Symbiosis and cohabitation/Symbiose et cohabitation

The impact of the intestinal microbiota in therapeutic responses against cancer[☆],[☆☆]

Impact du microbiote intestinal dans les réponses aux thérapies contre le cancer

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A B S T R A C T

Accumulating evidence points to the impact of the gut microbiota in regulating various chronic inflammatory disorders such as cancers. The intestinal microbiome is not only influencing the spontaneous course of colon malignancies but also acts at distant sterile sites of neoplasia, mostly playing a detrimental role. By providing microbial-associated molecular patterns and potentially antigens sharing molecular mimicry with tumor antigens, our commensals modulate the local and the systemic immune tonus, eventually influencing tumor microenvironment. Complicating this algorithm, therapeutic interventions alter the delicate balance between the epithelium, the microbial community, and the intestinal immunity, governing the final clinical outcome. This seminar focused on the impact of the intestinal composition on the immunomodulatory and therapeutic activities of distinct compounds (alkylating agents, platinum salts and immunotherapies) used in oncology. This research opens up "the era of anticancer probiotics" aimed at restoring gut eubiosis for a better clinical outcome in cancer patients.

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R É S U M É

Récemment, l'impact du microbiote intestinal dans diverses pathologies inflammatoires chroniques, dont le cancer, a été mis en évidence. Le microbiome intestinal régule l'évolution spontanée des tumeurs malignes du côlon et aussi la carcinogenèse extra-intestinale, jouant principalement un rôle délétère. En exprimant des motifs moléculaires associés aux microbes et, potentiellement, des antigènes partageant un mimétisme moléculaire avec des antigènes tumoraux, nos commensaux modulent le tonus immunitaire local et systémique, et peuvent influencer le microenvironnement tumoral. Compliquant cette interaction, les traitements contre le cancer altèrent l'équilibre entre épithélium, microbiote et immunité intestinale, dictant ainsi la réponse clinique. Ce

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séminaire se concentre sur l'impact de la composition du microbiote intestinal sur les propriétés thérapeutiques et immunomodulatrices de différents agents (agents alkylants, sels de platine et immunothérapies) utilisés en oncologie. Ce champ de recherche ouvre les portes vers « l'ère des probiotiques anti-cancer » visant à restaurer une eubiose intestinale, de manière à améliorer la réponse clinique des patients atteints de cancer.

1. Introduction

Our scientific community gained insights on the importance of the intestinal resident microflora for the host's health and pathophysiology over the past decade [1–3]. Overwhelming our mammalian genome and spread over a 200-m^2 surface, the gut microbiome exerts a variety of different fundamental functions. Our commensal community participates in the degradation of nutrients, the elimination of xenobiotics, the growth and the differentiation of epithelial cells of the gut barrier, the local peristaltism, the colonization resistance and the eradication of pathogens as well as, last but not least, the maturation of our immune system [4]. Indeed, the intestine represents the largest compartment of the immune system. It ensures tolerance of food and commensal antigens at portals of entry. Bacteria belonging to *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* phyla are the most prevalent together with members of the *Archaea* kingdom [4]. Recent advances in sequencing technologies as well as culturomics have allowed the deep characterization of the human microbiota, thus greatly improving our knowledge on the role of the microbiome in human health and disease. Human microbiome project consortium studies (Meta-HIT and HMP) [5,6] demonstrated that healthy individuals have not only a high degree of bacterial diversity, dependent on their habitat (intestine, oral cavity, skin or vagina), but that there is also a remarkable inter-individual variability at the level of the species. However, there is a certain constancy that preserves both the function and the bacterial gene profiling associated with specific tissue sites to exert specific functions.

As the field is making considerable progress in defining a "healthy" gut composition of our symbiotic microflora, attempts to describe abnormal compositions of this prokaryotic community in various immunopathologies highlighted associations or causative roles of "intestinal dysbiosis" (deviated repertoire from the normal gut microbiome) accumulated in a variety of chronic inflammatory disorders, including cancers [7].

2. Antitumor immunity and the cancer-immune set point

We are facing a paradigm shift in oncology in that targeting tumor cells is considered necessary, but not sufficient to cure cancer. We need to mobilize the immune system and elicit long-term memory T cell responses protective against the residual disease [8]. To achieve this goal, we expect "the cancer-immunity cycle" to be fully operational. Dendritic cells are specialized antigen-presenting cells patrolling and sampling the surrounding tissues that can take up and process proteins (called antigens) from cancer cells, migrate to tumor draining lymph nodes where they educate CD8$^+$ and CD4$^+$ specific T cells. Those T cells differentiate into effector and memory T lymphocytes. Then, they migrate to tumor lesions and play their scavenging role by killing tumor cells or reprogramming the tumor microenvironment. However, this ideal scenario is jeopardized by various mechanisms evoked by tumor cells to escape cancer immunosurveillance. In fact, more than 70% of human malignancies are devoid of T cell infiltration, because T cells are simply not educated, excluded from tumor beds, or embedded into an inflammatory and immunosuppressive milieu, which inhibits their functions. We learned from clinical trials using immune-targeted monoclonal antibodies (mAbs) that clinical success is associated with tumor invasion by Interferon-γ (IFNγ)-producing CD8$^+$ T cells (CTLs). However, mounting anticancer CTLs responses capable of infiltrating tumor deposits depend on a variety of crucial factors.

The immunity of a person is influenced by a complex set of parameters, including tumor, host, and environmental factors that govern the threshold, the strength, the duration and timing of anti-cancer immune responses, called the "cancer-immune set point" [9,10]. Our team participated in showing that the microbiome contributes to the cancer-immune set point, the very threshold beyond which an immune response is ensured and controls the success of immune-targeted antibodies and immunomodulatory chemotherapies [11]. Antibodies targeting "immune checkpoint inhibitors", such as cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) or Programmed cell Death-1 (PD-1) molecules, are now considered as the backbone of anti-cancer treatment modalities, as indicated by the burgeoning of FDA approvals in many histological indications.

3. Microbiome and cancer incidence

Commensal microbial communities inhabiting the intestine appear to play an unappreciated role in intestinal and extra-intestinal carcinogenesis [12]. Pioneering studies performed in germ-free, gnotobiotic, or antibiotic-treated rodents have revealed an unsuspected role for commensals in tumorigenesis. In the genesis of colon cancer or hepatocarcinoma, microbes can mediate direct transforming activities [13,14] by providing toxic metab-

olites, oncogenic products or by inducing inflammation [15–17]. Recently, the development of breast and ovarian neoplasias was linked to receptors detecting "danger signals" (called Toll-Like-Receptor [TLR]) like those expressed by microorganisms. Intestinal microbes can indeed provoke IL-6 or IL-17-driven systemic inflammation through an interaction between host TLR5 and bacterial flagellin, a TLR5 ligand. Likewise, other observations support a beneficial role for bacteria in combatting cancer. Prolonged antibiotic treatment with a combination of metronidazole and ciprofloxacine subsequently tripled breast cancer (BC) incidence in proto-oncogene HER2/neu driven-transgenic mice [18]. In humans, epidemiological studies suggest a dose-dependent association between antibiotic use and risk of BC [19–22].

4. Chemotherapy efficacy is impacted by the gut microbiota

The beneficial role of the intestinal microbiota was firstly reported by Paulos et al., who showed that total body irradiation facilitates the efficacy of adoptive T cell transfer [23]. Indeed, irradiation promoted the translocation of Gram negative commensals and the release of lipopolysaccharide, thereby inducing a TLR4-dependent activation of antigen-presenting cells. Following this initial observation, other groups reported the beneficial role of commensals in influencing the efficacy of cytotoxicants in tumor bearers. Hence, cyclophosphamide and platinum salts lost their capacity to reduce tumor growth in mice that have been raised in germ-free conditions or have been sterilized with a combination of broad-spectrum antibiotics. In germ-free or antibiotics-treated animals, innate and adaptive immune responses (including tumor–specific CTL responses) were compromised compared with littermates reared in specific pathogen-free (so called "normal") conditions [24,25].

Cyclophosphamide (CTX) is a DNA-alkylating agent known for its immuno-modulatory and anti-angiogenesis properties [26]. Cyclophosphamide tumoricidal activity depended upon its ability to induce the translocation of selective Gram positive bacteria niching in the small intestine, such as *Enterococcus hirae* or *Lactobacillus johnsonii* into secondary lymphoid organs [24]. Cyclophosphamide disrupts the gut barrier integrity perturbing the intestinal homeostasis (both epithelial and immune compartments), leading to host immunization against some bacterial strains. Cyclophosphamide induced the activation of CD4$^+$ effector lymphocytes called "pathogenic Th17" (pTh17) coproducing IFNγ and IL-17, and helping tumor-infiltrating T cells to control tumor growth. Interestingly, broad spectrum antibiotics as well as vancomycin (which kills Gram positive bacteria) and colistin (which eliminates Gram negative bacteria), all compromised the polarization of pTh17 in the spleen and the full-blown anticancer activity of CTX *in vivo* in mastocytoma- and sarcoma-bearing mice, supporting the notion that the efficacy of CTX was microbiota-dependent. Among the translocating Gram positive bacteria, *E. hirae* induced the most potent IFNγ and IL-17 CD4$^+$ T cell responses and stimulated cognate tumor-specific CD8$^+$

T cells [27]. In addition, *E. hirae* reduced immunosuppressive intratumoral T regulators and IL-17-producing $\gamma\delta$ T cells. Mono-association of antibiotics-treated mice with *E. hirae* greatly improved tumor growth reduction by CTX, and this effect was blocked by the depletion of CD8$^+$ T cells or the neutralization of IFNγ.

Moreover, we identified the cytoplasmic sensor nucleotide-binding oligomerization domain 2 (NOD2) as a gut immune checkpoint regulating the efficacy of CTX. Indeed, the tumoricidal activity of CTX was greatly ameliorated in mice presenting a genetic defect in the intestinal NOD2 expression. The characterization of the gut microbiota in NOD2-deficient animals highlighted that a Gram-negative bacterium, *Barnesiella intestinihominis* preferentially found in the proximal colon, was overrepresented after chemotherapy with CTX. A cause–effect relationship between the abundance of *B. intestinihominis* in the colon and the superior anticancer efficacy of CTX in NOD2-deficient mice was subsequently brought up. Mice that have been mono-associated with *B. intestinihominis* exhibited more abundant polyfunctional Th1 CD4$^+$, CD8$^+$ and $\gamma\delta$ T cells in the spleen that could also be found in tumor beds. Moreover, when CTX was combined with *B. intestinihominis* in mice with antibiotics-induced dysbiosis, conditions that normally blunt the tumoridical activity of CTX, the restoration of CTX tumoricidal activity was observed against a variety of transplantable cancers. The adjuvant effects of *B. intestinihominis* depended upon CD8$^+$ T cells-, IFNγ-, but not IL-17. Finally, the clinical relevance of these findings point out the adjuvanticity of distinct intestinal commensals during chemotherapy that was shown in non-small cell lung and ovarian cancer patients resistant to platinum salts, and treated with metronomic CTX. In patients who developed *E. hirae* and *B. intestinihominis* specific-memory IFNγ responses, we observed a longer survival in two independent cohorts.

The demonstration of the importance of the gut microbiota in the efficacy of chemotherapies was corroborated by Trinchieri's group [25]. Iida et al. reported that gut bacteria accounted for the release of reactive oxygen species (ROS) by tumor-infiltrating hematopoietic cells during platinum-based anticancer therapies. The expression of an enzyme responsible for ROS production and induced by chemotherapy was attenuated by the antibiotics. In antibiotics-treated mice, the reduced myeloid-cell production participated in the reduced effect of oxaliplatin tumoricidal activity against lymphoma or colon carcinoma (compared to SPF mice). Altogether, these studies support the notion that the gut microbiota is affecting the therapeutic effects of various cytotoxicants currently used in the oncological armamentarium.

5. Gut bacteria and efficacy of anticancer-immunotherapies

Immunotherapy with anti-interleukin-10 (IL-10) plus cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN) is very effective in controlling the subcutaneous growth of colon cancers in mice. Previous work indicated that Tumor Necrosis Factor α (TNFα) is indispensable for the necrotizing effects of immunothera-

py in this model. Interestingly, Iida et al. showed that antibiotics blunted TNFα release and the efficacy of anti-IL-10/CpG [25]. Importantly, *Alistipes shahii* was found overrepresented in the colons of cancer-bearing mice responding to therapy and correlated with local TNFα release. The cause–effect relationship between gut microbes and TNFα secretion in the tumor microenvironment was evidenced using the mono-association of germ-free mice with *A. shahii*. The immunotherapeutic response of colon cancers to anti-IL-10/CpG was improved in tumor bearers that received an oral gavage with *A. shahii*, as compared to antibiotics-treated mice. Here, *A. shahii* led to an increase in TNFα production by intratumoral myeloid cells, and the neutralization of TNFα abolished its therapeutic effect. Thus, *A. shahii* impacted myeloid immune effector cell functions to improve the outcome of immunotherapy.

In parallel, other studies showed the involvement of the gut commensals in boosting the efficacy of other immunotherapy approaches (such as those utilizing immune checkpoint blockers). Ipilimumab, a mAb neutralizing CTLA-4, and inducing increase overall survival in metastatic melanoma [28,29], was shown to also rely on the intestinal microbiota to reinvigorate intratumoral T cells. The antitumoral efficacy of anti-CTLA4 mAbs depended, at least in part, on the non-enterotoxin-producing strains of *Bacteroides fragilis* [30]. After blockade of the CTLA-4 immune checkpoint, *B. fragilis* was overrepresented in the ileum, followed by a splenic, Th1 cell memory response against *B. fragilis* polysaccharide A. Anti-CTLA-4 mAbs reduced the growth of subcutaneous sarcomas and colon cancers in mice kept on normal chow, yet failed to do so in germ-free mice or mice treated with broad-spectrum antibiotics. This defect was overcome by mono-association with *B. fragilis* as well as by adoptive transfer of *B. fragilis*-specific CD4+ T cells. *B. fragilis* stimulated the production of IL-12 by bone-marrow-derived dendritic cells *in vitro*. Moreover, neutralization of IL-12 prevented the anticancer effects of *B. fragilis* in the context of CTLA-4 blockade *in vivo*. Interestingly, dendritic cells from tumors of *B. fragilis*-mono-colonized mice exhibited a more mature phenotype than controls with respect to the expression of MHC class II and the co-stimulatory CD80 and CD86 molecules. The clinical relevance of these findings was brought up by analyzing the gut microbiota of metastatic melanoma patients before and after ipilimumab. The non-supervised hierarchical clustering of patients stools based on the 16S rRNA sequencing of gene amplicons identified three distinct clusters of gut microbiome composition, based on abundance of *Bacteroides*, and *Prevotella* genera. By performing fecal microbial transfer (FMT) of patients' feces falling into each stool cluster into tumor-bearing germ-free mice subsequently treated with the mouse anti-CTLA-4 mAb, we demonstrated that the microbial composition of the cluster enriched in immunogenic *Bacteroides* species was able to promote *B. fragilis* colonization and amplification over the courses of mAb and to restore the anti-CTLA-4 efficacy in these germ-free animals that were resistant to therapy.

Moreover, the antitumoral efficacy of anti-PD1 or its ligand PD-L1 mAbs was shown to be driven by *Bifidobac-terium longum* and *B. breve* species in mice [31]. Sivan and colleagues compared the antitumor CTLs responses in genetically similar C57BL/6 tumor bearers purchased from two different vendors and differing at the level of the microbiota composition. *Bifidobacteriales* were indeed found to be particularly abundant in the colon of mice that exhibited reduced growth of transplantable melanomas and improved CTL-mediated immunosurveillance. The selective transfer of *B. breve* or *B. longum* into mice that usually are devoid of these strains was sufficient to reduce melanoma natural growth and restored anti-melanoma specific T cell responses. *B. breve* and *B. longum* stimulated the maturation of dendritic cells both *in vitro* and *in vivo*. As a consequence, the frequency of tumor-specific CTLs residing in melanoma lesions increased in mice carrying *B. breve* or *B. longum* and such T cell-infiltrated tumors responded more vigorously to anti-PD-L1 mAbs than did melanomas evolving in sterile mice or mice bearing a gut microbiome devoid of immunostimulatory *Bifidobacteriales*.

Bringing up the final proof-of-concept of the impact of the gut microbiota in the clinical benefit to PD-1 blockade in patients, three reports recently highlighted the immunostimulatory role of a highly diverse microbiome in advanced cancer patients receiving a second- or third-line FDA/EMA approved therapy with nivolumab or pembrolizumab [32–34]. The first study conducted in lung and renal carcinoma patients unveiled that antibiotic-induced dysbiosis compromised the anti-PD1 mAb-mediated efficacy [34]. Exploring the gut microbiota composition, the authors unraveled the overrepresentation of the commensal Akkermansia muciniphila at diagnosis in stools of patients proned to develop a favorable clinical outcome. In parallel, a memory Th1/CTL cell response against *A. muciniphila* monitored in blood by the recall response to the commensal and specific IFNγ release by CD4+ and CD8+ T cells was associated with a better clinical outcome during anti-PD1 therapy. To establish a cause–effect relationship, the authors followed the following procedure. Sterile mice were colonized with fecal microbiota collected at diagnosis from advanced patients, prior to tumor inoculation and therapy with anti-PD-1 mAbs. Unlike stools from responder patients, those from non-responding patients compromised the tumoricidal activity of anti-PD-1 mAbs. Finally, adding *A. muciniphila* (alone or with *Enterococcus hirae*) by oral gavage permitted to restore sensitivity to anti-PD1 mAbs in mice colonized with non-responding patients' derived feces. Likewise, Gopalakrishnan et al. conducted a similar study in metastatic melanoma patients and showed that patients who responded to the immunotherapy had a "favorable" gut microbiome (defined by high diversity and abundance of *Clostridiales/Ruminococcaceae*) associated with an enhanced systemic T cell immune response, accompanied by a high density of immune cells bearing hallmarks of antigen processing and presentation in the tumor microenvironment [32]. In contrast, patients who failed to respond to the immunotherapy presented an "unfavorable" gut microbiome (defined by low diversity and high abundance of *Bacteroidales*) associated with a suppressive and regulatory immune tonus in the periphery and

infiltrate in tumor deposits. Finally, a third group of investigators corroborated these findings in melanoma patients and recipient mice colonized with human stools [33]. In the third report, a scoring of "dysbiosis" in the stool predicted resistance of melanoma patients to PD-1 blockade, encompassing *Bifidobacteria, Coriobacteria*, and *Enterococci*. In the four patients who harbored *A. muciniphila* and 100% responded (while the expected response rate was 40% in the overall cohort).

Altogether, these studies allowed us to conclude that the gut microbiome markedly influenced the outcome of cancer treatments in mice and patients. By shaping the immune tone of the metaorganism and transforming non-inflamed immune cell-excluding tumors into immunostimulated microenvironments, the intestinal microbiome constitutes a novel regulator influencing the "cancer-immune set point".

6. Conclusive remarks

The field of anticancer probiotics and manoeuvers aimed at restoring a balanced gut ecosystem is born in oncology. The intestinal microbiome appears to dictate part if not the whole peripheral immune tone, and controls "the cancer immune set point" in debilitated cancer patients (affected by antibiotics, co-medications, co-morbidities), favoring, in best cases, the elicitation of an efficient tumor immunesurveillance culminating in intra-tumoral dendritic cell activation and proliferating anti-cancer-lymphocytes. We surmise that novel diagnosis tools aimed at evaluating patients' gut dysbiosis (and based upon metagenomics, culturomics, PCR, mass spectrometry or metabolomics) will be developed by companies and will pave the way to patients' stratification to allow the large-scale prescription of anticancer probiotics endowed with immuno-stimulatory functions.

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Glossary

Ag: MHC class I or II-associated tumor antigens are 9–10mer or 15–20mer-small peptides capable of inducing CD8+ or CD4+ T cell immune responses respectively.

CTLs: Cytotoxic T cells are CD8$^+$ T cells capable of recognizing and killing transformed cells (such as cancer cells) specifically, in an MHC class I-restricted manner.

"Danger": "Danger" signals are released after tissue injury (from self-tissues, called "damage-associated molecular patterns") or after exposure to microorganisms/pathogens (from infected tissues, called "pathogen-associated molecular patterns") or commensals (called "microbe-associated molecular patterns"). These patterns are recognized by receptors (pattern recognition receptors, such as TLR or nucleotide-binding oligomerization domain (NOD)) expressed by innate immune cells such as macrophages, dendritic cells, polymorphonuclear neutrophils, and NK cells. These interactions initiate innate immune responses.

Dendritic cells: Dendritic cells are innate myeloid immune cells, derived from bone marrow or the embryonic yalk salk, specialized in the priming of adaptive immune responses. They patrol in tissues, sample, take up and process antigens (DNA, RNA, proteins from cells they engulf), then mature (up regulation of MHC, costimulatory and adhesion molecules) and migrate to lymph nodes and educate CD4$^+$ and CD8$^+$ MHC restricted antigen-specific T cells.

Dysbiosis: Dysbiosis corresponds to an imbalance of the qualitative and quantitative composition of the microbiota.

FMT: Fecal microbial transfer consists in oral (or rectal) gavage of recipient mice with "autologous or allogeneic" microbiota from a donor.

IFN-γ: Interferon-γ is a cytokine produced by activated Th1/CTL cells and coordinating several cellular programs such as facilitating pathogen killing and microbicidal functions, antigen processing and presentation, blocking cell proliferation, and gearing up Th1/CTL immune responses.

Immunotherapies: Immunotherapies are treatments aimed at boosting the functions of the immune system, dampening regulatory T cells and stimulating effector cells in cancer indications, including monoclonal antibodies (mAbs) (immune checkpoint blockade, ADCC-mediating ones targeting tumor antigen and antigen presenting cells), cells (like dendritic cells or CAR-T cells), therapeutic vaccines, harnessing "danger signals" (adjuvants to vaccines or dendritic cells), cytokines (T cell growth factors, dendritic cell homeostasis, direct tumoricidal activity)...

Immune checkpoint: Immune checkpoint inhibitors are molecules expressed by tumor cells and/or immune cells engaging immunosuppressive immune receptors in order to restrain cognate T cell-based immune responses. These regulatory proteins shut down effective immune responses.

ICB: Immune checkpoints blockers are monoclonal antibodies (mAbs) that inhibit the interaction between immune checkpoint inhibitors (ligands) and their specific receptors (inhibitory receptors). Repressed immune responses are unleashed. The main ICBs include anti-CTLA4 and anti-PD1 mAbs that prevent binding of CTLA-4 or PD-1 to CD86 or PD-L1/PD-L2, respectively.

Phylum: Phylum is a phylogenic rank in the taxonomy of bacteria and commensals. It contains bacteria from different classes, orders, genus, and species.

Regulatory T cells or Tregs: Regulatory T cells or Tregs are immunoregulatory T cells. They inhibit effector T cell responses, preventing excessive proliferation, secretion of effector cytokines, cytotoxicity, and positive feedback loops in immune responses.

TLR: Toll-Like-Receptors are receptors expressed by innate cells that sense "danger" signals (DAMPs, MAMPs, and see above) to trigger immune responses through cell signaling culminating in the activation of NF-κB or IRF pathways.