the most common commensal (species that live together for mutual benefit) species. Furthermore, there are significant differences in the bacterial populations of the oral, gastric, and gut reflecting the physiological variables such as oxygen gradient, pH, and the presence of antimicrobial peptides (AMPs) (5).

The composition, diversity, and functionality of gut microbiota do not remain static throughout life as they keep on changing over time. When we lose diversity of the microbiota we also lose metabolic functions (6).

Although the composition of the human gut microbiota is dependent on many factors such as host genetics, dietary lifestyle, ethnicity, immune status, and medication, its composition is determined in birth and infancy (5). Microbes from their mother and the environment are introduced to infants as soon as they are born. The birth process itself is a major source of this

exposure. Infants born vaginally receive bacteria that are similar to the maternal vaginal microbiota (mostly *Lactobacillus* and *Prevotella*), whereas those born through cesarean acquire bacteria that are similar to the skin microbiota (predominantly *Staphylococcus*) (7).

As a result, the previously mentioned factors can be modified/manipulated to influence the composition of the gut microbiota, thereby influencing the development of diseases, e.g., cancer (1).

2 Importance of Microbiota and Its Impact on Cancer

Considering the fact that microorganisms are constantly interacting with the host's tissues, it is worth noting that the microbiota plays an important role in the host's body function (8). The host provides resources and a living environment for the microbes, while the microbes protect the host from invading pathogens (9).

This homeostatic interaction between the host and microbiota allows microbes to either live in a favorable environment and produce essential metabolites (needed for defense), metabolize nutrients, and protect the host from invading pathogens. Therefore, they play a crucial role in disease prevention by retarding the pathogen's growth, inhibiting its colonization, and removing toxins that could be harmful to the host (8).

The disruption of the symbiotic interaction between host cells and commensal microorganisms results in dysbiosis (1). Microbial dysbiosis, defined as an imbalance in the intestinal microbial community, has been related to a variety of disease states, including metabolic and inflammatory diseases such as Type 1 Diabetes, Rheumatoid Arthritis, and several types of cancers (10, 11).

Dysbiosis could be caused by the addition or removal of community members and changes in the relative abundance of microbes (2). It occurs when the gut loses beneficial microbes and becomes enriched with pathogenic and cancer-promoting microorganisms, which increases cancer-related processes such as angiogenesis, loss of apoptosis, and cell proliferation (12). Thus, these changes in the composition of the microbiota have an impact on tumor growth (13).

Overall, the gut microbiota has a significant impact on the onset and progression of cancer through a variety of mechanisms, e.g., altering the host´s deoxyribonucleic acid (DNA), establishing and sustaining a pro-inflammatory environment, and influencing the host´s immunological responses (14). Nonetheless, the mechanisms through which bacteria invade tumors are poorly known (8, 9).

When dysbiosis is installed, bacterial pathogens can proliferate and release a huge number of toxins, which causes host DNA breaks, contributing to genomic instability, tumor initiation,

and progression in predisposed cells. This is the case of colibactin and cytolethal distending toxins (CDT), both of which are generated by *Escherichia coli* and have deoxyribonuclease activity (Figure 1) (15, 16).

A microorganism implicated in the pro-cancer processes is *Helicobacter pylori.* This bacterium lead to DNA damage accumulation and induces cytotoxin-mediated degradation of the p53 proteasome in gastric epithelial cells that triggers cell proliferation and potentially cancerogenic changes in the affected host's cells (Figure 1) (15, 17, 18).

Furthermore, other bacteria can promote cancer growth by blocking immune effectors that ordinarily inhibit carcinogenesis. *Fusobacterium nucleatum*, for example, inhibits the host's Natural Killer (NK) cells to recruit Myeloid-derived suppressor cells (MDSCs) at the site of infection, indirectly supporting cancer development (Figure 1) (15, 19). Figure 1 gives an overview of the gut microbiome's pro-tumoral actions.

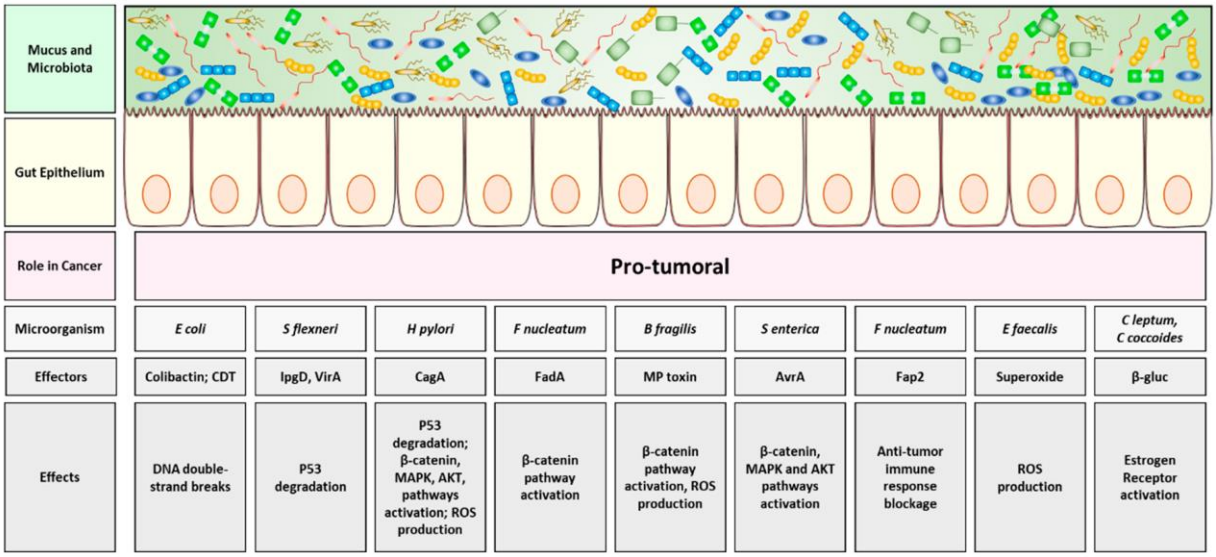

Figure 1: The gut´s microbiota pro-tumoral effects (15)

However, some bacterial species have an unexpected ability to invade and colonize solid tumors, which may result in tumor growth retardation or even tumor elimination (20).

Several microbiome-derived molecules participate in anti-tumor activity (21). Butyrate and propionate, normally produced in the gut by butyrogenic anaerobic, e.g., *Faecalibacterium prausnitzii* or *Clostridium spp*., and *Propionibacterium*, respectively, inhibit the host's tumor cells, resulting in an anti-cancer effect (Figure 2) (15, 21-23).

Another example is the bacterial lipopolysaccharide (LPS), which helps in the activation of the host's cell surface receptor – toll-like receptor 4 (TLR4), thereby activating an immune T cell-mediated response against a variety of cancer cells. Further, the Ferricrome metabolite secreted by *Lactobacillus casei* is capable of inducing apoptosis in tumor cells via c-Jun N-terminal kinase pathway activation, and *Lactobacilli spp.* may stimulate the host's immune cells, such as dendritic cells (DCs) or the T Helper 1 (T_H1) response, as well as NK cells, resulting in the eradication of malignant cells (Figure 2) (15, 21, 24). Figure 2 illustrates the anti-cancer effects of the gut microbiome.

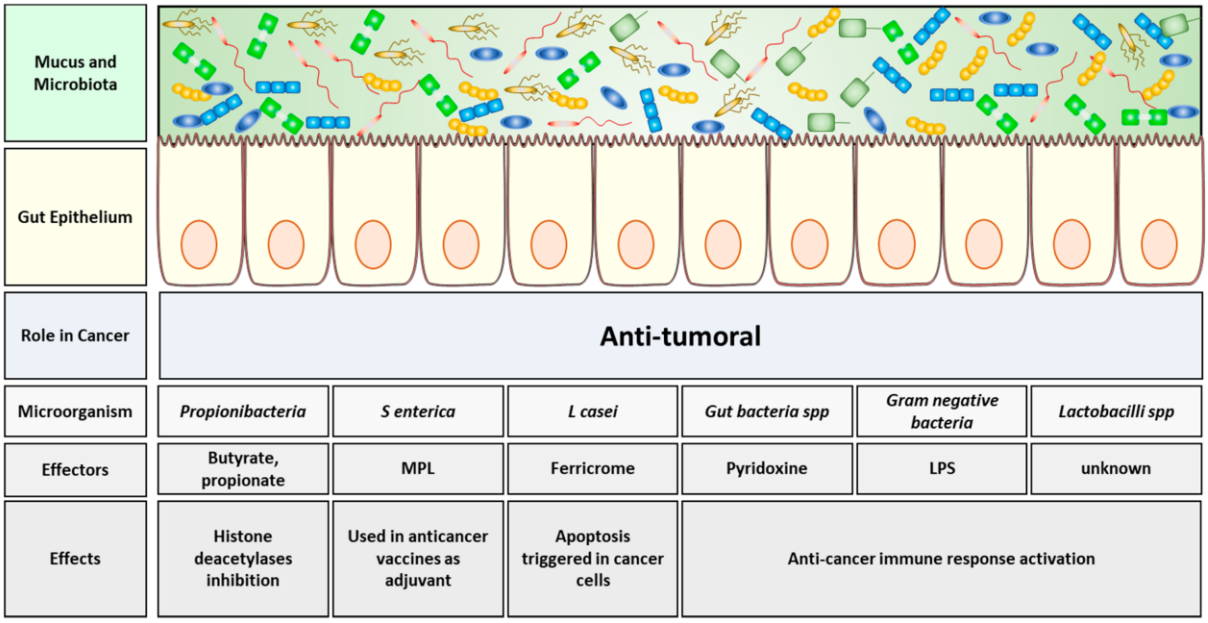

Figure 2: The gut´s microbiota anti-tumoral effects (15)

Therefore, either tumor-promoting and anticancer effects can be seen in the presence or absence of specific microbial species (25). Figure 3 depicts bacteria's dual activity as carcinogenic (blue side) or anticancer agents (pink side) (10).

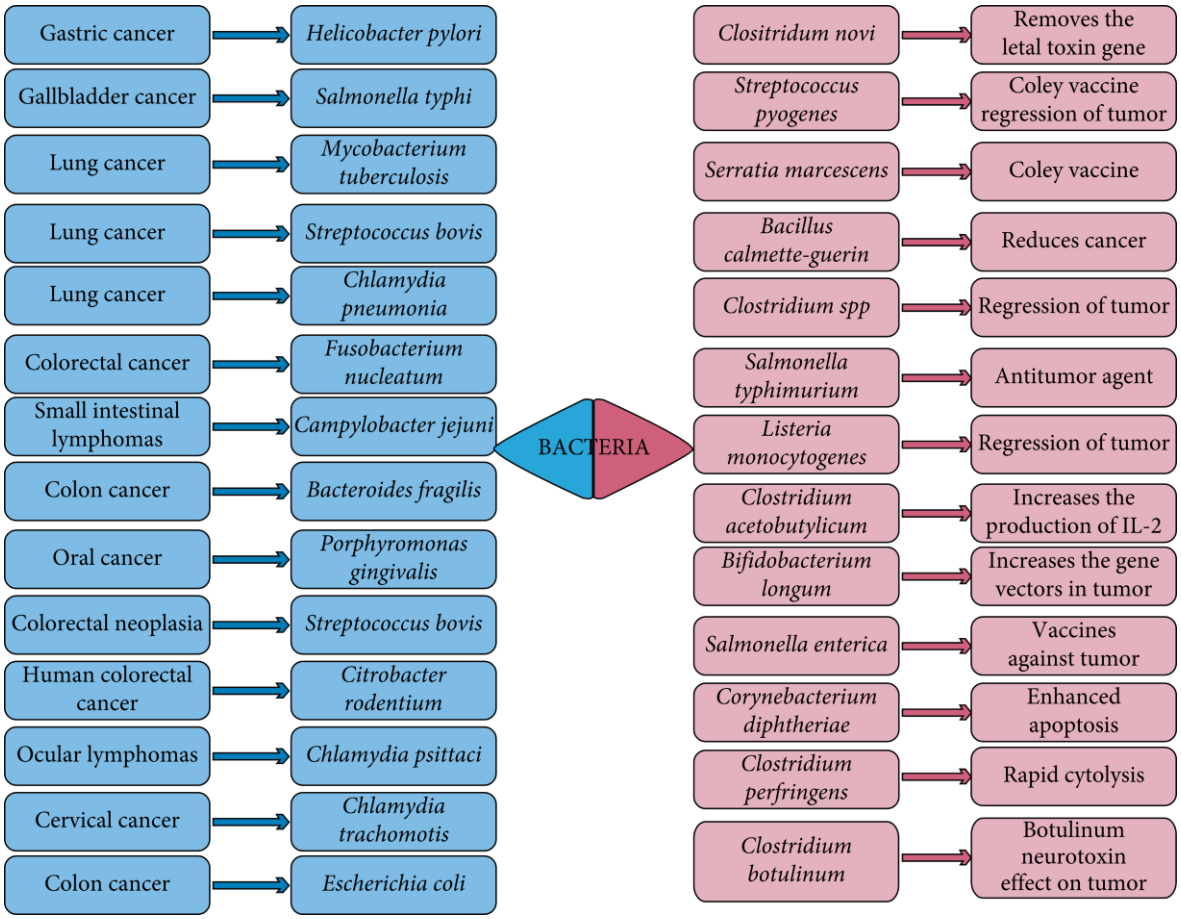

Figure 3: Bacteria's dual activity - carcinogenic (in blue) or anticancer agents (in pink) (10)

In addition to gut microbial dysbiosis, many studies have been conducted to determine the connection of oral microbial dysbiosis and cancer, as well as the role of the oral microbiota in *in-situ* or in distant tumor progression (10, 26).

Studies have found a significant association between bacteria in the oral cavity with the progression of different carcinomas, e.g., colon cancer. Thus, these bacteria are capable of being present in the colon where they can cause changes to the resident bacteria composition. This will result in intestinal dysbiosis, which stimulates the immune system, causing an inflammatory reaction and the establishment of colon cancer (10).

There are different types of microorganisms, other than bacteria, that contribute to cancer. One example is the *Human papillomavirus* (HPV), widely documented for its role in oropharyngeal cancer progression. Furthermore, abnormalities in fungi and oral parasites may also be associated with carcinogenesis, although more studies are needed to confirm this (26).

3 The Microbiome and Its Association with Various Types of Cancer

It was shown that each type of tumor has a unique microbiome composition (27). In fact, the use of different methods for microbiome profile determination, such as metagenomics and transcriptomics analysis, allows the characterization of the microbiome in different types of cancer (21).

3.1 Oral Cancer

A number of bacterial species in the oral cavity have been associated to the development of oral cancer by causing persistent inflammation (10). However, many other factors may contribute to oral cancer, including excessive alcohol consumption, smoking cigarettes, an unhealthy diet, and poor oral health (8). According to research, the five major phyla found in a healthy human oral cavity are *Fusobacteria*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Firmicutes*, now named *Fusobacteriota, Bacteroidota*, *Actinomycetota*, *Pseudomonadota* and *Bacillota,* respectively (28).

Researchers have highlighted the relationship between oral fungi and viral microorganisms in the development of mouth cancer. HPV-16, a significant causative agent of countless carcinomas connected to oropharyngeal squamous cells, and *Candida albicans*, which is responsible for nearly 30% of oral malignancies, are two examples (29, 30).

3.2 Liver Cancer

The hepatic portal vein is responsible to transport metabolites, pathogen-associated molecular patterns (PAMPs), and antigens from gut bacteria to the liver. Thus, gut microbiota influences not only the entire gut but also lateral organs, e.g., the liver (8, 31).

Studies have shown individuals with advanced fibrosis have lower quantities of *Bacteroides* and higher amounts of *Prevotella* bacteria, with the species *Prevotellacopri* acting as a good indicator of advanced liver fibrosis and chronic liver illness. While *Fusobacteria, Firmicutes, Proteobacteria, Actinobacteria,* and *Bacteroidetes* are the most common bacterial phyla in liver cancer (21).

Further, secondary bile acids (Bas), LPS, and lipoteichoic acid are some of the gut bacteria-derived chemicals that have been demonstrated to either accelerate carcinogenesis or decrease anti-tumor immunity in the liver (Figure 5) (31-33). It was found that eliminating *Clostridium* XIV, increasing primary bile acids, and lowering Bas slow the progression of liver cancer (32).

3.3 Breast Cancer

After lung and bronchus cancer, breast cancer (BC) is the major cause of death among women all over the world. BC is related with estrogen alterations. High estrogen levels have been shown to deregulate many regulatory pathways, resulting in overexpression of BC-causing factors (21).

Although the breast's microbiome contribution to BC is unknown, the gut microbiota appears to play a key role in the disease pathogenesis. The gut microbiota secretes bioactive metabolites, such as reactivated estrogens, Short-chain fatty acids (SCFAs), amino acid metabolites, and Bas. These metabolites resemble human hormones and travel through the bloodstream to distant locations of action, and so, influence BC (27).

Researchers have reported a unique microbiome in breast tissue, and the presence of numerous bacterial species that play important roles, such as supporting neonatal development and immune system maturation (10). There is a significant difference in the persistent microbiota composition between benign and malignant breast tissue samples (21).

3.4 Pancreatic Cancer

Pancreatic cancer is ranked as the 7^{th} leading cause of cancer mortality in the world (34). Pancreatic cancer refers to tumors that begin in pancreatic cells, including exocrine tumors. In general, it is divided into two types: pancreatic ductal adenocarcinoma (PDAC), which accounts for around 95% of pancreatic malignancies, and endocrine tumors, which are responsible for about 5% of pancreatic cancers (35).

The pancreas has been assumed to be a sterile organ, however recent research has revealed the presence of bacteria populations in both normal pancreatic tissue and PDAC tumor samples. Besides the intratumoral dysbiosis, a recent study has found some differences in the oral and intestinal microbiota between PDAC patients and healthy people (Figure 4) (36).

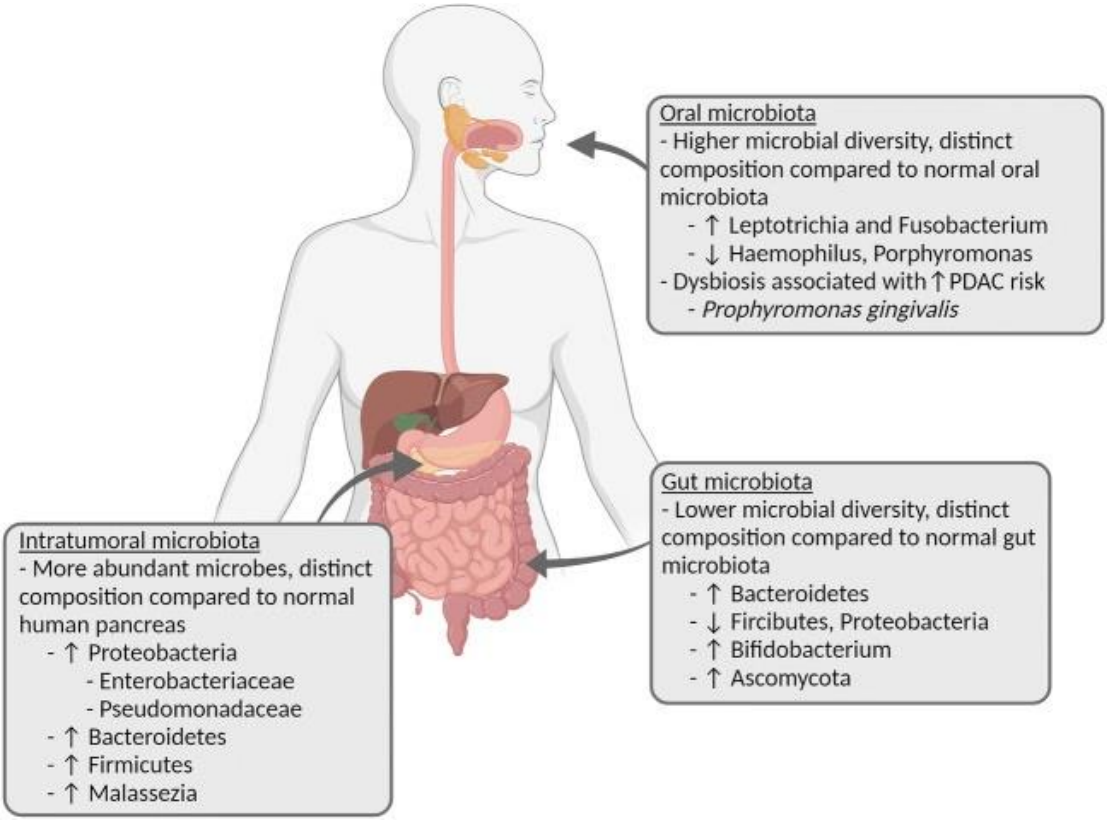

Figure 4: Microbiota in Pancreatic Cancer (36, 37)

3.5 Lung Cancer

Chlamydophila pneumoniae is an intracellular bacterium that infects cells in the human respiratory system and predisposes to lung cancer development. Studies suggest that *C. pneumoniae* was found in 230 cases of lung cancer, and patients infected with *C. pneumoniae* were 1.6 times more susceptible to develop lung cancer (27).

Lung cancer is recognized to be directly connected to persistent inflammation. Some of the bacterial infection mechanisms that induce lung cancer include gene damage and neoplastic transformation, which are triggered by inflammatory mediators, nitric oxide (NO), and other types of reactive oxygen species (ROS). *Chlamydial* infections induce an increase in NO release, which can cause an inflammatory response, thereby leading to the development of lung cancer (27, 38).

3.6 Gastric Cancer

Despite there are multiple predisposing risk factors, such as family history, bad eating habits, alcohol, and smoking, *H. pylori* infection has the strongest connection with gastric cancer (27). *H. pylori* is a helicoidal gram-negative microaerophilic bacteria that can infect the stomach mucoid lining. Although most people that are *H. pylori* -positive may be asymptomatic, its infection is associated with long-term effects, such as gastric ulcers, gastritis, and gastric cancer (25, 27).

H. pylori infection has been related to the development of gastric cancer through a variety of processes, including the production of virulence factors, such as cytotoxin-associated gene A (CagA), an oncoprotein that impairs normal epithelial cell division in the gastric mucosa, and vacuolating cytotoxin gene (VacA), that causes chronic gastric inflammation, endoplasmic reticulum stress, autophagy, and oxidative stress in gastric epithelium (Figure 5) (25, 27, 31).

3.7 Colorectal Cancer

The colorectal cancer (CRC), one of the most important cancers in the world, is the third most common cancer in both genders. In CRC, modifications in the bacterial composition of the gut's microbiota have been identified, and they are responsible for dysbiosis in colorectal carcinogenesis. In addition to the significant microbial diversity in the tumor area, researchers discovered an increase of virulence-associated bacterial genes in the tumor microenvironment (TME), reinforcing that microbiome plays an important role in CRC formation and progression (8, 10, 21, 39).

Even though no disease-specific microbiota profile has been established, when compared to healthy people, CRC patients have lower bacterial diversity and richness. B*acteroides fragilis*, *Clostridium septicum*, *E. coli*, and *Fusobacterium spp.*, as well as numerous microbial metabolites, have been related to the development of CRC (8, 10, 40).

The gut microbiome can directly or indirectly affect CRC by secreting metabolites, invading tissues, and regulating the host immune response (13). Table 1 summarizes some of the potential mechanisms used by different intestinal bacteria to promote CRC development (41).

Table 1: Mechanisms of action of intestinal bacteria in the development and progression of Colorectal Cancer

Mechanism	Bacterial species	References
Inflammatory response	*Bacteroides fragilis, Fusobacterium nucleatum, Helicobacter pylori*	(42, 43)
Immune response	*Fusobacterium nucleatum, Akkermansia muciniphila*	(19, 44, 45)
DNA damage	*Escherichia coli, Enterotoxigenic Bacteroides fragilis*	(46-48)
Modulation of cell proliferation	*Enterotoxigenic B. fragilis, F. nucleatum*	(49, 50)

Curiously, the risk of CRC in colitis patients is tenfold higher, and this inflammation is caused by *Enterobacteriaceae* family members such as *Enterococcus faecalis* and *E. coli* when they are dysregulated. These strains are more than 100-fold higher in CRC. Furthermore, the synthesis of CDT and colibactin by *Campylobacter jejuni* and *E. coli* has been associated with CRC in mice models (1, 21, 51).

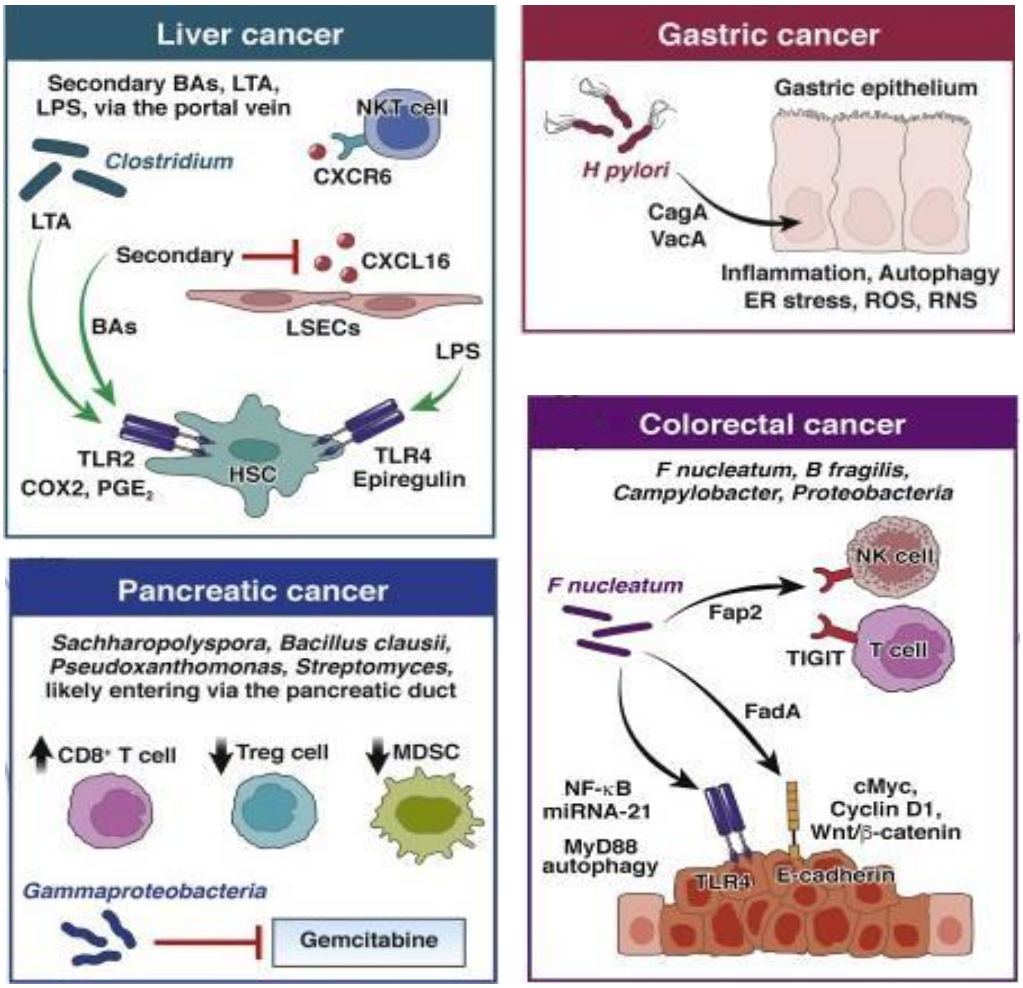

Figure 5: Effects of gut microbiota on the development of tumors (31)

4 Crosstalk Between Microbiota and Tumor Immunity

The gut microbiome prevents infection by intestinal pathogens through the establishment of a niche, altering its environment, competing for resources, and controlling host immunological defense. This process begins at birth, during the formation of the microbiome, affecting immune system maturation (13).

Immunity is the ability of an organism to defend itself against microorganisms. The immune function can be divided into two main types: innate immunity (also known as “natural immunity” or “innate resistance”) and adaptive immunity (also known as “acquired immunity”) (52).

The immune system provides us protection by combining multiple complex and integrated mechanisms from innate, humoral, and cell-mediated immunity (53).

4.1 Innate Immune System

The innate immune system (IIS) is the first line of response to bacterial invasion or an aseptic tissue injury and is known to respond quickly to pathogen, by either eliminating it or restraining it until the adaptive immune response, which is slower but more targeted, can be formed (5).

This system consists of physical, chemical, and biological barriers, as well as cellular components and soluble molecules (54). The skin and mucosal epithelium, are the most important barriers to the human body, since the immune cells in these barriers are capable of sensing, identifying, and phagocytosing pathogens to eliminate them (53).

Briefly, the intestinal immune system has two distinct roles: the capacity to react to potentially pathogenic bacteria, and microbial products while also maintaining tolerance to a variety of commensal intestinal microbes (55).

The capacity to react against potentially harmful organisms is conferred by the cells of both hematopoietic and non-hematopoietic origin, such as DCs, monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, NK cells, and NK T cells, and epithelial cells lining the respiratory, GI, and genitourinary tracts (56). Phagocytosis, the release of inflammatory mediators, the activation of complement system proteins, and the production of

acute-phase proteins, cytokines, and chemokines are the main mechanisms identified for the innate immunity (54).

Pattern Recognition Receptors (PRRs) are proteins that recognize molecules commonly found in pathogens (so-called PAMPs) or molecules released by damaged cells (so-called damage-associated Molecular Patterns—DAMPs), and they are considered part of the IIS (57).

PAMPs are highly conserved and repetitive structures found in a wide range of bacteria or their metabolites, but not in host cells. PAMPs may include bacterial, viral, or parasite products such as LPS, mannose residues, and teichoic acids, which are commonly found on the surface of microorganisms. DAMPs, on the other hand, are often derived from tissue damage that causes necrotic death in host cells and includes complement products, reactive oxygen intermediates, and stress molecules. The recognition of bacterial PAMPs by PRRs present on intestinal epithelial cells (IECs) promote local immune responses (56).

PRRs include TLRs, C-type lectin receptors (CLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), and cytosolic sensors of DNA and RNA (58). The engagement of PRRs on the innate immune cells induces co-stimulatory signals for the adaptive immune cells (particularly T lymphocytes), and they activate microbicidal and pro-inflammatory responses required to eliminate or contain infectious agents (57).

The recognition of microorganisms via PRRs helps to maintain the vital balance between the host and microorganisms. However, inappropriate PRRs activation can result in aggressive immune responses, inflammatory illness, and autoimmunity (58).

An example of an important PRR are the TLRs, a family of type I transmembrane receptors, present on innate immune cells, especially on macrophages, DCs, and B cells, as well as cells from different tissues, such as endothelial cells, epithelial cells, and fibroblasts (59, 60). The TLRs are distinct from the other PRRs involved in opsonization, complement activation, and phagocytosis because of their important involvement in binding to pathogens and beginning the inflammatory response. TLR engagement on DCs causes their maturation and promotes their migration to lymph nodes. It is possible that all TLRs operate in concert with multiple binding molecules to acquire maximum sensitivity and specificity. TLRs recognize distinct microbial structures, e.g, TLR2 targets bacterial lipoteichoic acids, whereas TLR3 recognizes double-stranded ribonucleic acid (dsRNA) (54).

4.2 Adaptive Immune System

When the IIS fails to eliminate harmful organisms, the adaptive immune system, through its specialized cells, protects by recognizing specific antigens of the pathogens (61). These cells are necessary to maintaining immunological homeostasis and the integrity of the gut mucosal barrier function (8, 13). The key hallmarks of the acquired response are specificity and diversity of recognition, memory, response specialization, self-limitation, and tolerance to organism-specific components (54).

Humoral immunity, which is mediated by antibodies, and cell-mediated immunity, are the two branches of the specific or adaptive immunity that can identify external and intracellular antigens. The most studied cellular and humoral components of the acquired immune system are: antigen-presenting cells (APCs) including macrophages, B cells, and DCs; B-lymphocytes comprising B cells in the process of development, mature B cells, plasma cells, and memory B cell; T cells across different stages of development, T_H cells, Cytotoxic T lymphocytes (CTL) and NK cells (56).

B and T cells are antigen-specific cells that are only activated when a high-affinity antigen is present. Antibodies are the antigen receptors on B cells, and they recognize the tertiary (three-dimensional folded) structure of a protein, whereas T cells recognize the primary structure (amino acid sequence), but only when they are linked to class I or class II major histocompatibility complex (MHC) proteins on the cell surface (60). While in the lymphoid organs the $CD4^+$ T cells are activated by antigens presented by MHC class II molecules at DC, the $CD8^+$ T cytotoxic cells are activated by DC that present peptide-MHC class I complexes (62).

Clonal expansion of lymphocytes in response to infection is essential for the formation of an effective immune response. Furthermore, cytokines produced by cells in innate and adaptive immune responses play important roles in recruiting and activating highly specific adaptive lymphocytes (61).

Pathogen elimination and host immunity will be achieved by a well-coordinated innate and adaptive immune response. However, failure to effectively differentiate self from non-self in innate and adaptive immunity can result in harmful immunologic responses including allergy, autoimmunity, and allograft rejection (52, 60).

4.3 The Crosstalk Between Microbiome and Immune Responses

The gut microbial community is critical for the proper functioning and stimulation of the host immune system, resulting in the development of tolerance for beneficial bacteria and an immunological response against gut pathogens (15).

The role of the microbiota has been studied in germ free (GF) mice, who exhibited a dysregulated immune system (5). The absence of a mucous layer, altered immunoglobulin A (IgA) secretion, reduced size and functionality of Peyer's patches, and draining mesenteric lymph nodes (mLNs) were observed in GF mice (63, 64). As a result, GF mice showed deficiencies in the gastrointestinal (GI) immune compartment, being more susceptible to infections (65).

The intestinal structure of the host is critical for the functioning of the gut microbiome. IECs and intraepithelial lymphocytes (IELs) constitute the single-layered epithelium of the intestinal mucosa. The IECs contain Paneth and goblet cells, the two major types of IECs, with an essential role in the gut´s homeostasis. Paneth cells secrete AMPs, which are innate immune effector molecules with bactericidal, anti-inflammatory, and anti-endotoxic capabilities, while goblet cells secrete mucus enriched with mucin glycoproteins, which in turn coat the epithelial layer. Further, the mucus layer has an inner layer that prevents bacterial adherence and invasion, and an outer mucus layer that offers an environment for the gut microbiota to grow (Figure 6) (58, 66).

The lamina propria (LP), a connective tissue layer underneath the mucosal layer, contains Peyer's patches as well as a variety of other immune cells such as APCs, innate lymphoid cells (ILCs), $CD4^+$ T, $CD8^+$ T and B cells. This gut-associated lymphoid tissue (GALT) is the body's largest immune system component, influencing immunological responses both locally and systemically (Figure 6) (63).

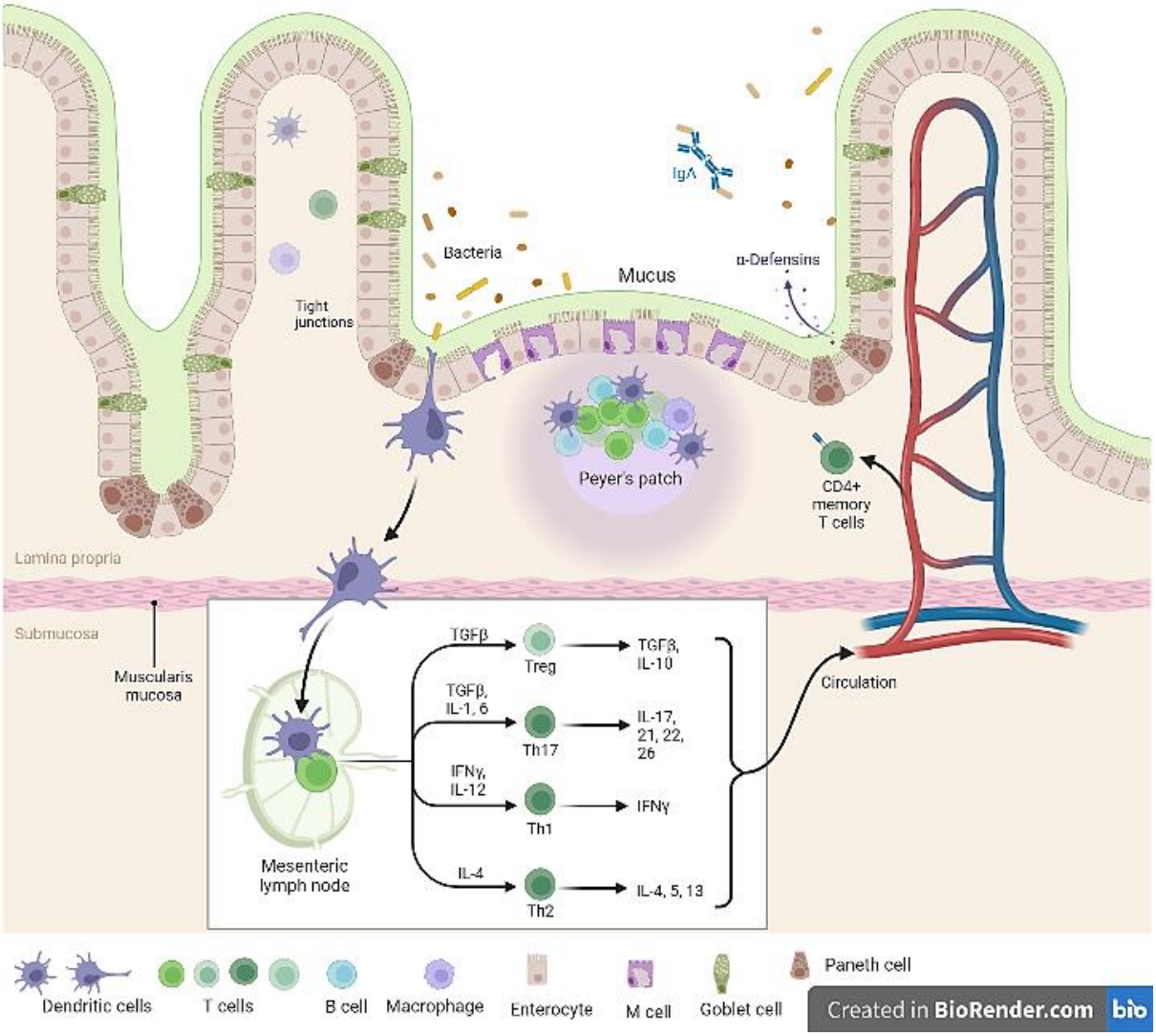

Figure 6: The microbiome and immunity. Created by BioRender.

Commensal organisms within the lumen of the gut influence the immune system, locally within the gut mucosa, and systemically, in draining mLNs (63). Goblet cells have been found to shuttle bacterial antigens from the lumen to APCs in LP, such as DCs and macrophages. DCs can sample bacterial antigens directly through dendritic interdigitation through the mucosal layer, or indirectly through processing and transphagocytosis by specialized IECs called M cells. Antigen-loaded APCs can then transport the antigens to lymphoid follicles within the LP or to mLNs, where they can stimulate naive T cells to produce $CD4^+$ T cells, particularly $CD4^+$ Tregs and T_H17 cells. Under homeostatic settings, APCs can leave the GALT and enter the circulation, allowing them to transport bacterial antigens systemically (31, 63).

The messengers that could carry signals from the gut and/or GALT to a distant tumor site include bacterial metabolites that enter the circulation and regulate gene expression in various cells; microbe-associated molecular patterns (MAMPs), which modulate innate immunity by signaling through PRRs; whole viable bacteria that can potentially translocate and affect immune responses in distant tumor tissues; immune cells which by sensing microbiota signals in the GALT could migrate and carry out immune-stimulatory or inhibitory functions in distant tumors; and cytokines that may be generated in the GALT, in response to microbial stimuli, and may then enter the circulation and regulate downstream immune activities systemically (31).

Firstly, metabolites produced by gut microbiota modulate for antitumor immunity. The gut microbiota produces or transforms a variety of metabolites, which are small molecules that can move from their initial position in the gut and influence the antitumor immune response both locally and systemically (67).

SCFAs, such as acetate, butyrate, and propionate, are one of the most well-studied classes of microbial metabolites that shape host immunity. These metabolites are the main source of energy for IECs, and they can influence cytokine production, macrophage and DCs activity, and B cell class switching (65). SCFAs may modulate local effects on the differentiation and activation of anti-inflammatory Treg cells or proinflammatory T_H1 and T_H17 cells (31).

SCFAs have been shown to increase immunity via IgA production by plasma cells, which acts by blocking bacterial adherence to epithelial cells. They have also been connected to promoting DCs formation in the bone marrow as well as their phagocytic abilities (63, 65). Further, SCFAs were discovered to be important physical and chemical barriers because drive paneth cells and goblet cells to create, respectively, AMPs and mucus to maintain the gut mucosal barrier's integrity (67).

Furthermore, SCFAs and indole derivatives have demonstrated substantial immunological and anticancer activity, as seen by an increase in lymphocytes in the peripheral blood, such as $CD4^+$ and $CD8^+$ T cells, as well as NK and NKT cells (67, 68).

SCFAs have also been related to suppressing tumor cell proliferation and inducing apoptosis. As example, SCFA propionic acid produced by *Akkermansia muciniphila* activates the cell

cycle inhibitor p21 via G protein-coupled receptor 43 and suppresses the inhibitor of apoptosis protein, and consequently induced apoptosis (67).

Some studies have also found that metabolites generated from the gut microbiota, such as BAs or tryptophan, have immunosuppressive effects. For example, bile acids enhance antitumor immune responses by activating and attracting antitumor immune cells such as NK T cells (68). Additionally, through the aryl hydrocarbon receptor, a critical regulator of innate and adaptive immune responses, tryptophan metabolites from the gut microbiota can substantially influence the host's immune system. Thus, tryptophan breakdown products can increase intraepithelial $CD4^+CD8\alpha\alpha^+$ T cells, changing the immunological response to tumors (58, 68).

Additionally, specific metabolites or bacterial byproducts can induce the DC to skew toward a Treg phenotype rather than a T_H17 phenotype. Tregs secrete interleukin (IL)-10, which creates a local anti-inflammatory cytokine environment, while T_H17 cells release cytokines that encourage IECs to establish tight junctions and boost paneth cell AMP production. IL-17 can also increase the production of inflammatory cytokines and recruit neutrophils from the circulation to the gut microenvironment (63).

The development and differentiation of the immune system, especially T cell development into T_H1, T_H2, and T_H17 cells, as well as Treg cells, has been demonstrated to be influenced by specific bacteria species. It is important to highlight that the ability of different members of the commensal microbiota to polarize T cell responses is not equal (58). Segmented filamentous bacteria (SFB), for example, can induce ILC3 to secrete IL-22, resulting in the formation of serum amyloid A in the terminal ileum, which then acts on DCs in LP to drive T_H17 polarization. Thus, SFB are particularly potent inducers of T_H17 cell differentiation (Figure 7) (65, 69).

On the other hand, polysaccharide A (PSA) synthesized *by Leuconostoc mesenteroides* strain NTM048 *or B. fragilis* works as an immunostimulant to improve mucosal barrier function and modulate systemic immune responses. PSA can be recognized by DCs in the small intestine and activate $CD4^+$ T cells to produce cytokines, thereby promoting T cell proliferation, enhancing T_H1/T_H2 cell balance, and promoting lymphoid tissue formation (67, 69).

Gut microbiota-derived signals have been also shown to modulate innate immune defenses by lymphoid stimulation in the spleen, modulation of neutrophil migration and function, induction and activation of macrophages, and stimulation of the maturation of NK cells functions. The absence of microbiota-derived signaling molecules impairs immunological function, increasing the risk of systemic infection (58).

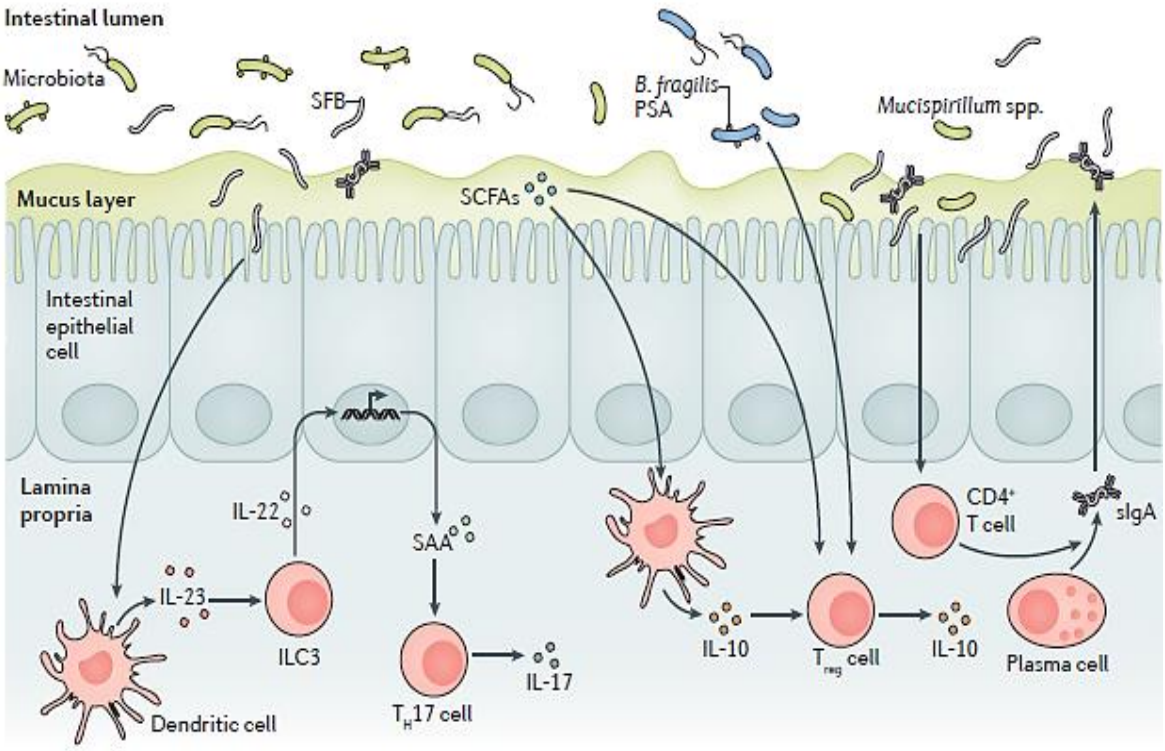

Figure 7: The microbiome shapes the immune system (69)

The microbial environment has a significant impact on the IIS. However, there is strong evidence that the IIS has an impact on the microbial environment as well (5).

The IIS may assist in the growth of beneficial microbiota members and the maintenance of a stable microbiota community. Changing the microbiota's intimate interaction with this system during homeostasis may influence or possibly induce a wide range of multifactorial disorders (70).

The immune system is one of the most powerful mechanisms to modulate the normal and dysbiotic microbiome. The recognition of microbial peptidoglycan by the nucleotide-binding

oligomerization domain-containing protein 2 – NOD2-, which contributes to intestinal homeostasis, and induces the production of AMPs and mucin, is one of the innate mechanisms of regulation. Other microbial compounds, such as flagellin and lipoproteins, trigger TLR5 in DCs and epithelial cells, increasing AMP epithelial expression. The NLRP6 inflammasome is activated by microbial metabolites, resulting in the secretion of IL-18 and AMPs that regulate microbiota composition. Furthermore, ILCs have lately been linked to the regulation of homeostatic microbial colonization. RORγt^{+} ILCs, for example, are the most abundant source of IL-22, which has been associated with AMPs synthesis in epithelial cells (Figure 8) (69).

B cells play an important role to maintain intestinal homeostasis by secreting IgA antibodies that can be directed against certain bacteria or even a flagella formation, a specific bacterial process. The production of secretory IgA (sIgA), which is mediated by T follicular helper – T_{FH} -, cells, as well as CD1d-driven activation of invariant NKT cells and secretion of anti-inflammatory cytokines, are examples of adaptive microbial regulatory mechanisms. Furthermore, the production of cytokines and antimicrobial compounds by IELs that express γδ T cell receptor (TCR) prevents bacteria from disseminating through the mucosal barrier following mucosal damage (Figure 8) (69).

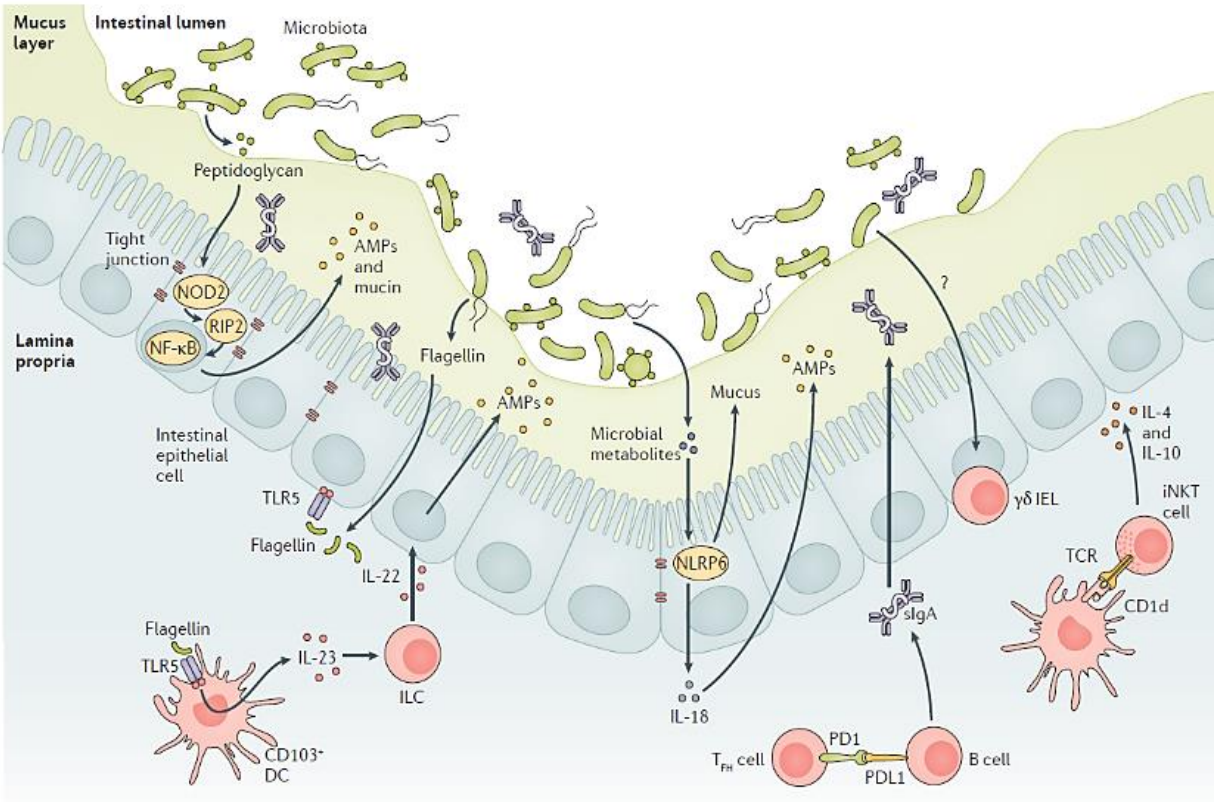

Figure 8: Innate and adaptive immunity in the regulation of microbial homeostasis (69)

4.3.1 The Crosstalk Between Microbiome and Immune Response in PDAC

When compared to the normal pancreas, the cancerous pancreas contains a significant abundance of the microbiome with a distinct bacterial composition. When compared to healthy controls, patients with PDAC showed an increased phylum *Proteobacteria* (such as *Gammaproteobacteria*) and a decreased phylum *Firmicutes* (such as butyrate-producing bacteria like *Eubacterium rectale, F. prausnitzii,* and *Roseburia intestinalis*) (35, 71). The enrichment of *Sachharopolyspora*, *Pseudoxanthomonas*, *Streptomyces,* and *Bacillus clausii* in the tumor was highly associated with longer survival (31).

Gammaproteobacteria has been shown to migrate from the gut to pancreatic tumors via the pancreatic duct, where they metabolize the active form of the drug Gemcitabine, reducing its efficacy. Kras expression in the pancreas appeared to result not only in the development of pancreatic tumors but also in a change in the microbiota composition, and in the enrichment of tumor-promoting bacteria. Furthermore, inflammation is critical for the development and progression of PDAC (Figure 5) (31, 72).

The microbiome is a powerful regulator of the tumor inflammatory microenvironment. In fact, it was demonstrated that bacterial dysbiosis associated with PDAC lead to innate and adaptive immunosuppression. Recent studies showed that gut microbiome depletion reduced MDSCs infiltration and reprogramming of tumor-associated macrophages to a tumor-protective M1-like phenotype. Furthermore, the binding of specific PRRs accelerated the progression of PDAC. TLR2 and TLR5 binding promoted PDAC and produced both innate and adaptive immune suppression, with TLR binding being required for the suppressive effects of the PDAC microbiota on macrophage programming (72).

4.3.2 The Crosstalk Between Microbiome and Immune Response in CRC

Specific populations of bacteria with pro-carcinogenic characteristics can trigger disease development by damaging DNA in the intestinal epithelium. After tumorigenesis begins, the intestinal environment changes, resulting in an increase in opportunistic bacteria and a decrease in pioneer strains. Higher mucosa permeability, bacterial translocation, and activation of components of both the innate and adaptive immune systems arise from a microbiota imbalance favoring opportunistic infections, culminating in chronic inflammation (39).

Many bacteria, including oral microbes, have been detected in colonic and stool-based studies of CRC, but three major oncomicrobes - microbes that trigger transformation events in host cells - play a role in CRC, *B. fragilis* , *F. nucleatum*, and *E. coli* (73).

E. coli produces genotoxins such as cytotoxic necrotizing factor, CDT, cycle-inhibiting factor, and colibactin, a metabolite that binds to DNA and forms DNA cross-links and interstrand breaks that destabilize cell division and increase mutagenesis (73). *F.nucleatum* and certain strains of *B. fragilis* have been mechanistically linked to the development of CRC by activating *β*-catenin signaling and by driving inflammatory responses (31). *B. fragilis* increase intestinal permeability and the production of inflammatory cytokines, such as IL-8 and TNF-α, by activating the transcription factors STAT3 and NF-κB (73, 74). The activation of NF-κB in tumor cells enhances antiapoptotic genes and promotes the survival and proliferation of tumor cells (68). Furthermore, *B. fragilis* causes phosphorylation of STAT3 and IL-17–producing T_H17 and γδ T cells in a multistep process. Both processes promote the recruitment of pro-tumorigenic myeloid cells that suppress cytotoxic antitumor immunity (73, 74).

F. nucleatum also modulates the host's NK and T cells by direct binding of its Fap2 to TIGIT, an inhibitory receptor presents in these cells. Thus, *F. nucleatum* can impair these cell function, reducing cytotoxicity, and promoting immune cell death, resulting in tumor immunosurveillance escape. Furthermore, $Fap2^{+}$ *F. nucleatum* also activates epithelial and myeloid cells and induces a pro-tumorigenic inflammatory response (Figure 5) (31, 73, 75).

Enrichment of *Fusobacterium* in the gut microbiota or in tumor tissues has been associated with an increase in *Campylobacter* species that contributed to tumor growth by eliciting a pro-inflammatory response mediated by IL-18 (31).

4.4 Microbiome Impact on Cancer Immunotherapy

Historically, there have been three pillars of cancer treatment: surgery, chemotherapy, and radiotherapy. Recently, immunotherapy has emerged as a possible fourth pillar, due to the rapid advancement of tumor immunity research (76). This therapy stimulates the immune system to fight cancer cells, however, it has been associated with a high rate of adverse reactions (68). Thus, it is necessary to optimize immunotherapies to improve the therapeutic effects.

The gut microbiome involvement influences the host immunity, thus is also expected they have a substantial impact on cancer therapeutic response (63). The microbiome modulates immunotherapy response by controlling the host's local and systemic immune responses (77). The composition of gut microbiota changes over time, and this variation influences the carcinogenesis, response, and toxicity to therapy (31). Importantly, the intestinal microbiota composition, which may be sensitive to treatment or prone to adverse reactions, has some attributes that might be used as biomarkers, known as 'immune potentiators', to predict immunotherapy prognosis. The presence of a given bacterial strain can promote immunotherapeutic efficiency by direct stimulation of immune cells or by the production of intermediate metabolites (40, 66, 68). For example, the composition of the gut microbiota can affect cancer patients' anti-tumor immunity and can be used as a biomarker to predict patient response to immune checkpoint blockade (ICB) therapy (13). Unfortunately, there are no biomarkers that can precisely predict clinical responses to this type of therapy, so it is important to continue the analysis of microbiota and their constituents, to allow the identification of biomarkers and therapeutic targets for the development of cancer immunotherapeutics (77).

4.4.1 Gut Microbiota and Immune Checkpoint Blockade

Immune checkpoints are molecules that must be stimulated or inhibited by specific immunological cells to trigger a specific type of immune response to a disease (21). ICB aims to restore and increase the anticancer response by reducing tumor cells' intrinsic immuno-inhibitory pathways, which are responsible for immune resistance (78).

Current immunotherapies, including immune checkpoint inhibitors (ICI) therapy, promote an immune response through the activation antitumor immunity mediated by T cells (77).

The cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand (PD-L1) antibodies, are some examples of ICIs that improve the body's immune response to cancer cells (21, 68). Targeting CTLA-4 (Ipilimumab), PD-1 (Nivolumab), or its ligand PD-L1 (Pembrolizumab) in advanced-stage diseases such as non-small-cell lung cancer (NSCLC), urothelial bladder cancer, and melanoma are particularly effective (40). Although this therapy has shown promising results in clinical trials, many tumors have developed resistance to this immune strategy, lowering the overall efficacy and restricting its use in cancer treatment (66).

Several studies have shown an important association between the diversity and composition of the gut microbiota and response to ICB in cancer patients, thus indicating that the gut microbiota has a crucial impact on the efficacy of ICI treatment (77, 79). According to studies, a high microbial diversity in the GI system is associated with improved effectiveness of ICI treatment. The composition of the intestinal microbiota has a significant impact on the efficacy of anticancer immune surveillance, which contributes to the therapeutic activity of CTLA-4 or PD-1/PD-L1-based cancer immunotherapy (77, 80, 81).

4.4.1.1 Microbiome Implications in CTLA-4 Based Immunotherapy

CTLA-4 antibody can compete with CD28 and CD80/86 ligands to block their binding, thus interfering with TCR signals and T cell activation and proliferation, acting as a tumor suppressor (68). Anti-CTLA-4 therapy for melanoma showed an increased CTLA-4 expression on Tregs in the TME. These findings suggest that tumors avoid immune clearance by upregulating CTLA-4, which controls Treg cells and suppresses immunological responses (66, 82).

A study compared the therapeutic efficacy of anti-CTLA-4 therapy in specific-pathogen-free (SPF), GF, and antibiotic-treated mice, to demonstrate that the gut microbiota was required for anti-CTLA-4 immunotherapy to be effective. The results showed that tumor growth in SPF mice was reduced as compared to GF and antibiotic-treated mice (40, 66). After treatment with the anti-CTLA-4 antibody, significant changes in the abundance of gut microbiota in mice were observed, with a relative rise in *Bacteroidales* and *Burkholderiales* and a decrease in *Clostridiales* (63, 66). *B. fragilis*, in particular, has been shown to improve anti-CTLA-4 efficacy when combined with *Bacteroides thetaiotaomicron* or *Burkholderia cepacia*, through a mechanism involving the activation of T_H1 cells in lymph nodes and the facilitation of intra-tumoral DCs maturation. These works demonstrated the relevance of gut microbiota in anti-CTLA-4-based immunotherapy (63).

4.4.1.2 Microbiome Implications in PD-1/PD-L1 Inhibitor-Based Immunotherapy

PD-1 is a checkpoint protein present in immunological T cells. The activation of PD-1 on T cells by PD-L1, which is overexpressed in some cancer cells, sets off a complex chain of reactions that inhibit T-cell responses, thus allowing cancer cells to escape to immunological responses (21, 66, 68). Monoclonal antibodies targeting either PD-1 or PD-L1 have been shown to boost the immune response to cancer cells (21).

In human melanoma patients, the effectiveness of anti-PD-L1 therapy is related to the commensal microbiota. Patients who responded to anti-PD-L1 therapy had a different fecal microbiota, and higher diversity of bacteria in their gut microbiota (enrichment of beneficial bacteria such as *Ruminococcaceae/Faecalibacterium*, and *Bifidobacterium* species) than non-responder patients (75). On the other hand, non-responder patients exhibited a smaller diversity of gut bacteria and a larger abundance of *Bacteroidales* members (63, 67).

Routy *et al*. demonstrated that a higher abundance of *A. muciniphila* is associated with a better immune response to the anti-PD-1 therapy in patients with lung or kidney cancer. They verified that IL-12, which is released in response to *A. muciniphila*, help in the recruitment of T cells to fight cancer (13). In addition, patients who respond to PD-1 blockade have a large number of circulating T_H17 cells. According to the research, this bacteria may improve antigen processing and presentation, as well as the activity of T_H17 cells presented in the circulation and TME. On the other hand, high numbers of activated Tregs and MDSCs characterize non-responder patients (77, 81).

4.4.1.3 The Gut Microbiota Remodel the Tumor Microenvironment to Improve ICI Efficacy

To optimize ICI responses, the gut microbiota can affect either innate and adaptive immunity, as well as tumor antigens. In this topic, intrinsic mechanisms by which specific bacterial species reprogram the TME to boost ICI efficacy will be discussed.

4.4.1.3.1 Gut Microbiota Modulate Innate and Adaptive Immunity to Enhance ICI Responses

In lymphoma, colon carcinoma, melanoma, and breast carcinoma a high-fiber diet, monocolonization with c-diAMP-producing *A. muciniphila*, or fecal microbiota transfer from ICI responders patients can activate the monocyte-IFN-I-NK-cell-DC cascade, improving antitumor responses and ICI efficacy (83). In B16F10 skin melanoma mouse model, a high-salt diet increases intestinal permeability and, as a result, intratumoral *Bifidobacterium* localization, as well as NK cell activation to generate antitumor immunity (Figure 9) (84, 85).

Several studies have shown that certain gut microbiota activate $CD8^+$ T cells in the systemic circulation or the TME. Interestingly, $CD8^+$ T cell infiltration in tumor is promoted by *Bifdobacterium*, *Enterococcus*, *Faecalibacterium*, *Ruminococcus*, and *Clostridiales*. Evidence from a clinical trial suggests that *Phyla Firmicutes* and *Actinobacteria* improve the activation of $CD56^+CD8^+$ T cells in the peripheral blood of ICI responders, and eleven strains (7 *Bacteroidale*s and 4 non-*Bacteroidales* species) increase the proportion of effector IFN^+CD8^+ T cells in the systemic circulation to improve ICI therapy efficacy (Figure 9) (67).

In melanoma, oral administration of *Bifidobacterium* boosted DC activation, which improved tumor-specific $CD8^+$ T cell responses and restored anti-PD-L1 therapeutic efficacy in mice with "unfavorable" gut microbiota, and *B. fragilis* improved CTLA-4 blockade's anticancer effects by triggering DC maturation and stimulating IL-12-dependent T_H1 cell immunological responses (86, 87). In melanoma, *Faecalibacterium* increases the $CD4^+$ T cell proportion and serum CD25 production in human patients while decreasing Treg cell proportion in peripheral blood (88). Furthermore, in adenocarcinoma and melanoma, eleven strains coupled with ICI effectively inhibited tumor growth by inducing IFN^+ $CD8^+$ T cells (Figure 9) (89).

In NSCLC, Renal Cell Carcinoma (RCC), and urothelial carcinoma, oral supplementation with *A. muciniphila* restores anti-PD-1 responses in fecal microbiota transplantation (FMT)

nonresponsive mice by promoting $CCR9^{+}CXCR3^{+}CD4^{+}$ T cell recruitment into tumor beds (80).

4.4.1.3.2 Gut Microbiota Modulate the Immunogenicity of Tumor Cells to Improve ICI Responses

The gut microbiota also improves the efficiency of ICIs by increasing the immunogenicity of tumor cells by providing tumor cross-antigens, such as the antigen epitope TMP1 and the antigen epitope SVY. The antigen epitope SVY expressed by the commensal *Bacterium Bifdobacterium* was shown to be identical to the antigen epitope SIY expressed by tumors, resulting in SVY-specific T cells detecting SIY and limiting tumor growth. They stimulated $CD8^{+}$ T cells and increase the efficacy of PD-1 blocking treatment (Figure 9) (67, 90).

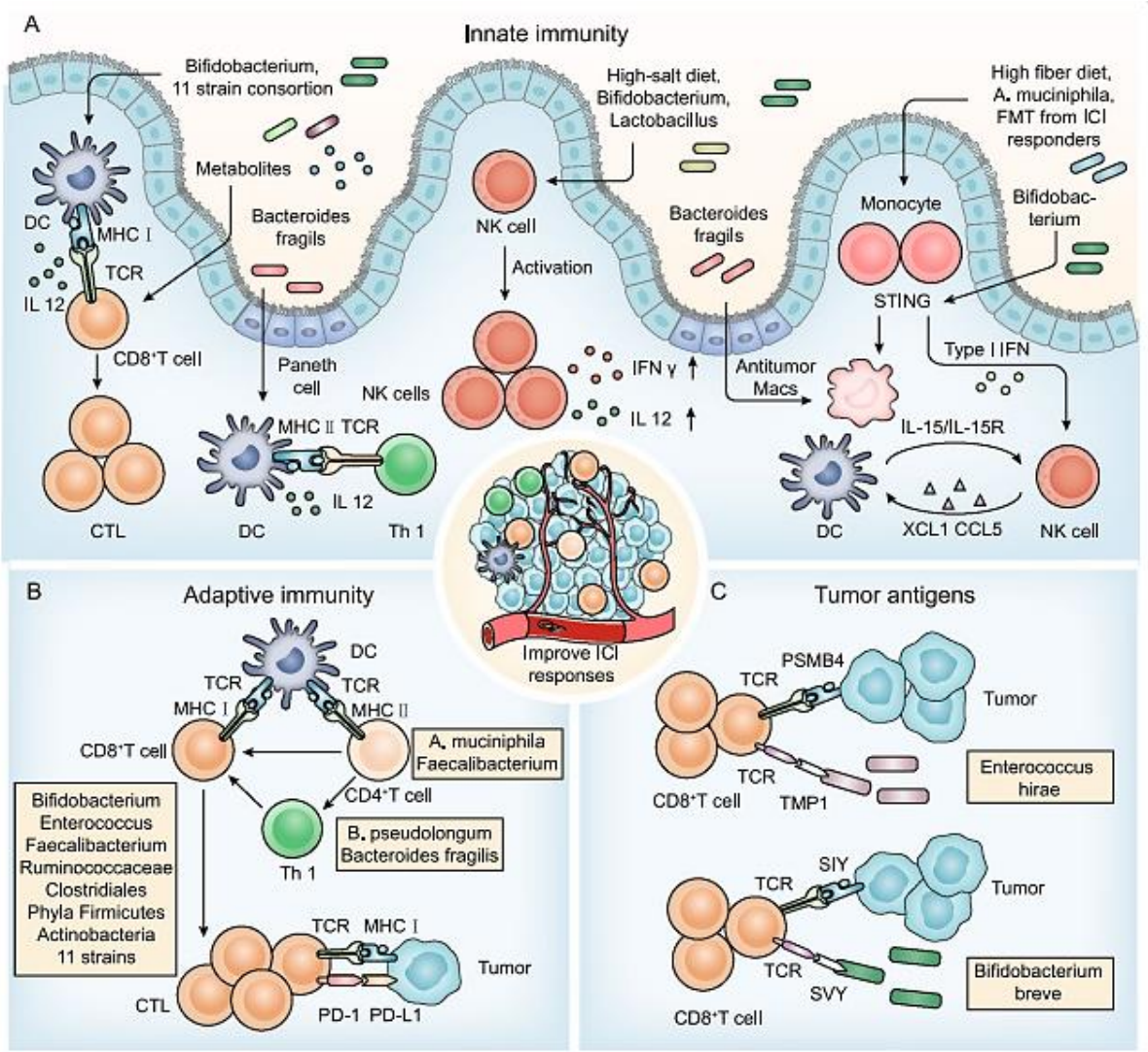

Figure 9: To optimize ICI responses, the gut microbiome modulates innate immunity, adaptive immunity, and tumor antigens (67)

4.4.2 Gut Microbiome and Efficacy of Other Immunotherapeutic Strategies

Although most of the studies are focused on the gut microbiome's interaction with ICI, there are some studies in other immunotherapeutic strategies.

4.4.2.1 Microbiome Implications in CpG-Oligodeoxynucleotide Immunotherapy

Unmethylated cytosine-phosphate-guanine (CpG) is a promising immune adjuvant that can stimulate a wide range of immune cells, leading to powerful innate and adaptive immune responses (91). It has been reported that the bacterial genome has an unmethylated CpG dinucleotide motif. When they appear in humans, these motifs will be detected as PAMPs,

initiating a TLR9/IL1 response-mediated signaling cascade that results in the production of pro-inflammatory cytokines (78, 91).

CpG DNA can be obtained naturally or synthesized with specific sequences. CpG oligodeoxynucleotides (ODN) are a synthetic short single-stranded DNA molecule that contains CpG motifs similar to those found naturally in bacteria. CpG ODN can be used to replace bacterial CpG DNA to achieve a very similar immunostimulatory effect for direct B cells and plasmacytoid dendritic cells activation to induce an innate and adaptive immune response. In fact, studies have shown that CpG ODN synthetic oligodeoxynucleotides have a strong immunostimulatory effect and can be used for several cancer treatments due to their anticancer activity (21, 91).

The efficacy of CpG-ODN in cancer treatment has been linked to several fecal microorganisms. Gram-negative, such as *Ruminococcus* and *Alistipes shahii*, and gram-positive, e.g., *Lactobacillus fermentum*, *Lactobacillus intestinalis*, and *Lactobacillus murinum* genera, was positively and negatively correlated to CpG-ODN-induced TNF production, respectively. *Alistipes shaii* improves the therapeutic benefits of CpG ODNs by stimulating myeloid cells to produce immunostimulatory cytokines, via the TLR4 signaling pathway (77, 78).

4.4.2.2 Microbiome Implications in Allogeneic Hematopoietic Stem Cell Transplantation

Allogeneic Hematopoietic Stem Cell Transplantation (AHSCT), a potentially curative procedure for a range of hematologic malignancies, has become increasingly popular from last decades (66). In AHSCT, a patient receives healthy stem cells from a donor's blood or bone marrow to replace their stem cells that have been damaged by radiation or heavy doses of chemotherapy. One of the benefits of AHSCT is that the patient's immune system is restored after the donated cells engraft (85).

Clinical studies have confirmed an impact of the gut microbiota in patients after AHSCT (63). In patients with hematologic malignancies, certain bacterial taxa are linked to AHSCT efficacy and a lower risk of Graft-Versus-Host Disease (GVHD) after treatment (65). A study was carried out to determining the bacterial composition of the feces of 64 patients 12 days after AHSCT, and it was concluded that a greater bacterial diversity was associated with lower GVHD-related mortality. Furthermore, higher levels of bacteria from the genus *Blautia* were associated with lower GVHD lethality (92).

4.4.2.3 Microbiome Implications in Adoptive Cell Therapy

Even though immunotherapy with ICI has improved the survival of patients with melanoma and NSCLC, a considerable number of individuals still have disease progression. For some patients, Adoptive Cell Therapy (ACT) may be an alternative treatment (93). ACT uses autologous immune cells, specifically T cells, that are harvested from the tumor – tumor-infiltrating lymphocytes (TILs) – or the peripheral blood – peripheral blood lymphocytes (PBLs), then *ex vivo* expanded, and re-infused back into patients after several weeks of expansion in culture, to mediate tumor destruction (76, 94).

In phase I/II clinical trials, TILs treatment produced significant objective tumor responses of around 50% in patients with metastatic melanoma (93).

A study was carried out analyzed the impact of the gut microbiome on the ACT efficacy, comparing tumor growth in C57BL/6 female mice with a diverse gut microbiota. ACT reduced tumor progression and fecal bacterial communities presented a more diverse range of *Bacteroidetes* taxa, namely *Bacteroides*, *Parabacteroides*, *Prevotella*, the *Rikenellaceae* family (95).

5 Strategies to Manipulate the Microbiome in Cancer

The remarkable impact of microbiome on oncogenesis and cancer therapy provides significant proof that modulation of microbial networks is a promising strategy for prevention and treatment of cancer (77). The discovery of viable microbial targets for preventive and therapeutic intervention will be made possible by a thorough understanding of how bacteria contribute to cancer (96).

Many different approaches can be used to manipulate specific microorganisms or the microbial ecosystem. These include strategies such as dietary modification, prebiotics, probiotics, antibiotics, FMT and innovative techniques such as nanotechnology, which will be discussed hereafter (77).

Antibiotics

Antibiotics have a significant impact on cancer patients' clinical outcomes through modulation of their microbiome. Depending on the cancer and drug, the use of antibiotics improves or decreases therapeutic efficacy. A customized antibiotic therapy that selectively removes certain bacteria has the potential to improve cancer treatment. However, antibiotics may also cause dysbiosis by reducing the diversity of gut bacteria if they are not sufficiently selective. For example, the use of broad-spectrum antibiotics frequently worsens clinical results (77). Thus, antibiotics should be used carefully in cancer immunotherapy patients (65). Clinical studies showed that antibiotic-treated patients have a lower response to immunotherapy, which is consistent with results from preclinical animal trials (97). In melanoma, colon cancer, or sarcoma antibiotic-treated mice with, the efficacy of CTLA-4 blockade is significantly reduced. In patients with NSCLC, RCC, and urothelial carcinoma, bacterial ablation reduces the therapeutic benefit of PD-1/PD-L1 blockade (77).

Furthermore, when compared to patients who did not receive antibiotics, patients under antibiotic therapy had considerably worse progression-free survival and overall survival rates. This shows that dysbiosis (via antibiotic use) may impair anti-tumor immune responses and ICB responses (63).

Moreover, PDAC patients benefit from bacterial ablation because numerous intratumoral microorganisms form an immunosuppressive TME by activating anti-inflammatory immune cells. Thus, antibiotics and PD-1 blockade promote antitumor benefits in PDAC patients (77).

Antibiotic treatment can also improve the efficacy of chemotherapy in cancer patients. *Gammaproteobacteria* are common in PDAC TME, and they deaminate Gemcitabine, rendering it ineffective as an anticancer drug (98). The co-treatment with an antibiotic (Ciprofloxacin) reduces the drug resistance caused by *Gammaproteobacteria* (72, 77).

Additionally, antibiotics could be used prior to FMT to promote microbial modulation by taking advantage of broad-spectrum reduction of gut microbiota (37).

Diet and Prebiotics

The modulation of the existing commensal community via prebiotics or dietary changes could promote the expansion of beneficial bacteria that require specific substrates (65).

Prebiotic is a substrate selectively used by the beneficial microbial communities to confer a health benefit effect (99). Prebiotics include fiber (e.g., soybean fiber), fructans (e.g., inulin), resistant starch (RS), and oligosaccharides (e.g., fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS)) (77, 99, 100).

Fiber is essential for improving GI barrier function. A low-fiber diet reduces the body's ability to defend itself against pathogens. When dietary fiber is clearly poor, gut microbes use mucus glycoproteins as sources of nutrients, weakening the intestinal mucus barrier. Thus, the consume of fiber prevents these negative effects (77). Inulin is another common prebiotic that has been shown to stimulate the growth of *Faecalibacterium* and *Bifidobacterium* species in gut microbiota (37, 63, 100).

Different studies showed that fiber or related prebiotics, which are fermented in the large intestine by commensal microbes to produce SCFAs, support the growth of beneficial gut microbes (e.g., *Lactobacillus spp.* and *Bifidobacterium spp.*). These bacteria produce lactate and acetate and increase butyrate levels by interacting with butyrate-producing microbes (e.g., *Eubacterium rectale, Roseburia spp.* and *F. prausnitzii*) (77, 99).

A long-term imbalanced diet has been related to cancer. It was observed that a diet low in fiber and high in protein from red meat is sufficient to promote CRC development (78). In genetically susceptible mice, a high-fat diet has been shown to promote tumor progression and a change in the gut microbiota (96).

Prebiotics and dietary modification could enhance ICI responses. In patients with metastatic NSCLC treated with ICIs, a high-fiber diet is connected to *Bifidobacterium* species enrichment and better clinical outcomes. ICI response was also improved in melanoma patients who consumed more fiber (97).

Thus, dietary changes could be an effective intervention to alter gut microbiota in cancer patients due to its safety profile, cost, and accessibility. However, this does not always result in a permanent compositional shift, and it is extremely difficult to maintain a new diet (37).

Probiotics

Probiotics are live microorganisms (individual/single or in combinations) that, when consumed in adequate quantity, could provide health benefits (77). Probiotic species are used to restore the homeostasis in the gut, and play an important role in preventing a wide range of diseases, including several types of tumors (8).

Probiotics are commonly administered as supplements or consumed in fermented foods (e.g., yogurt) (77). Further, probiotics are mainly represented by lactic acid-producing microbes, such as *Lactobacillus* and *Bifidobacterium* species, that support the expansion of a healthy microbiota (69, 77).

According to research, the administration of probiotics containing strains of *Lactobacillus acidophilus* and *Bifidobacterium lactis* increase the abundance of butyrate-producing bacteria (e.g., *Faecalibacterium, Clostridiales*) in mucosal and fecal samples, while decreasing the abundance of CRC-associated genera (e.g., *Fusobacterium, Peptostreptococcus*) (NCT03072641) (101).

Researchers have also found that a probiotic supplement containing *Lactobacillus rhamnosus GG* (LGG) can reduce colon tumor incidence by inhibiting the expression of inflammatory proteins; *L. casei BL23* can improve immune response by lowering Treg levels in mice with colitis-associated cancer; and *Lactobacillus plantarum* can prolong survival of tumor-bearing mice by enhancing effector $CD8^+$ T cells functions, $CD4^+$ T cells differentiation, and NK cells intratumoral infiltration (78, 102, 103). Curiously, when compared to untreated controls, the administration of *L. casei* also improved the clinical outcomes of patients with early bladder cancer (77).

Moreover, *L. acidophilus* administration reduced tumor size in a mouse model of BC by altering the levels of certain cytokines (e.g., INF-γ, IL-4, TGF-β) and increasing the number of TILs (104).

Modulation of gut microbiome with probiotics has also been shown to improve immunotherapy efficacy. Probiotics were found to improve *Firmicutes*/*Bacteriodes* ratio, enrich *Lachnospiraceae*, and decrease *Muribaculaceae* in the gut, as well as increase fecal butyrate levels when used in conjunction with anti-PD1 (105). Specifically, *Bifidobacterium* and *B. fragilis*, have been shown to improve the efficacy of anti-PD-L1 and anti–CTLA-4 therapy. Oral administration of *Bifidobacterium* increases DC activation and improves tumor-specific $CD8^+$ T cell response, whereas *B. fragilis* activates anti-tumor T_H1 cells with suspected cross-reactivity to bacterial antigens and tumor neoantigens (86, 87).

Fecal microbiota transplantation

FMT is one of the most recent strategies to modulate the microbiome (79). FMT involves transferring the entire fecal microbial community, including bacteria, viruses, fungi, and their metabolites, from healthy donors into recipients' GI tracts via oral lyophilized pills, colonoscopy, or gastroscopy in order to restore the balance and functions of gut microbiota (37, 67, 78).

FMT has been widely used and it´s the only effective strategy to treat recurrent and refractory *Clostridioides difficile* infection (RCDI) with incredibly high response rates, and it also shows therapeutic potential against GVHD, neuropsychiatric diseases (e.g., depression and Parkinson's disease), or gut disorders (e.g. inflammatory bowel disease and ulcerative colitis) (77, 78). FMT was approved by the Food and Drug Administration in 2013 as a biological therapeutic regimen for the treatment of RCDI (100). Furthermore, the success of FMT in RCDI has given rise to the hope that this procedure may be similarly effective in treating other dysbiosis-related diseases, such as CRC (69).

Coupling FMT with immunotherapy is mainly under preclinical investigation. Transplanting feces from responders of anti-PD-1 treatment into tumor-bearing mice with depleted microbiota to improve PD-1/PD-L1 blockade efficacy. On the other hand, the same mice reconstituted with feces from patients who had not responded to anti–PD-1 therapy did not respond to PD-1 blockade. However, additional FMT from responder patients could reverse the non-responder phenotype in mice (31, 75, 78).

Several clinical trials, mostly in patients with metastatic melanoma, are currently ongoing to assess the safety and efficacy of the combination of FMT with ICI treatment (67).

A phase I clinical trial using FMT to improve the outcome of anti-PD-1 therapy on metastatic melanoma cancer patients who have failed to respond to immunotherapy has proceeded (NCT03353402). In this study, researchers performed FMT from a complete response donor and reinduction anti-PD-1 therapy on ten melanoma patients who were unresponsive to PD-1 blockade. Three of the ten patients showed tumor volume reduction, including two partial responses and one complete response (67, 79). This study demonstrated that combining FMT with reintroduction of anti-PD-1 therapy in patients with refractory metastatic melanoma was safe, feasible, and potentially effective (106).

However, there are significant concerns about the long-term safety of FMT (67). Despite its success, FMT has a limitation because the entire gut microbiota is transferred along with the therapeutic bacteria species (15). Pathogenic bacteria, parasites, bacteriophages, and multidrug-resistant bacteria can all be transferred unintentionally through feces (31). In order to reduce the risk of infection, the transplanted fecal matter should be thoroughly screened for pathogens, which is especially important for immunocompromised patients (77).

6 Use of Nanotechnology to Immunomodulate Microbiome in Cancer

The previously mentioned approaches are unable to selectively act on the targeted microbiome, which may result in limited and uncertain therapeutic effects on cancer (100). Researchers have gained interest in the use of nanoparticles (NPs) to modulate the microbiota or tumor associated bacteria in order to improve cancer treatment (107). Indeed, nanotechnology has marked a new era of microbiome modulation (100).

Since a drug can only have a therapeutic effect if it is present in the correct amount and form, NPs used as carriers may increase the local drug concentration inside and around tumor cells. This effect also reduces the risk of toxicity on healthy cells. To overcome the specific toxic effect of conventional drug delivery, the drug-carrying NPs deliver the drug directly into its targeted area (organ, cellular, and subcellular level of specific tissue), reducing the amount of drug required for therapeutic efficacy. As result, the use of NPs in drug delivery opens new avenues for improving drug delivery and changing cancer management (108, 109).

Several nano-based systems composed of various materials and thus exhibiting diverse properties have been proposed and classified in polymeric, lipidic, metallic, and inorganic nanocarriers (Figure 10). Some examples of NP are liposomes, polymeric NPs, micelles, and dendrimers (110).

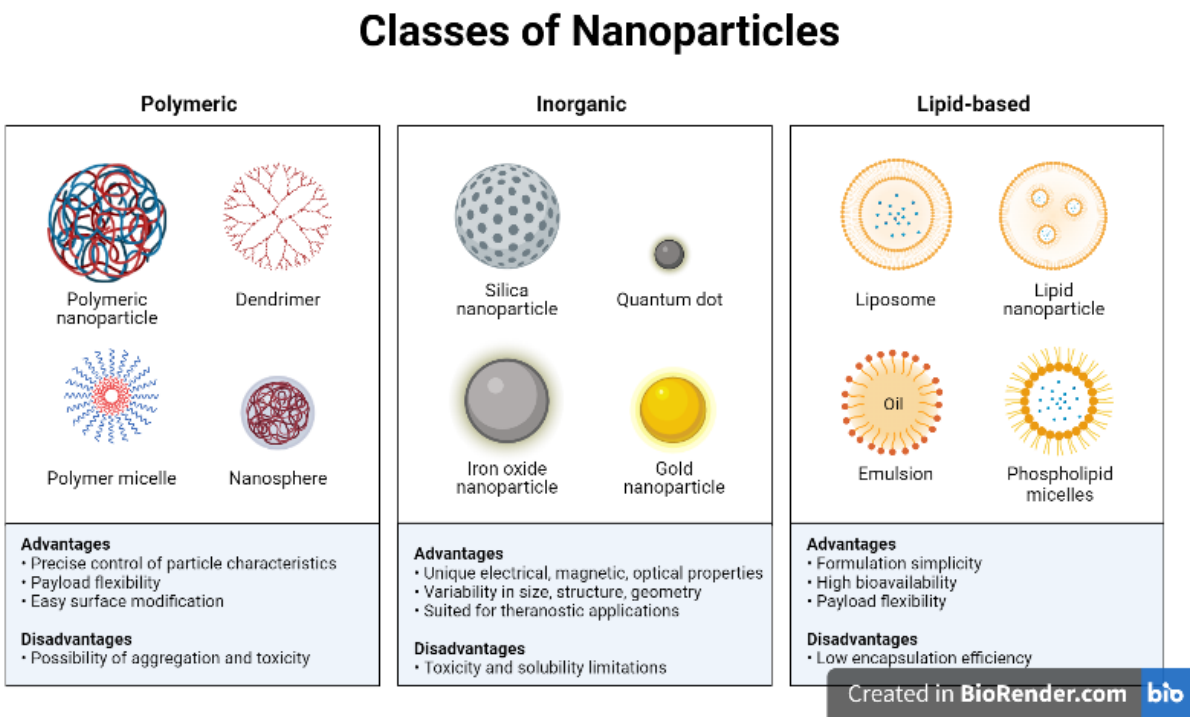

Figure 10: Classes and subclasses of NPs (adapted from (111))

Liposomes

Liposomes are based from phospholipids that comprise a hydrophilic head and a hydrophobic tail. They form vesicle-like structures capable of entrapping therapeutics in their core when using aqueous solvents. Different hydrophobic and hydrophilic interactions between lipids and lipid-water result in bilayered structures. Further, liposomes are classified into three types based on their size and layer number: multilamellar vesicles, large unilamellar vesicles, and small unilamellar vesicles (112).

Liposomes have the potential to be used as delivery systems because they provide a slow and sustained release, which improves the accumulation of entrapped molecules. Additionally, as they modulate biodistribution and pharmacokinetics, they have the potential to reduce cytotoxicity of incorporated molecules. Moreover, liposomes have been proposed as delivery platforms for vaccines, anticancer drugs, and gene therapy due to their biocompatibility, biodegradability, and ability to cross lipid bilayers and cell membranes (110).

One of the major disadvantages of conventional liposomes is their rapid elimination from the bloodstream by mononuclear phagocytes of the reticular endothelial system (RES). PEGylation polymers are a popular solution to this problem. PEG chains raise the concentration of hydrated groups on the surface, which sterically inhibits both electrostatic and hydrophobic reactions with plasma proteins to reduce internalization by the RES (112). PEG coatings on NP protect the surface from aggregation, opsonization, and phagocytosis, extending the time of systemic circulation (113).

Polymeric nanoparticles

Polymeric NPs are typically highly stable and can efficiently entrap and/or adsorb both hydrophilic and hydrophobic molecules. Polymer properties like biocompatibility, low toxicity, and biodegradability have made polymeric NPs an appealing delivery strategy (110).

Many biodegradable polymers have been used as drug delivery agents, to increase therapeutic efficacy while reduce off-target toxicity. Several polymers, such as poly (lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA), have received significant attention due to their biocompatibility with chemotherapeutic agents and controlled release properties. Other naturally occurring polymers, such as sodium alginate and chitosan, have also been used as drug delivery agents (112).

Polymeric micelles

Polymer micelles are self-assembled spherical nanocarriers formed in an aqueous medium by amphiphilic block copolymers with a hydrophobic core and a hydrophilic surface. Due to their hydrophobic core, polymer micelles have been investigated as drug delivery systems (DDS) for poorly water-soluble drugs. Polymeric micelles have been shown to increase the bioavailability of hydrophobic molecules by protecting them from degradation *in vivo*. Polymeric micelles also have low toxicity, a long circulation time, and high levels of accumulation in tumor areas (110).

Dendrimers

Dendrimers are hyperbranched spherical nanocarriers composed of a central core, branching monomers, and functionalized peripheral groups. Dendrimers can be created through the convergent or divergent polymerization of branching units, resulting in a structure with a hydrophilic surface and a hydrophobic central core. The main physicochemical characteristics of dendrimers are low viscosity, hyperbranched molecular topology, high density of chemical functionality, and multiple end groups that can be chemically functionalized (110).

With the rapid development of nanotechnology in areas such as physical characterization (e.g., size, shape, surface charge), surface modification (e.g., cell membrane coating, functional moiety conjugation, physical adsorption), release control (e.g., stimuli-responsive release, retained release, multi-step release), and so on, various types of nanomedicines (e.g., liposome, micelle) have been developed as efficient targeting DDS. This provides tools for precise microbiome modulation to improve cancer therapy outcomes (100).

The size of NP is an important design parameter that can be tailored to direct particle distribution *in vivo*. Several biological phenomena can be influenced by size, including circulation half-lives, extravasation through leaky vasculature, and macrophage uptake. NPs with 5 nm of diameter, for example, are rapidly cleared by the kidneys after intravenous administration. The liver, for example, has noncontinuous endothelium with vascular fenestrations measuring 50-100 nm, resulting in nonspecific accumulation of larger particles. Moreover, splenic filtration accounts for retention of particles >200 nm, due to the 200–500 nm size range of interendothelial cell slits. Particles in the micrometer range (2-5 μm) have been shown to readily accumulate within lung capillaries, potentially providing a distinct advantage when targeting one of the most common sites of metastatic disease. Furthermore, the liver, spleen, and lungs' resident macrophages play a significant role in particle uptake. Typically, NPs that measure ~ 100 nm last longer in circulation. The propensity of NPs to extravasate through fenestrations in tumor vasculature is increased by long blood half-lives (114, 115).

Apart from the size, different NPs shapes have been reported. They can have distinct characteristics that significantly alter circulating lifetimes, cell membrane interactions and macrophage uptake, which in turn affect biodistribution among the different organs (115). Although it has been proposed that non-spherical particles may be beneficial as they have increased blood circulation time, due to reduced phagocytosis by unspecific cells, they also demonstrated decreased cellular uptake, when compared to spherical NPs. According to Gratton *et al*., rodshaped NPs have the best uptake performance, followed by spheres, cylinders and finally cubical NPs (110).

The NP's circulation half-life is also highly influenced by shape. For example, Discher and colleagues, demonstrated that filamentous polymer micelles (filomicelles) have longer circulating lifetimes (>1 week after administration) than spherical counterparts (2-3 days), owing largely to these particles' proclivity to align with blood flow (114).

Another design feature that can be tailored to extend circulation lifetimes and selectively enhance accumulation at specific sites of interest is the surface charge of NPs. The charge of NPs affects opsonization, circulation times, and interactions with resident macrophages in organs of the mononuclear phagocyte system. Positively charged NPs are more susceptible to sequestration by macrophages in the lungs, liver, and spleen, as well as a higher rate of nonspecific uptake in the majority of cells, whereas neutral and slightly negatively charged NPs have longer circulation lifetimes and less accumulation in organs, such as the liver and spleen (114).

Interestingly, a study showed that cationic liposomes are preferentially internalized by tumor-associated angiogenic endothelial cells compared with normal vasculature. Thus, for effective delivery of NPs into tumors, the surface charge of the NPs must be neutral or slightly negative during intravenous administration but should change to a positive charge upon arrival at the tumor site. As a result, the design of zwitterionic NPs with switchable charges based on environmental stimuli will be ideal for maximizing tumor drug accumulation in the tumor (114).

Beyond effective antibiotic targeting delivery by DDS, other advanced forms of nanomedicine that control commensal bacteria and their byproducts have demonstrated promise in enhancing cancer therapy (Figure 11) (100).

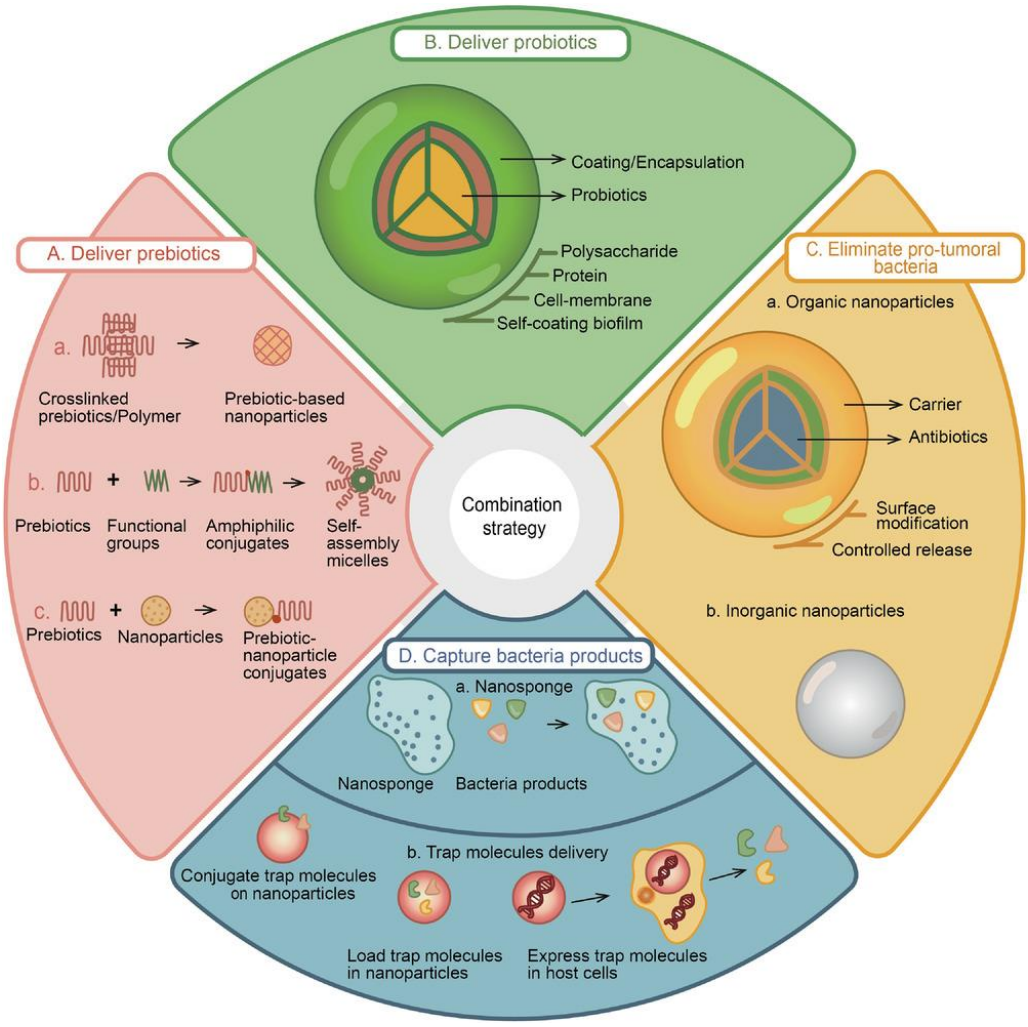

Figure 11: Drug delivery systems for microbiota modulation to improve the outcomes of cancer therapy (100)

6.1 Targeted Chemotherapy and DDS

NPs can use several distinct physiological features of the TME to improve chemotherapy outcomes, including acidic pH, ROS, overexpression of specific enzymes, and a lack of intratumoral oxygen or hypoxia (116).

pH-Responsive Nanosystems

The pH of the body varies hugely. The physiological pH is 7.4, whereas in tumor site it ranges from 5.7 to 7.8. This physiological difference enables the development of pH-sensitive nanocarriers. These systems typically rely on structural or size changes in response to acidity, or the breaking of a bond sensitive to acidic pH, to allow for controlled release of the cargo drug at the tumor site (116, 117).

For example, in physiological pH, platinum pro-drug conjugated polymeric NPs form large nanoclusters (NCs), which improves their accumulation at the tumor site (118). However, when NCs are exposed to acidic pH, an amide bond is cleaved and tiny polyamidoamine are released, allowing a greater tumor penetration and cellular internalization (116).

Enzyme-Sensitive Nanosystems

The overexpression of various enzymes in tumors has been used to create enzyme-sensitive nanosystems. Exploiting this, a triple-layered nanogel was designed. This nanogel degrades in the presence of a lipase secreted by *Staphylococcus aureus*, releasing the encapsulated antibiotic – Doxorubicin – into TME. This triggers a specific release in cancerous cells and thus a selective cytotoxic effect, in the H22 tumor-bearing mice model (107, 119).

One of the biggest concerns in this field is the heterogeneous expression of target enzymes in different types of cancer. Thus, a better understanding of enzyme expression patterns at tumor sites will allow the development of more effective and precise enzyme-responsive DDS (116).

ROS-Responsive Nanosystems

The elevated presence of ROS, a byproduct of several physiological processes, is also notable in the physiology of the tumor site. Several types of NPs, such as cerium oxide, carbon, and manganese NPs, have been identified as promising for the treatment of ROS-related diseases (116).

Hypoxia-Responsive Nanosystems

It has been observed that hypoxic TME creates a reducing environment with a higher concentration of nitroreductases and azoreductases. Thus, nitroaromatic, quinone, and azobenzene derivatives have been used as hypoxia-sensitive moieties in the development of TME-responsive nanomedicine (116).

Hypoxia-activated prodrugs (HAPs), which are nontoxic compounds designed to be reduced to cytotoxic compounds in a hypoxic environment, have been widely reported for cancer therapy. Furthermore, nanocarriers have been designed to improve the accumulation of HAPs at the tumor sites (116, 120).

6.2 Elimination of Pro-Tumoral Bacteria by DDS

Antibiotics are commonly used to kill pathogenic bacteria and to modulate the microbiome. Nonetheless, the use of broad-spectrum antibiotic can result in antimicrobial resistance and dramatic changes in the commensal microbiota (121). With the growing need for targeted antibiotics, antibiotics delivered by nanosystems are promising to selectively eliminate pro-tumoral bacteria and avoid dysbiosis (Figure 11) (100).

Recent nanotechnologies can target specific bacteria implicated in gut cancers (e.g., *H. pylori* and *F. nucleatum*) to facilitate antibiotic delivery (121).

H. pylori is a potential target for preventing gastric cancer. In a study performed by Angsantikul *et al.*, a PLGA NP, encapsulating the antibiotic Clarithromycin, was coated with gastric epithelial cell membranes (AGS cells) to bind to *H. pylori*. This method took advantage of the interaction between receptors on AGS cells and adhesins on the surface of *H. pylori*, which allow the bacteria to adhere to the gastric epithelium and thus release the antibiotic (Figure 12) (122). This study showed how well-known nanotechnology functions (such as cell-membrane coatings, drug encapsulation, and navigating and targeting in complex microenvironments) can provide the technological foundation for cancer-causing microbes to be targeted and killed as a preventive strategy (121).

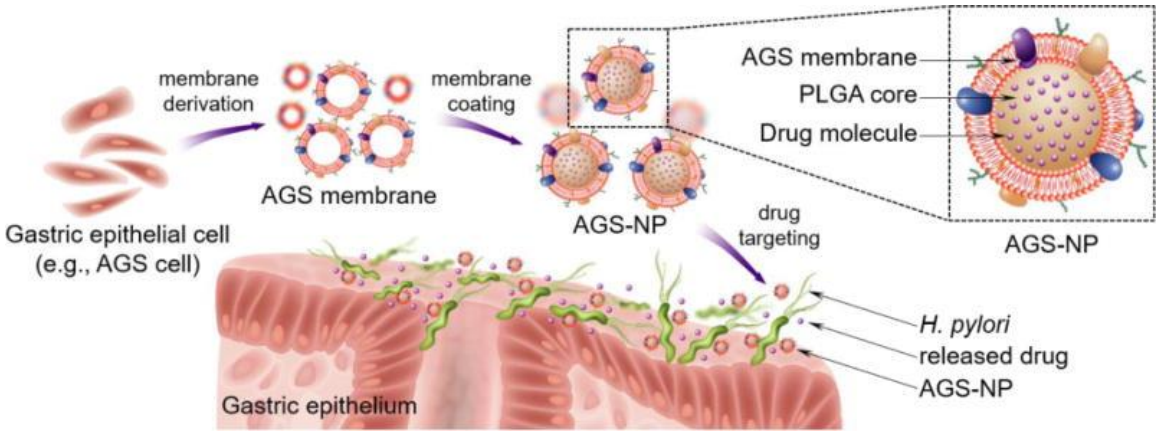

Figure 12: Preparation of poly(lactic-co-glycolic acid) (PLGA) nanoparticles coated with epithelial cell membranes and loaded with antibiotics for *Helicobacter pylori* targeting and killing (121)

Additionally, Zhang *et al*. developed Irinotecan (IRT) – loaded dextran NPs covalently linked to azide-modified phages and delivered it in CRC models (Figure 13). This research revealed promising results, in particular an inhibition of pro-oncogenic *F. nucleatum* growth, the proliferation of butyrate-producing *Clostridium butyricum* due to dextran presence, and a significant improvement in the effectiveness of CRC chemotherapy treatments, reducing the side effects (105, 107, 121, 123).

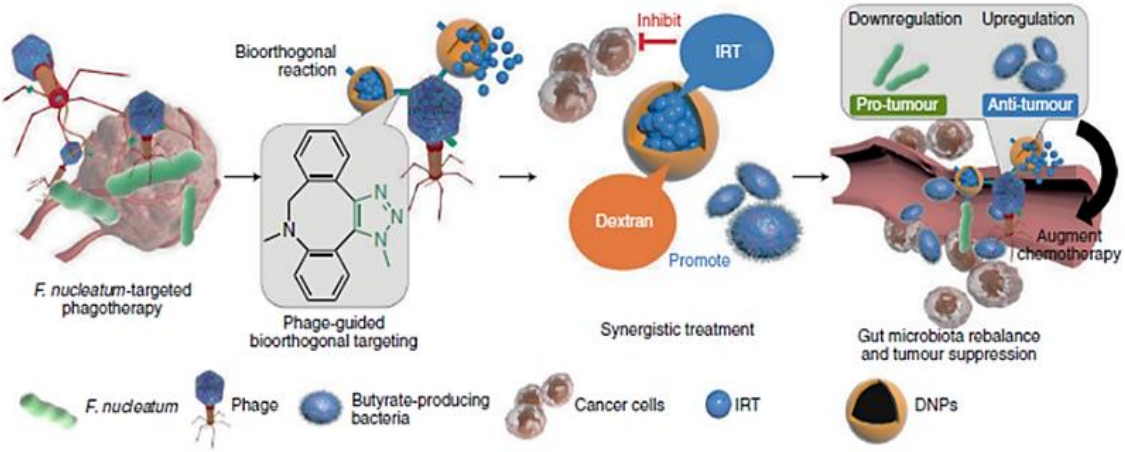

Figure 13: Phage-based targeting to deliver prebiotics, upregulate anti-cancer commensal bacteria and chemotherapeutics to kill cancer cells (121)

6.3 Nanotechnology to Modulate Microbiome Metabolites

As referred, bacterial metabolites could play important roles in cancer development, and nanotechnologies should be used to interact with them. Potential approaches to manipulate microbial products include nanotechnologies that can directly inactivate, bind or otherwise block bacteria that produced toxins and metabolisms with pro-oncogenic effect (107).

As example, LPS is one of the most abundant products in the gut microbiota that has been linked to the development of CRC and resistance to anti-PD-L1 therapy. In order to overcome their oncogenic effect, Song *et al*. encapsulated the coding sequence of LPS-targeting fusion protein (which blocks specifically the tested LPS) into lipid-protamine-DNA NPs (107, 124). By expressing a protein that traps LPS (LPS-Trap protein), the LPS-trapping NP system selectively blocks LPS inside tumors. This LPS-trapping system boosts antitumor T cell-mediated immunity and reverses the immunosuppressive TME, increasing the efficacy of PD-L1 blockade (77). In addition, LPS trap system decreased liver metastasis in primary CRC (66).

Additionally, nanomedicine may prevent the pathogenetic adhesion of bacterial products by adsorbing them. For example, Wang *et al.* developed an hydrogel to retain toxin-absorbing nanosponges. Nanosponges were constituted by red blood cell membrane-coated NPs that can absorb pore-forming toxins produced by methicillin-resistant *Staphylococcus aureus* (125). Thus, NPs can be designed to bind or adsorb bacterial products, as well as to release or intracellularly express inhibitors of bacterial products (Figure 11) (100).

6.4 Improving the Efficiency of Pro- and Prebiotics Delivery

Nanotechnology can also be used to deliver prebiotics that specifically regulate the proliferation or metabolism of commensal bacteria with known anti-cancer effects (121). As illustrated in the Figure 11, prebiotics can be used as sustained release materials to prepare NPs for drug delivery, conjugated with functional groups to form self-assembly micelles, or conjugated directly to NPs (100).

The material that facilitate the growth of tumor suppressor bacteria might be included in the NPs formulation. For example, it has been shown that dextran-based NPs increased the proliferation of *C. butyricum*, a bacteria known to secrete SCFAs, allowing to suppress growth of CRC. This strategy might be applied to specifically promote the growth of bacteria that inhibit the tumor growth without promoting microbiota imbalance (107).

In terms of probiotics, a study using *E. coli Nissle* (EcN) 1917 showed that this probiotic played a positive role against the invasion of pathogens. EcN inhibits the invasion of *C. jejuni* in HT-29 cells via enhancing the intestinal barrier function (126). Further, EcN also relieves the intestinal barrier dysfunction by retaining Claudin-1 expression and inhibiting microbiota dysbiosis. Moreover, it is important to note that when compared to free EcN, encapsulation by alginate and chitosan NP efficiently maintains the viability of EcN in simulated gastric and intestinal fluid (100, 126, 127).

According to Figure 11, in addition to the commonly used carriers for probiotic delivery, namely polysaccharides and proteins, novel approaches to encapsulate probiotics, such as cell-membrane and self-coating biofilm, have been proposed (100).

6.5 Microbe-inspired Nanotechnologies

In the emerging field of cancer immunotherapy, live attenuated bacteria are becoming increasingly important in biotechnology and medicine. Researchers describe a new strategy for developing cationic NP-coated bacterial vectors capable of efficiently delivering oral DNA

vaccine for effective cancer immunotherapy (128). By coating live attenuated bacteria with synthetic NPs self-assembled from cationic polymers and plasmid DNA, the protective NP coating layer is able to effectively escape phagosomes, significantly improve bacteria acid tolerance in stomach and intestines, and greatly promote bacteria dissemination into blood circulation after oral administration (128).

The hybrid vector demonstrated remarkable T cell activation and cytokine production when DNA vaccines encoding autologous vascular endothelial growth factor receptor 2 (VEGFR2) were administered orally. Successful inhibition of tumor growth was also achieved by efficient oral delivery of VEGFR2 with NP coated bacterial vectors due to angiogenesis suppression in the tumor vasculature and tumor necrosis (128).

This research shows that coating live attenuated bacteria with polymeric NPs is a promising strategy for developing efficient and versatile DNA vaccine vectors. NPs can be further tailored and functionalized for various delivery applications using a diverse range of bacterium options. In the emerging era of personalized nanomedicine, this method of delivering DNA vaccines could become a very promising therapeutic modality for treating a wide range of cancers (128).

6.6 Nanoparticulate Cancer Vacines

Given the well-established role of DCs in inducing CTL immunity, several studies have been conducted to investigate the use of DC-based cancer vaccines in tumor immunotherapy (110).

Cancer vaccines based on NPs can be targeted to DCs *in vivo* and induce maturation of these cells, leading to improved immune responses. NPs are able to deliver several tumor-associated antigens (TAAs) and adjuvants simultaneously, enabling a coordinated activation of DCs. Briefly, TAAs are presented to $CD8^+$ and $CD4^+$ naive T cells via MHC class I and class II molecules, which recognize the processed antigens via TCRs. Activated $CD8^+$ T cells divide into CTLs, which can destroy tumor cells, and memory T cells, that are important to prevent recidivism and metastasis. $CD4^+$ T cells can differentiate into T_H1 cells, which will enhance CTL activity and activate innate immune cells, such as NK cells, granulocytes, and macrophages, all of which play a role in tumor destruction (110).

6.7 Therapeutic Combination Delivery Based on DDS

One of the most attractive features of a nanosystem is its ability to co-deliver various therapeutic agents for combined therapy. For example, prebiotics are suitable candidates to combine with other cancer therapeutic agents to achieve synergistic effects (100).

Antimicrobial approaches that complement or synergize with antibiotics have been developed. For example, inorganic NPs kill bacteria primarily through ROS release, which can synergize with antibiotics to increase the death of cancer-causing bacteria. In another example, nanotechnology-mediated delivery of antibiotic combinations was used to destroy *H. pylori* in greater quantities than individual antibiotics alone. The combination of Amoxicillin, Clarithromycin, and Omeprazole in a single chitosan-based NP reduced the *H. pylori* burden in mice more than individual NP antibiotic formulations. As a result, the synergistic effect of various drugs improves chemotherapy outcomes (121).

6.8 Challenges

Nanotechnologies for microbiome intervention face the same challenges as current cancer nanotechnologies such as scale-up, targeting efficiency and biodistribution, toxicity and side effects, and overcoming delivery challenges arising from tumor heterogeneity between patients. These issues can be addressed by improving specific nanomaterial design benchmarks namely nanomaterial selection and attributes such as surface charge, size, and shape (129). For example, regarding the carrier material toxicity, studies have shown that inorganic NPs are less biocompatible and pose more health risks, since inorganic NPs may alter or disrupt the commensal microbiota (100).

As nanotechnology-based microbiome interventions are developed, future research should focus on these concerns, especially toxicity, side effects, and downstream effects of the commensal microbiota, given the implications for cancer development, progression, and treatment (121). Further, the interactions of the innate and adaptive immune system with nanotechnology-based microbiome modulators are still under investigation (112).

7 Conclusions

The commensal microbiota has a significant impact on the host´s immune system, and it is recognized as a factor that can influence anti-tumor immunity and therapeutic outcomes. It is critical to determine which bacteria are relevant in humans, their abundance, and the impact of their products on cancer progression, as well as to understand their interactions with the human immune system. The microbiome could be used as an additional prognostic or predictive biomarker for treatment outcomes. There is still a lack of understanding about the whole range of microorganisms and their specialized activities in different cancer types.

The microbiota's role on cancer has motivated efforts to manipulate the microbiome to prevent and treat cancer. Microbial manipulation can be used as an adjunct to cancer therapies or as a stand-alone therapy after standard therapy fails. Antibiotics, prebiotics, diet, probiotics, and FMT are all being studied to manipulate the microbiota. As such, important variables must be considered in each of these strategies.

Facing several challenges presented by conventional therapy, nanotechnology was the only treatment option available for patients. The development of nano-based systems has provided strategies for protecting incorporated agents such as biomolecules (nucleic acids, peptides, and proteins), which are generally rapidly degraded when administered *in vivo*. To achieve an optimized release profile, therapeutic agents can be embedded, encapsulated, or even adsorbed or conjugated onto nanosystems, which can be then modified and associated with other adjuvants. Indeed, the well-known versatility of nanotechnology strategies enables the precise design of multifunctional nanocarriers. These, in turn, can be functionalized by ligands of various types to promote targeted delivery of their cargo at both the cellular and subcellular levels. Thus, nanotechnology emerges with several advantages, namely, the need for a lower dosage, greater specificity, better pharmacokinetic parameters, and greater targeting efficiency. Although there are several challenges in modulating the microbiome for cancer treatment, the nanotechnological strategies have been showed promising results.

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