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Perspective

Microbiome and Anticancer Immunosurveillance

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Anticancer immune responses can be considered a desirable form of autoimmunity that may be profoundly shaped by the microbiome. Here, we discuss evidence for the microbiome's influence on anti-tumor immunosurveillance, including those that are indirect and can act at a distance, and we put forward hypotheses regarding mechanisms of how these effects are implemented. These may involve cross-reactivity between microbial and tumor antigens shaping T cell repertoires and/or microbial products stimulating pattern recognition receptors that influence the type and intensity of immune responses. Understanding how the microbiome impacts natural cancer immunosurveillance as well as treatment-induced immune responses will pave the way for more effective therapies and prophylactics.

Introduction

The relationship between cancer and microbiota has intrigued the biomedical community since the late 19th century following William Coley's partially successful attempts to cure sarcomas by local injection of bacteria, referred to as "Coley's toxin." Since then, experimental and clinical oncologists have been attempting to isolate microbial agents or products to treat malignant disease with some success, such as treatment of superficial bladder cancer based on an attenuated form of *Mycobacterium bovis* (Kiselyov et al., 2015), an FDA-approved oncolytic herpes virus for the treatment of melanoma (Greig, 2015), and initial clinical trials exploring treatment of pancreatic cancer with *Listeria monocytogenes* (Le et al., 2015).

It also appears that our naturally occurring microbiome affects cancer development. Oncogenic viruses (such as Papilloma virus, the agent responsible for cervical carcinoma), bacteria (exemplified by *Helicobacter pylori*, the agent responsible for non-cardia gastric carcinoma), and helminthes (such as *Schistosoma hematobium* causing bladder cancer) can be targeted by appropriate antibiotics to avoid or abort cancers. A more subtle relationship between the microbiome and malignancy exists as well (Garrett, 2015). All human body surfaces and all cavities that communicate with the exterior are inhabited by complex, individualized, and variable ecosystems of microorganisms (bacteria, viruses, protozoa, fungi, and archae) whose composition is influenced by host genetics, feeding habits, life style, and early-life microbial exposure. Such microbiota facilitate the absorption of nutrients, produce vitamins, contribute to barrier functions, and displace pathogenic microorganisms, yet also mediate profound effects on disease, be it through local effects, such as the influence of intestinal microbiota on inflammatory bowel disease or that of the oral microbiota on periodontitis, or through long-distance effects on a priori sterile organs such as the cardiovascular, endocrine, and central nervous systems (Belkaid and Hand, 2014).

In this perspective, we will review the influence of the microbiota on cancer and cancer therapies, with a focus on how interactions between the microbiota and the immune system impact cancer progression and treatment, including immunotherapy. While there have been interesting studies documenting proximal direct effects of microbes on immune cells in colon cancer, for example, the evidence that the bacterium *Fusobacterium nucleatum* produces a protein that engages an immunoreceptor on T and natural killer (NK) cells to block their cytotoxic activity on tumor cells (Mima et al., 2015; Gur et al., 2015), we will focus here on the capability of the microbiota to affect the development of cancers by more distal, systemic means. We will discuss the literature investigating the impact of the natural microbiome on anticancer immune responses (Figure 1), both in the presence and absence of therapies, and we will put forward more speculative hypotheses for potential mechanisms of these interactions that we hope will stimulate further exciting work in this area.

Microbiome and Natural Immunosurveillance

Microbiota, Tumor-Associated Antigens, and Immune health

Leukocytes patrol tissues to eliminate invading pathogens and dying, dead, or senescent cells, both of which are important for

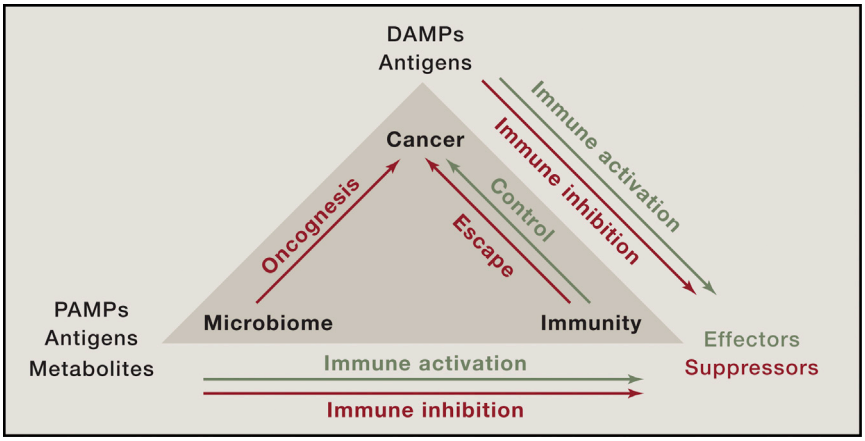

Figure 1. Triangulation between the Microbiome, the Immune System, and Cancer

Danger-associated molecular patterns (DAMPs) and tumor antigens in the tumor can activate antitumoral immune effectors, whereas cytokines and chemokines present in the tumor microenvironment can induce or attract immunosuppressive immune populations. The overall equilibrium between these two phenomena will impact the clinical outcome. The immune system of the patient (host) is modulated by microbiota-derived pathogen-associated molecular patterns (PAMPs), antigens, and metabolites that imprint both the effector and suppressive arms of immunity. Through its influence on immunity the microbiota contributes to the immune control or escape of distant tumors. Microbiota-derived metabolites can also contribute, through direct cell-autonomous carcinogenic mechanisms, to the development of local tumors.

warding off inflammation. In addition, immune mechanisms participate in the early abortion of malignant transformation, not only by destroying cells infected by oncogenic viruses, but also by recognizing cells that express tumor-associated antigens (TAAs) (Vesely et al., 2011). Analyses of high-dimensional datasets from The Cancer Genome Atlas (TCGA) revealed the potential impact of viral antigens on anticancer immunosurveillance (Rooney et al., 2015). For example, the expression of granzyme A and perforine, reflecting the intratumoral presence of cytotoxic T lymphocytes (CTLs) was higher in Epstein Barr virus $(EBV)^+$ versus EBV^- stomach cancers, as well as human papilloma virus $(HPV)^+$ versus HPV^- head and neck, bladder, uterine, and cervical cancers (Rooney et al., 2015). Following on data for tumors driven by endogenous retrovriuses (ERVs) obtained in immune-deficient mice (Young et al., 2012), ERVs were found to be reactivated in some human malignancies (such as stomach adenocarcinomas) coupled to local invasion by CTLs (Rooney et al., 2015). Hence, ERVs may constitute one particular class of TAAs.

One may consider that the immunogenicity of a cancer cell, and therefore its susceptibility to immunosurveillance, requires a combination of antigenicity (*i.e.* the presence of TAAs) and adjuvanticity, that is, the degree to which the immune system is called to action by danger-associated molecular patterns (DAMPs). (Kroemer et al., 2015). Within this framework, the general state of the immune system ("immune health") with respect to general immunosurveillance matters for clinical outcome, and this may be influenced by local or systemic alterations in the microbiome. The influence and mechanism of microbiome-mediated changes in TAA presentation and overall "immune health" in cancer progression are important areas for future study.

Under-Exposure to Microbes—a "Cancer Hygiene Hypothesis"

According to the "hygiene hypothesis," reduced exposure to infections may lead to the spectacular rise of allergies and some autoimmune diseases such as type 1 diabetes and systemic lupus erythematosus that has been observed over the last century in developed and developing countries (Strachan, 1989; Thorburn et al., 2014). Epidemiological studies indicate that the number of childhood infections inversely correlate with the probability of developing chronic lymphoid leukemia during adulthood (Parodi et al., 2013), and higher socioeconomic status (that exacerbates hygiene) correlates with an increased incidence in Hodgkin lymphoma (Gutensohn and Cole, 1981). These and other findings can be blended into a "cancer hygiene hypothesis" to postulate that the increase in the incidence of particular cancers may result from the non-physiological under-exposure to certain microbial species linked to the modern lifestyle and the consumption of sterilized and processed food (Oikonomopoulou et al., 2013). As a variation of this hypothesis, it can be speculated that, in analogy to autoimmune diseases (such as systemic lupus erythematosus and rheumatoid arthritis, which occur more frequently in women than in men), part of the sex preference of some cancers is conditioned by gender-specific differences in the gut microbiome (Dominianni et al., 2015; Markle and Fish, 2014).

There is circumstantial evidence that dysbiosis caused by repeated antibiotic medication can enhance the frequency of some cancers. While it may be not surprising that certain antibiotics modulate the incidence of gastric and colorectal carcinomas, it is important to note that a large epidemiological study in humans (125,441 cases and 490,510 matched controls) suggest that antibiotic exposure may also affect the frequency of lung cancer (increases upon use of repeated courses of penicillin, cephalosporins, or macrolides), prostate and bladder cancer (increases with penicillin use), suggesting a relationship between the microbiome and carcinogenesis (Boursi et al., 2015). The repeated use of tetracyclines and sulphonamides also increases the risk of breast cancer (Boursi et al., 2015), validating the results of a prior study enrolling 2.1 million women (Friedman et al., 2006). These findings either suggest that certain antibiotics have direct effects on host cells that in the end are carcinogenic (tetracyclines, for instance, inhibit replication of mtDNA) or that certain antibiotics favor shifts in the composition of the microbiota that favor the development of tumors. In the absence of further mechanistic insights, these results might be viewed as an additional support of the cancer hygiene hypothesis, yet have to be interpreted with caution because repeated use of antibiotics may indicate the presence of immune defects that would be the primary cause of both bacterial infections and enhanced cancer incidence. Apparently contradicting this

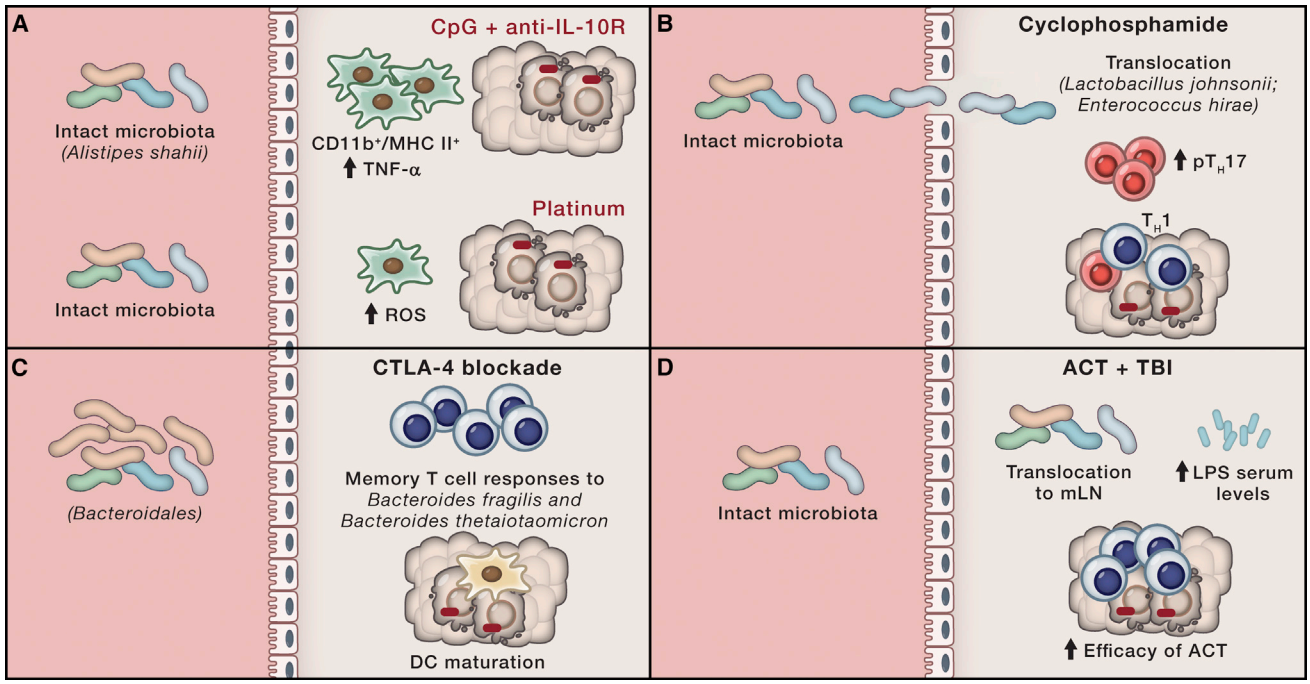

Figure 2. Chemo-Immunotherapy Effects Modulated by the Microbiota

(A–D) The gut microbiota participates to the efficacy of anti-tumor chemotherapy (platinum and cyclophosphamide), immunotherapy (CpG+anti-IL-10, CTLA-4 blockade) (A and C) and combined total body irradiation (TBI) adoptive T cell transfer therapy (ACT) (D) through several immune mechanisms: modulation of myeloid-derived cells production of TNF-α and reactive oxygen species (ROS) in mice (A), stimulation of pathogenic T_H17 (pT_H17) and T_H1 by translocated gram-positive bacteria in mice (B), enhancement of Bacteroidales-specific memory T cell responses in mice and patients and of DC maturation in mice (C), TLR4-dependent improved efficacy of ACT in a mouse model when combined to TBI that causes intestinal injury and results in translocation of commensal bacteria to mesenteric lymph nodes (mLNs) and in elevated lipopolysaccharide (LPS) serum levels (D).

possibility, antibiotics can accelerate the development of cancers in mice: this has been shown for the treatment of proto-neu transgenic mice that spontaneously develop mammary carcinomas with metronidazole plus ciprofloxacin (Rossini et al., 2006). Similarly, mice treated with a combination of broad-spectrum antibiotics (ampicillin, vancomycin, neomycin, and metronidazole) show accelerated development of lung metastases after intravenous injection of melanoma or non-small-cell lung cancer cells, correlating with a defective induction of a γδT17 cell response. This effect could be reversed by adoptive transfer of γδT cells from normal mice or by supplementing recombinant IL-17A protein, providing a formal demonstration that defective immune surveillance was responsible for the antibiotic-mediated acceleration of cancer development (Cheng et al., 2014). These results support the notion that antibiotic use can stimulate cancer development via the subversion of immunosurveillance.

Recently, more direct evidence in favor of the capacity of commensal bacteria to promote tumor immunosurveillance was provided by comparing the growth of melanomas and their infiltration by IFN-γ producing CTLs in C57BL/6 mice from two different providers, Jackson Laboratories (JAX) and Taconic Farms (TAC) (Sivan et al., 2015). TAC mice with poor immunosurveillance exhibited a relative loss of *Bifidobacterium* species. Oral feeding of TAC mice with *Bifidobacterium* or their cohousing with JAX mice restored defective processing and presentation of tumor antigens by dendritic cells (DCs), re-established infiltration of melanomas by CTLs and reduced malignant growth.

Altogether, these observations indicate that intervening with the microbiota in mice can alter the outgrowth of transplantable tumor cell lines bearing strong immunogenic antigens. In addition, it appears that the microbiome influences the response to anticancer therapies, as will be discussed in the following section.

Microbiome and Therapy-Induced Immunosurveillance

Microbial Disruption Boosts Therapeutic Efficacy

Over the past decade it has become clear that the long-term effects of anticancer drugs cannot be considered only autonomous to the targeted cells, but also involve an obligatory immune component (Galluzzi et al., 2015; Zitvogel et al., 2013). The microbiome appears to play a role here. The chemotherapeutic agents oxaliplatin and cyclophosphamide are both much less efficient in reducing the growth of tumors, not only in immunodeficient (as compared to immunocompetent) mice, but also in germ-free or broad-spectrum antibiotic-treated (as opposed to specific pathogen-free) mice. Upon depletion of commensal bacteria, the combination of oxaliplatin and CpG oligonucleotides loses its capacity to stimulate tumor infiltration by $CD11b^+$ MHC class II^+ myeloid cells producing TNF-α, which in turn are required for the anticancer effects (Figure 2A) (Iida et al., 2013).

The presence in the gut of several bacterial species (such as *Alistipes shahii*) was positively correlated with the TNF response, while *Lactobacillus* species (such as *L. fermentum*) decreased

this response (Iida et al., 2013). In the case of cyclophosphamide, the generation of a specific subset of "pathogenic" T helper 17 (pT_H17) cells was inhibited in germ-free mice, perhaps due to the absence of the translocation of $Gram^+$ bacteria from the intestinal lumen into secondary lymphoid organs (Figure 2B) (Viaud et al., 2013). Cyclophosphamide induces the translocation of specific bacteria (in particular *Lactobacillus johnsonii* and *Enterococcus hirae* but not *Escherichia coli* or *Lactobacillus plantarum*) into mesenteric lymph nodes and the spleen, and it only triggers T_H1 memory responses against those commensals that cross the mucosal barrier. Importantly, cyclophosphamide potently increases the permeability of the intestine to intravenously injected fluorescein isothiocyanate (FITC)-dextran of 70 kDa (Viaud et al., 2013), as it induces the invasion of intestinal bacteria into lungs, liver, spleen, and kidneys (Green et al., 2015). Usually, an effective gut-vascular barrier avoids the translocation of microbial antigen into the bloodstream and blocks the entry of the microbiota (Spadoni et al., 2015). The exact mechanisms through which cyclophosphamide would cause permeabilization of the gut-vascular barrier are elusive, although it is common knowledge that local inflammation and dysbiosis favor leakiness of the gut (Marchiando et al., 2010). Both cyclophosphamide and ipilimumab, a monoclonal antibody that targets an immune checkpoint protein, induce apoptosis of a fraction of enterocytes and colonocytes, but only the former causes translocation of bacteria into mesenteric lymph nodes and spleens, indicating that intestinal epithelial cell apoptosis induction as such cannot explain the phenomenon (Vétizou et al., 2015; Viaud et al., 2013). Cyclophosphamide does induce a gut dysbiosis by altering the ratio of Firmicutes and Bacteroides (Viaud et al., 2013; Xu and Zhang, 2015), but this is a rather late phenomenon succeeding the immune modulations accompanying cyclophosphamide injections. Hence, the exact contribution of these changes to its capacity to breach the mucosal barrier and affect systemic immunity (or vice versa) has not been elucidated.

Recent advances in immunotherapy for cancer involves inhibition of pathways that downregulate the immune responses toward tumors. These therapeutic agents are referred to as immune checkpoint blockers (ICBs) and target immune inhibitors such as CTLA4 and PD-1. ICBs have recently revolutionized the treatment of melanoma (which involves the blockade of CTLA4 and/or that of the interaction between PD-1 and PD-L1) and non-small-cell lung cancer (based on blockade of PD-L1) (Sharma and Allison, 2015). In mouse models, the efficacy of such ICBs is strongly dependent on the gut microbiome. CTLA4 blockade lost its therapeutic activity against fibrosarcomas in mice that were either raised in a germ-free environment or that had been raised in specific pathogen-free conditions and then treated with multiple broad-spectrum antibiotics to sterilize the gut (Figure 2C) (Vétizou et al., 2015). This defect was overcome by gavage with *Bacteroides fragilis*, by immunization with *B. fragilis* polysaccharides, or by adoptive transfer of *B. fragilis*-specific T cells, suggesting a therapy-relevant cross-reactivity between microbial and tumor antigens recognized by the same T cell receptor (TCR). Accordingly, both in mice and in patients, T cell responses specific for distinct *Bacteroides* species (*B. fragilis* and *B. thetaiotaomicron*) were associated with the administration (in humans) and efficacy (in mice) of CTLA-4 blockade. Moreover, fecal microbial transplantation from humans to mice confirmed that injection of melanoma patients with antibodies against CTLA-4 favored the outgrowth of *B. fragilis* with anticancer properties (Vétizou et al., 2015).

The microbiome also affects the therapeutic efficacy of PD-L1 blockade. Injection of a blocking antibody against PD-L1 was much more efficient in reducing the growth of melanomas in mice containing a high abundance of *Bifidobacterium* in their gut than in mice lacking this genus. *Bifidobacterium*-treated mice exhibited significantly improved tumor control compared with their untreated littermates, and this effect was mediated by $CD8^+$ T cells. DCs purified from mice that had been treated with *Bifidobacterium* were particularly active in presenting a melanoma-derived peptide antigen to T cells for stimulation of their proliferation and IFN-γ production, suggesting that *Bifidobacterium* improves the anticancer immune response through an effect on DCs (Sivan et al., 2015). Hence, the mechanistic bases of the microbial contribution to the mode of action of distinct checkpoint blockers share common features but might also somewhat differ. While both studies describe the gut microbiota-dependent intratumoral maturation of DCs, the first study (on anti-CTLA4) suggests a role for cross-reactive T cell epitopes present on bacteria and cancer, the latter (on anti-PD-L1) postulates an effect on innate immunity leading to a gut microbiota-dependent resetting of antigen presenting cell functions.

Altogether, the aforementioned examples illustrate that anticancer therapies aiming at reinstating immunosurveillance are profoundly influenced in their efficacy by the gut microflora that can act at distance, on a range of a priori sterile tumors.

Conceptualizing Putative Mechanistic Links between Gut Microbiota and Anticancer Immunosurveillance

The findings summarized above support a complex triangulation between microbiome, immunosurveillance, and malignancy, in which, at least in some specific cases, the microbiome conditions anticancer immune responses over some distance. Thus far, only the impact of the intestinal microbiota has been studied at this level, although it may be possible that other microbiota (such as the oral or cutaneous ones, yet devoid of a large microbial diversity) also mediate long-range effects. In order to generate hypotheses about how the microbiota mediate long-range effects, it may be useful to theoretically frame their putative influences into two categories that are linked to the aforementioned framework of immunogenicity resulting from a combination of antigenicity and adjuvanticity, in which a theoretical "signal 1" links to the antigenicity of cancers, and "signal 2" links to their adjuvanticity (Figure 3).

Linking Gut Microbiota to Tumor Antigenicity

As a "signal 1 hypothesis," one might postulate that microbial proteins might be sufficiently similar to tumor antigens to elicit immune cell activity via antigenic mimicry or cross-reactivity. If microbial antigens remain confined in the intestinal lumen and biofilm, for T cell antigens, this hypothesis requires the T cell to be primed by microbial antigen as an intra-epithelial or lamina propria T cell entering into direct contact with local DCs and then traveling to distinct extra-intestinal sites to recognize tumor antigens expressed by mutated or epigenetically deranged (pre-) neoplastic cells. This scenario may enter into open conflict with

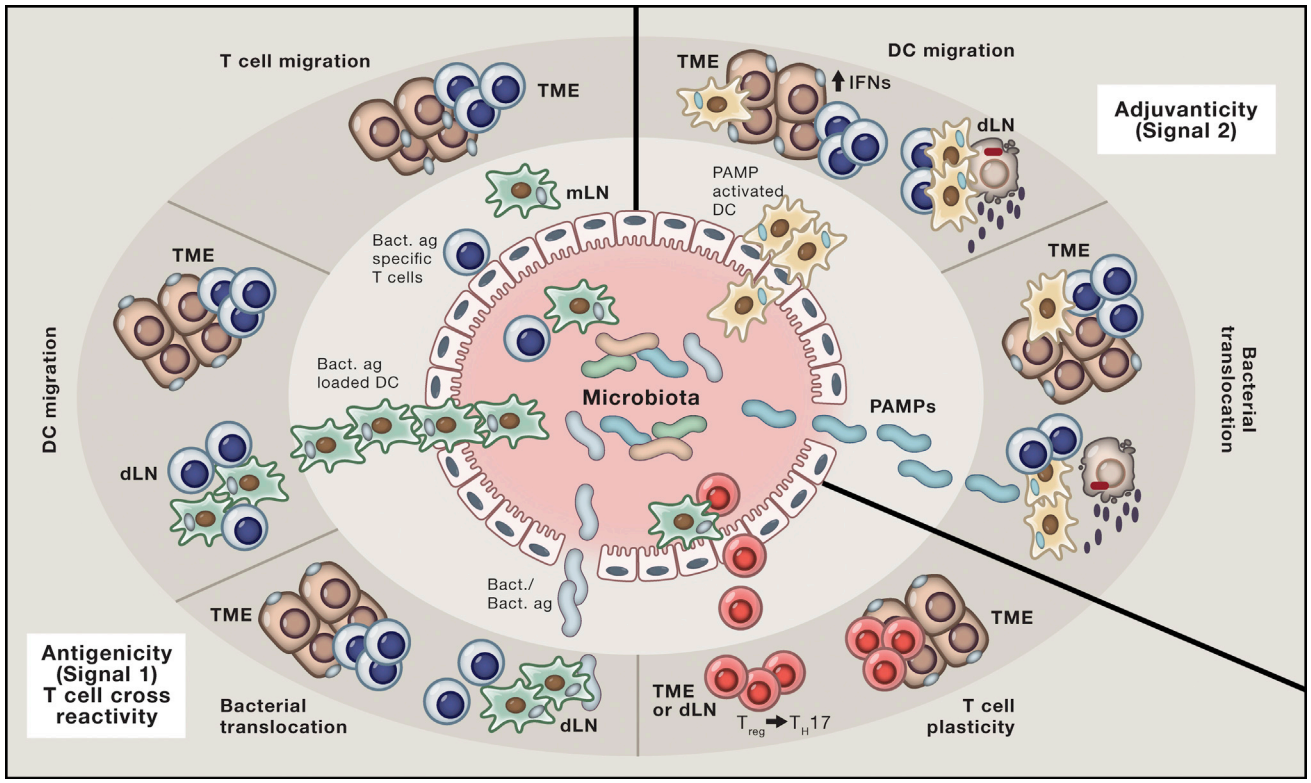

Figure 3. Proposed Mechanisms Accounting for the Microbiome Distant Effects on the Tumor Microenvironment

T cells and antigen presenting cells (APCs) can migrate from the gut to mesenteric lymph nodes (mLNs) as well as to other mucosal sites and inflammatory sites, including tumors and their draining LN (dLN). Intestinal injury caused by chemotherapy, immunotherapy, or antibiotics can lead to the translocation of bacteria, bacterial antigens and bacterial PAMPs to mLN and, through the circulation, to distant sites including tumors and tumor-draining LN. Microbiota-imprinted immune cells migration and bacterial translocation can influence the immune response to tumors either through the priming of bacterial antigen-specific tumor antigen-cross reactive T cells (TCR mediated = signal 1) or through the modulation of the immune tonus (PAMPs mediated = signal 2).

the notion that T lymphocytes act in a thoroughly compartmentalized fashion (Kroemer et al., 1993), in part due to tissue-specific education by DCs that induce the expression of homing receptors causing T cells to traffic locally (Sigmundsdottir and Butcher, 2008). Nonetheless, it is conceivable that chemokine gradients generated by growing tumor deposits could compete with the mucosal inductive site, thereby attracting or rerouting T lymphocytes primed in the gut toward cancer. Notably, transplantable sarcomas and RET melanomas can secrete 10–100× more CCL25 than the small intestine, causing intratumoral accumulation of CCR9-expressing T cells (Jacquelot et al., 2016). Moreover, the concept of a ''common mucosal immune system'' supports the idea that B and T cells primed locally in an initial mucosal site can seed distantly to other mucosal or lymphoid tissues. By seeding distal sites with antigen-specific effector or memory phenotypes, this mucosa-derived immunity would protect the host against any pathogen encountered in the future, irrespective of the portal of entry (Wilson and Obradovic, 2015), thus establishing ''functional connectiveness'' within the immune system (Kiyono and Fukuyama, 2004; Kunisawa et al., 2008; McGhee et al., 1992). This notion could explain the potential efficacy of immune checkpoint blockers such as anti-PD1/PD-L1 Ab against lung cancers in the context of intestinal *Bifidobacterium*-dependent T cell function resetting (Sivan et al., 2015).

However, whether T lymphocytes primed in the common mucosal immune system might be geared to peripheral sites of inflammation and cause immunopathologies (such as arthritis, encephalitis, or diabetes) remains an open conundrum. A number of mouse models of chronic inflammation residing in distant, non-mucosal tissues, where an impact of the gut microbiota was demonstrated, outlined the pro-inflammatory role of intestinal T_H17 cells (Lee et al., 2011; Wu et al., 2010; Yang et al., 2014), suggesting that T_H17 primed in the intestine could traffic to peripheral lesions, undergo functional plasticity, and mediate immunopathologies. Fate determination of cells that had produced IL-17A before their conversion by environmental cytokines using a reporter mouse strain (Hirota et al., 2011) unraveled that T_H17 cells exhibit functional plasticity depending on whether the inflammatory conditions are acute, chronic, or resolved (McGeachy and Cua, 2008). Alternatively, Kaede transgenic mice, which universally express a photoconvertible fluorescent reporter (Tomura et al., 2008), allow visualization of immigration to and emigration from the intestine. Unexpected broad movements of leukocytes have been observed at steady state, with evidence of a splenic relocation of gut-derived T_H17 primed by segmented filamentous bacteria in mice at the onset of their genetically determined arthritis (Morton et al., 2014; Wu et al., 2010). Hence, fate mapping of IL-17A-producing T cells

educated in the gut are awaited in tumor bearers to conclude on their relevance in anticancer immunosurveillance.

An alternative mechanism by which the signal 1 hypothesis might be implemented is via microbial antigens, rather than T cells, traveling through the organism. Translocation of microbial proteins and even entire microorganisms from the intestine to mesenteric lymph nodes, the spleen, and other sites has been documented (Abt et al., 2012; Wheeler et al., 2014), and this breach of the mucosal barrier may prove essential for instances in which antigenic mimicry determines the long-range effects of the microbiome on immunosurveillance. In such a scenario, translocating microbial antigens would trigger an initial immune response in secondary lymphoid organs, hence priming T cells that then would migrate to tumor sites to participate in immunosurveillance. Peptidoglycans, conserved bacterial constituents shed during bacterial division, regulate various functions of host physiology and immunity at distant sites from the gut through widely expressed receptors (Wheeler et al., 2014). Yet another possibility is that microbes or microbial antigens do not penetrate beyond the lamina propria and instead are locally captured by $CD103^+$ $CD11b^+$ dendritic cells that migrate to the draining lymph node to present relevant antigen to T cells.

It should be noted that commensal-specific Tregs are capable of switching to effector phenotypes upon infection-related disruption of mucosal barriers (Hand et al., 2012). The conversion of induced Foxp3 Treg (iTreg) into inflammatory T_H17 (Raffin et al., 2013) could explain the transition between instructive intestinal immunity and peripheral immune responses (Komatsu et al., 2014). Indeed, several studies in mice have shown that iTreg were absent in germfree mice and that both commensal bacteria and their metabolites, such as the short-chain fatty acid (SCFA) butyrate, were necessary for their development (Arpaia et al., 2013; Atarashi et al., 2011; Furusawa et al., 2013). The modulation of T cell function by the microbiota, through butyrate, toward tolerance (IL-10 secretion) rather than inflammation (IL-17 secretion) has also been proposed for IL-10/IL-17 double-secreting T cells (Saito et al., 2015). Thus, an interesting scenario that has been invoked in the putative relationship between infection and autoimmunity (Ruff and Kriegel, 2015) consists of a switch from a tolerogenic to an auto-aggressive phenotype, if such Tregs recognize self-antigens as well.

Linking Gut Microbiota to Tumor Adjuvanticity

Signal 2 relies on the perception of one additional non-antigenic "co-stimulus" (or more realistically, multiple co-stimuli causing a plethora of secondary signals) that determines (1) whether (or not) signal 1 triggers an immune response and (2) of what specific nature this response is, guiding it toward the acquisition of specific phenotypes by $CD4^+$ T cells (such as T_H1, T_H2, T_H17, pT_H17, or Treg phenotypes) or $CD8^+$ T cells (such as T_C1 and T_C2 phenotypes). A general "signal 2" hypothesis can accommodate all effects of the microbiome on the host organism that are not linked to antigenic mimicry between microbial and tumor antigens and that lead to "bystander activation" of TAA-specific T lymphocytes. During co-evolution with microorganisms, the host has developed multiple strategies to detect invasion by microbes by means of so-called pathogen recognition receptors, also called pattern recognition receptors (PRRs), which sense the presence of molecules that are extraneous (different in their chemical properties from host-encoded molecules) or ectopic (in an inappropriate subcellular compartment) (Medzhitov and Janeway, 2000). Generally speaking, the activation of PRRs may break tolerance by overriding tolerogenic signals of auto-antigen (and perhaps tumor-antigen) specific lymphocytes (Horwitz et al., 1998). By activating PRRs, which are mostly expressed by innate immune effectors, microbes can stimulate the production of cytokines and interferons, which in turn set the proclivity to inflammatory, immunostimulatory, or immunosuppressive reactions and determine the immune "tonus," namely the tendency to mount T_H1/T_C1 reactions (dominated by the production of IL-2 and IFN-γ) or other types of reactions (such as T_H2 reactions dominated by the production of IL-4 and IL-13 or T_H17 reactions accompanied by the secretion of IL-17), knowing that T_H1/T_C1 (and to some extent T_H17 reactions) are particularly important for anticancer immunosurveillance (Coussens et al., 2013). These conditions may occur across a distance because (1) microbial products may attain draining lymph nodes or other locations in the body; (2) myeloid innate effectors may travel between distinct organs; and (3) beyond a certain threshold of activation, cytokine cascades have paracrine and endocrine functions, thereby mediating long-range effects.

As a particular variation of the signal 2 hypothesis, it should be mentioned that microbiomes have a major impact on the metabolome. Conservative estimates suggest that close to half of the metabolites found in human plasma are not derived from host cell-intrinsic reactions but actually from the microbiome (Martin et al., 2007). Obviously, such effects may cause long-distance alterations of whole-body physiology, either by impacting metabolic circuits and their neuroendocrine regulation or by triggering a series of metabolite-specific receptors, some of which are expressed by immune effectors.

Although both the signal 1 and the signal 2 hypotheses can be conceived in the absence of microbial translocation (or that of microbial products), it is tempting to speculate that at least a transient disruption of gastrointestinal barrier functions is a primary factor in shaping the relationship between the gut microbiome, the immune system and cancer. Such a disruption of epithelial barrier integrity is now emerging as an important factor in the pathophysiology of local inflammation, for instance in the context of inflammatory bowel disease (Maloy and Powrie, 2011) and chronic hepatitis (Lin et al., 1995), as well as in the pathogenesis of systemic diseases such as obesity (Gummesson et al., 2011) and its comorbidities, allergy (Islam and Luster, 2012), arthritis (Meier and Plevy, 2007), and acquired immunodeficiency (Burgener et al., 2015). It will be particularly important to understand which chemotherapeutic agents induce leaky guts and whether this effect would enhance or suppress immunosurveillance circuits.

Putative Cross-Reactivity between Microbial and Tumor Antigens

The signal 1 hypothesis invokes the theory of molecular mimicry which postulates that certain antibodies (or TCRs) that recognize self-antigens had originally been generated against xenoantigens. Such cross-reactivities between microbial and self-antigens have been widely documented in the context of autoimmune diseases, which in turn are often characterized by

Table 1. Cross-Reactivity of Tumor-, Self-, or Pathogen-Specific TCR to Microbiota-Derived Peptides/Epitopes

Disease	Antigen	Immune Response	Microorganism	Homology Assessment	Reference
Cancer					
melanoma	Melan-A/MART-1	A2/CD8	*Burkholderia cepacia; Escherichia coli*	PS-SCL[1]	Dutoit et al., 2002; Rubio-Godoy et al., 2002
melanoma	MAGE-A6	DR/CD4	*Mycoplasma penetrans*	sequence homology search	Vujanovic et al., 2007
melanoma	mutated CSMD1	−/CD8	*Burkholderia pseudomallei*	sequence homology search	Snyder et al., 2014
Autoimmunity					
MS model	MBP	DR2/CD4	*Mycobacterium avium; Escherichia coli*	motif homology search	Harkiolaki et al., 2009
Sjögren's syndrome	Ro60	DR3/CD4	*Capnocytophaga ochracea*	sequence homology search	Szymula et al., 2014
SLE	SmD	DR3/CD4	*Vibro cholerae; Streptococcus agalactiae*	pattern search	Deshmukh et al., 2011
Infection					
HIV-1[2]	gag p24	DR4/CD4	*Bifidobacterium bifidum*	sequence homology search	Su et al., 2013
HIV-1	gp41	antibody	*Bacterial RNA polymerase*	IgHV repertoire by NGS	Williams et al., 2015
Influenza					
EBV[3]	BZLF1	B8/CD8	*Staphylococcus aureus*	sequence homology search	Misko et al., 1999

[1]Positional Scanning-Synthetic Peptide Library.
[2]TCRs assessed in this study were derived from memory T cells from HIV-unexposed individuals.
[3]These CTLs were also cross-reactive to a self-peptide derived from a serine/threonine kinase.

changes in the composition of the microbiome (Sánchez et al., 2015).

In a simple thought experiment, it appears logical that pre-existing T lymphocytes that recognize a microbial peptide and that constitute immunological memory would be particularly apt for eliminating tumor cells that by chance express a cross-reactive TAA. Aligning peptide sequences of TAAs with those from the microbiome yields significant homologies (Table 1). However, at this point, it has not been determined whether such overlaps reflect more than a statistical artifact and whether they are relevant for the recognition of tumor antigens. Indeed, there are three types of uncertainty that overshadow the interpretation of homologies (or even identities) among peptides shared between the microbiota and the tumor and that are identified by DNA or RNA sequencing, namely, (1) the very existence/presence of the microbial peptide as a potential antigen, (2) the antigenic relevance of the tumor RNA-encoded peptide, and (3) the functional connection between both. Methodological challenges of measuring T lymphocyte responses and the discrete nature of TAAs renders it difficult to directly assay for cross-reactivity.

What is the strongest evidence that cross-reactivity between microbial and tumoral antigens may stimulate immunosurveillance? In a mouse model, adoptive transfer of *B.* fragilis-specific $CD4^+$ T cells can reduce the growth of MCA205 fibrosarcomas while TLR2/TLR4 agonists failed to do so (Vétizou et al., 2015); however, the cross-reactive epitope has not been determined. Analogous cross-reactivities have been documented in some detail for autoimmune diseases. For example, molecular mimicry and cross-reactivity involving *Escherichia coli* and human subunit E2 epitopes of the pyruvate dehydrogenase complex (PDC) may trigger the initiation of *E. coli*-associated anti-mitochondrial immune response in primary biliary cirrhosis (Matsumura et al., 2002). It is generally assumed that the disease may then advance via the phenomenon of epitope spreading, which consists of the development of autoimmune responses to endogenous epitopes subsequent to the release of self-antigens (Floreani et al., 2015). More information is available from a mouse model of autoimmune uveitis expressing a transgenic TCR specific to residues 161–180 of interphotoreceptor retinoid binding protein (IRBP), a retinal autoantigen. In this model, a microbiota-dependent non-cognate (though yet-to-be-identified) antigen in the intestine activates retina-specific T cells in the gut lamina propria for IL-17A production, thereby precipitating uveitis (Horai et al., 2015). This exemplifies the possibility that normal commensal microbes can accidentally prime T cells breaking immune privilege (of the eye). However, the aforementioned model may be criticized for its reliance on a transgenic TCR (Horai et al., 2015), requiring further validation in a more realistic (non-transgenic) setting. Furthermore, whether such a scenario may also apply to immune privilege within tumors has yet to be determined.

In addition or alternatively to cross-reactivity, it is possible that inflammatory microbial/host microenvironments regulate the quality of effector lymphocyte differentiation independently of cognate antigen (Lochner et al., 2011; Longman and Littman, 2015)—a component of signal 2, as will be discussed below.

Pattern Recognition Receptors and the Immune Tonus

Cells from the innate immune system are required for anticancer immunosurveillance, be it natural or therapy-induced. This has been amply documented for DCs (Broz et al., 2014; Ma et al., 2013; Ruffell et al., 2014) as well as, depending on the tumor

model, for other cell types including macrophages (Coussens et al., 2013) and NK cells (Vivier et al., 2011). Implicating microbiota in this arm of immunosurveillance, germ-free mice exhibit reduced functions of such innate immune cells when compared to animals raised in specific pathogen-free conditions. This has been documented for the release of TNF-α by lipopolysaccharide (LPS)-stimulated macrophages (Souza et al., 2004). Moreover, microbial activation of splenic DCs from germ-free mice yields comparatively low levels of TNF-α, IL-6, IL-15, and type I interferons, resulting in a secondary impairment of NK cell function (Ganal et al., 2012). These defects in innate immune effectors may account at least in part for impaired immunity against respiratory or systemic infection by viruses, reduced numbers of T and B cells, diminished levels of IgA and IgG antibodies, and a strong skewing toward the T_H2 $CD4^+$ T cell helper subset (Abt et al., 2012; Ichinohe et al., 2011; Yurkovetskiy et al., 2015). The primary cause of this defect in immune tonus has not been elucidated in molecular terms, although it has become clear that introduction of microbes to germ-free mice restores the T_H1 and T_H17 compartments (Sommer and Bäckhed, 2013), which in turn are important for anticancer immunosurveillance (Vesely et al., 2011).

Accumulating evidence suggests that the microbial regulation of immune tonus involves—at least to some extent —sensing of microbial products by a range of specific receptors expressed by innate immune effectors. PRRs recognize microbial structures (pathogen-associated molecular patterns, PAMPs) as well as host-intrinsic danger-associated molecular patterns (DAMPs). The importance of PRRs for determining microbiota-regulated immune responses has been well defined in the non-obese diabetic (NOD) mouse model of type 1 diabetes (T1D). Germ-free conditions accelerate the manifestation of T1D in NOD mice, in particular in myeloid differentiation primary response gene 88 (MyD88)-deficient mice. Colonization of *MyD88*$^{-/-}$ mice with a variety of intestinal bacteria prevents T1D, unless TIR-domain containing adaptor-inducing IFN-β (TRIF), which operates downstream of Toll-like receptor 4 (TLR4, which transmits anti-diabetic signals), was ablated as well. Reduction in T1D incidence caused by TLR2 deletion was reversed by germ-free conditions, suggesting that TLR2 mediates pro-diabetic signals (Burrows et al., 2015). These results indicate that distinct TLRs can mediate signals that aggravate or attenuate autoimmune processes.

TLR4 has also been involved in the microbial regulation of anticancer immune responses (Figure 2D). Whole-body irradiation causes the translocation of commensal microflora from the gut into mesenteric lymph nodes and elevated LPS levels in the sera, thereby stimulating the tumor growth-inhibitory action of adoptively transferred tumor-specific $CD8^+$ T cells. This positive effect of whole-body irradiation is lost upon antibiotic sterilization of the gut, inhibition of LPS by polymyxin B or knockout of components of the LPS receptor system including CD14 and TLR4 (Paulos et al., 2007). In this model, the LPS-responsive cells are most likely immature DCs, which acquire the capacity to activate transferred T cells. It is intriguing to speculate that myeloablative chemotherapeutic regimens that are clinically used to improve the outcome of adoptive cell transfer might stimulate similar mechanisms.

The only known ligand for TLR5 is flagellin, a primary structural component of the flagellum that can be expressed by gram-negative as well as gram-positive bacteria. Patients that are homozygous and heterozygous carriers of a deleterious polymorphism in TLR5 (*TLR5*^{R392X}) exhibit reduced survival after diagnosis of luminal breast cancer but increased survival after diagnosis of ovarian cancer (Rutkowski et al., 2015), indicating that the link between TLR5 signaling and malignancy is highly influenced by the cancer type. This dependency has been attributed to differences in the IL-6 responsiveness (knowing that ovarian cancers respond to IL-6-mediated immunosuppression, while breast carcinomas do not) because the commensal microbiota increases systemic levels of IL-6 via TLR5 signaling. Thus, in ovarian cancer, inhibition of TLR5 signaling reduces the IL-6-dependent recruitment of myeloid-derived suppressor cells into the tumor and improves survival. In contrast, in breast cancer, inhibition of TLR5 enhances the local production of IL-17, which depends on the commensal microbiota, thus reducing survival (Rutkowski et al., 2015).

Altogether, the aforementioned results suggest that common genetic polymorphisms in PRRs, which are present at a high frequency within the general population (Casanova et al., 2011) may influence tumor progression through altered sensing of the microbiota. Nonetheless, there is a caveat to this interpretation. Indeed, most if not all PRRs can also detect endogenous (host-derived) DAMPs, and the impact of TLR3 and TLR4 polymorphisms on breast cancer outcome in the context of adjuvant chemotherapy has been attributed to interactions with DAMPs rather than microbial products (Apetoh et al., 2007; Sistigu et al., 2014; Vacchelli et al., 2015). Hence, loss-of-function mutations in PRRs may impact cancer immunosurveillance not only by affecting the response to microbial PAMPs, but also by operating in response to endogenous DAMPs. That said, the endogenous DAMP for TLR5 has not (yet) been identified, suggesting a major, if not exclusive, role of the microbiome in modulating its activity.

The microbiota can influence the generation of particular T cell subtypes, hence determining the immune tonus at the level of the equilibrium between different cytokine production patterns. We refer to the reader to the following reviews for more information on aspect of the microbiome in cancer immunosurveillance (Gagliani et al., 2014; Perez-Chanona and Trinchieri 2016).

Altogether, there is ever-accumulating evidence that the microbiota shifts systemic immunity to different response types that may well be relevant to anticancer immunosurveillance or its suppression. However, at present, it remains to be determined which PRRs (including TLRs and other receptor types) determine the link between eubiotic versus dysbiotic states and the immune tonus.

Immunological Effects of Microbial Toxins and Metabolites

Bacteria may produce toxins or metabolites that affect cancer cells in a direct fashion or indirectly, via effects on their immunosurveillance. A number of microbial toxins participate in oncogenesis, as this has been demonstrated for cytotoxin-associated gene-A (CagA) and vacuolating cytotoxin A (VacA) produced by *Helicobacter pylori* in the context of gastric cancer, as well as for

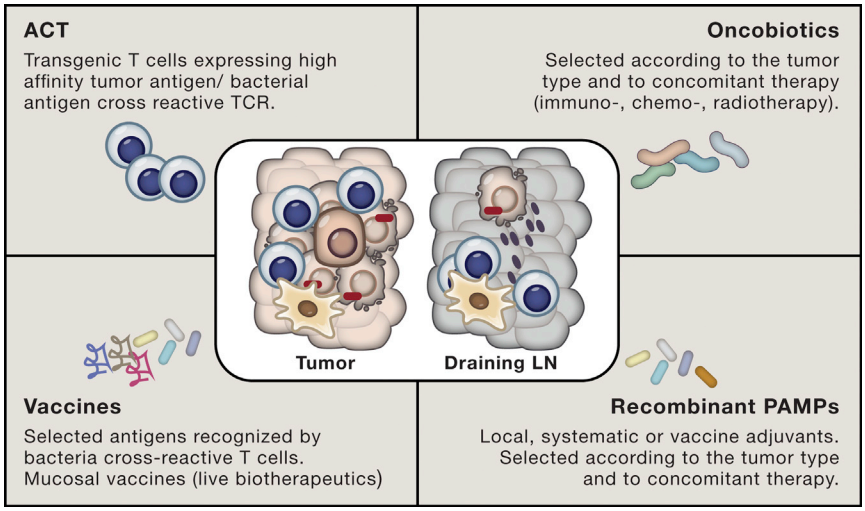

Figure 4. Implementation of Microbiome-Guided Anti-Cancer Therapeutic Strategies

Based on mouse tumor models and patient cohort studies, different commensal species or their derivatives (antigens and pathogen-associated molecular patterns, PAMPs) can guide the development of anti-cancer therapeutic strategies that rely on their antigenicity (signal 1) and/or adjuvanticity (signal 2). These include adoptive T cell transfer (ACT), vaccination, administration of PAMPs, and live biotherapeutics (probiotics, oncobiotics) and can be used as stand-alone interventions or combined with chemo-, radio-, or immune-therapy.

multiple bacterial protein toxins associated with colon carcinogenesis (Rosadi et al., 2016). The oncogenic effect of such protein toxins is likely direct and cell autonomous.

In terms of longer-range effects of the microbiome, bacterial metabolites influence the dialog between the immune system and cancer cells. As an example, deoxycholic acid (DCA) is secondary bile acid produced solely by the 7α-dehydroxylation of primary bile acids carried out by anaerobic gut bacteria from the genus *Clostridium*. DCA can be considered as a microbial co-carcinogen that not only contributes to colon carcinogenesis, but that also participates to the development of liver cancer, presumably by inducing the senescence-associated secretory phenotype of hepatic stellate cells, thereby stimulating pro-inflammatory and tumor-promoting reactions in a mouse model of obesity-associated hepatocellular carcinoma (Yoshimoto et al., 2013). DCA may well cooperate with other bacterial products, including LPS, in promoting hepatocellular carcinoma (Dapito et al., 2012).

SCFAs are generated by microbial fermentation of dietary polysaccharides in the gut, in particular certain *Clostridia* species (and especially those falling within clusters IV, XIVa, and XVIII). SCFAs constitute an important energy source for colonocytes and also function as signaling molecules, modulating intestinal inflammation, and metabolism. SCFAs, in particular acetate, propionate, and butyrate, favor histone H3K27 acetylation and increased expression of the Treg-specific transcription factor gene, Foxp3, thereby boosting Treg functions (Furusawa et al., 2013). Through this mechanism, certain bacterial species may promote immunologic tolerance and protect from inflammatory and allergic diseases (Honda and Littman, 2012). Although one might intuitively speculate that Treg-stimulating microbiota should favor oncogenesis by immunosuppression, several studies suggest that saccharolytic fermentation and butyrogenesis actually have a negative impact on colon carcinogenesis (O'Keefe et al., 2015). The reasons for this apparent discrepancy have not yet been elucidated.

It should be noted that SCFAs are probably not the only bacterial metabolites that may affect systemic immune responses. Evidence also exists for a role of nicotinic acid (Singh et al., 2014) and proprionate (Bindels et al., 2012). Other bacterial metabolites such as homoserine lactone, *N*-acetylmuramic acid and *N*-acetylglucosamine are known to be overtly immunosuppressive (Rojo et al., 2015). However, their possible role in oncogenesis and tumor progression has not yet been investigated.

Outlook

As summarized in this perspective, there is accumulating yet fragmentary evidence that the microbiome can affect anticancer immunosurveillance in multiple ways. Although the known associations most probably only constitute the "tip of the iceberg" of a pathophysiologically important and complex network of interactions, it is tempting to advance the following general speculations: (1) there may be cross-reactivity of tumor-, pathogen-, or self-specific T cells with microbiota-derived sequences that contribute to the immune response in the corresponding pathological conditions, (2) the "Westernized" lifestyle (a combination of hypercaloric and high-fat diet coupled to the reduction of healthy food items, excessive hygiene, and sedentary lifestyle) can affect the microbiome, which in turn modulates the immune-inflammatory system, and alterations in the microflora may mediate, at least in part, the increased cancer risk of this lifestyle, (3) Targeted interventions on the microbiome by pre- or pro-biotics might be used for cancer prevention, either in particularly high-risk populations or at a massive scale, and (4) Specific manipulations of the microbiome might be introduced into the clinic as an adjuvant regimen to increase the efficacy (and ideally to reduce the side effects) of existing cancer treatments including chemotherapy, radiotherapy, and immunotherapy (Figure 4).

The current challenge is to dissect the mechanistic bases of these microbially mediated positive effects and to translate them into clinical reality.

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