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Harnessing the Intestinal Microbiome for Optimal Therapeutic Immunomodulation

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Abstract

Distinct cytotoxic agents currently used in the oncological armamentarium mediate their clinical benefit by influencing, directly or indirectly, the immune system in such a way that innate and adaptive immunity contributes to the tumoricidal activity. Now, we bring up evidence that both arms of anticancer immunity can be triggered through the intervention of the intestinal microbiota. Alkylating agents, such as cyclophosphamide, set up the stage for enhanced permeability of the small intestine, facilitating the translocation of selected arrays of Gram-positive bacteria against which the host mounts effector pTh17 cells and memory Th1 responses. In addition, gut commensals, through lipopolysaccharide and other bacterial components, switch the tumor microenvironment, in particular the redox equilibrium and the TNF production of intratumoral myeloid cells during therapies with platinum salts or intratumoral TLR9 agonists combined with systemic anti-IL10R Ab respectively. Consequently, antibiotics can compromise the efficacy of certain chemotherapeutic or immunomodulatory regimens.

Introduction

The microbiome represented by tens of trillions of microbes residing in our gut and their genomes has coevolved with the host for an optimal mutualism in performing crucial

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functions (1). Intestinal colonization by commensal microorganisms regulates host metabolism of glycans and fat (2), promotes immune development, and controls pathogens (3) as well as contributes to the development of the enteric nervous system in the mid-distal small intestine (4). Specifically, distinct human bacterial strains capable of modulating adiposity, intestinal metabolite composition, or expansion of regulatory T cells (Tregs) have been identified (5). Moreover, characterization of the enterotypes or hierarchical configuration of the human gut bacterial communities revealed differences between healthy and diseased individuals (6–8). Importantly, fecal microbial transplantation established causal relationships between dysbiosis and colitis or metabolic disorders (9, 10). Surprisingly, not only long-term dietary intake but also rapid shifts in consumption of diets readily alter the human gut microbiome (11).

Given the versatile nature and plasticity of the composition of the intestinal bacterial and fungal content, it is conceivable that any therapeutic compound compromising the integrity of the intestinal barrier and/or mucosal immunity will compromise the symbiosis of this compartment and cause symptoms as well as distant immune dysfunctions.

Since many decades, it is well established that chemotherapeutics and radiotherapy damage the intestinal epithelium, causing various degrees of mucositis and debilitating side effects (vomiting and diarrhea). By exerting their proapoptotic activity against rapidly proliferating cells of the intestinal barrier, they not only affect the numbers and functions of CD45-negative stem/progenitor cells, goblet cells, Paneth cells, enteroendocrine cells, crypt fission, and crypts (12) but also generate gut microbial dysbiosis (13, 14) and bacterial trans-location and/or systemic exposure to bacterial products (15). Hence, the involvement of microbiota in the gastrointestinal toxicity of irinotecan (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin) used to treat colorectal and other cancers has been largely reported (16), prompting the use of prophylactic antibiotics to ameliorate tolerability. An important example of the impact of bacteria in regulating immunopathologies has been brought up by hematologists during allogeneic bone marrow transplantation. Histocompatibility complex differences between donor and recipient cause GVHD, which is somewhat reduced by prophylactic antibiotic decontamination of the intestinal microbiota and transplantation in germ-free conditions. Recently, investigators demonstrated that manipulation of the gut microbial diversity (by reintroduction of Lactobacillales, and *Lactobacillus johnsonii* in particular) reduces intestinal inflammation generated by the conditioning and may improve clinical outcome in mice and human after allo bone marrow transplantation (17).

Main Results

Besides the potentially deleterious role of a dysbiotic microbiota in driving inflammation and cancer, distinct commensal bacterial communities may have a protective effect in reinstating anticancer immunosurveillance. We and others have recently reported that some chemotherapeutics [such as cyclophosphamide (CTX) and platinum salts] can modulate the tumor microenvironment, triggering innate and cognate anticancer immune responses through the intervention of gut microbiota (Fig. 1).

CTX (2-[Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide) is an alkylating agent belonging to the nitrogen mustard family. It is a prodrug that requires metabolic activation through the hepatic cytochrome P-450 to generate 4-hydroxycyclophosphamide and aldophosphamide, the latest being further decomposed into phosphor-amide mustard (the active compound) and acrolein. CTX is being used as an anticancer agent since 1958. It is widely used at high dosing in regimens of conditioning before bone marrow transplantation and mobilization of progenitors. At low dosing, CTX is used for its immunostimulating and antiangiogenic properties.

What are the consequences of "sterilizing" the intestinal environment on the anticancer efficacy of chemotherapeutics?

The primary observation was that the antitumor effects mediated by CTX in specific pathogen–free (SPF) mice were significantly decreased in germ-free animals or in mice treated with broad-spectrum antibiotics (ATB; ref. 18). Several ATB regimens were compared for their ability to compromise the antitumor activity of CTX against transplantable sarcomas and mastocytomas. Vancomycin (ATB exclusively killing Gram-positive bacteria) severely blunted the CTX-induced control of MCA205, whereas colistin (ATB targeting Gram-negative bacteria) had a partial inhibitory effect (18). Similarly, an imipenem-based ATBx regimen altered the tumoricidal activity of oxaliplatin or cisplatin against the EL4 lymphoma (19), mimicking the lack of efficacy of these cytotoxic drugs in gnotobiotic mice inoculated with EL4. We corroborated these results in a spontaneous lung carcinoma model mimicking human tumorigenesis (20). KP (*KrasLSL-G12D/WT; p53*Flox/Flox) mice received a prophylactic CTX-based chemotherapy on a weekly basis in the presence of vancomycin starting on day 77 after inoculation of adenoviruses expressing Cre recombinase. In this preclinical model, we recapitulated that the eradication of Gram-positive bacteria compromised the efficacy of CTX-based chemotherapy (18).

What inhibitory effects do ATB mediate on CTX-induced systemic immunity?

We and others reported that metronomic dosing of CTX promotes immunostimulatory effects participating in the antitumor activity of this alkylating agent. An extensive characterization of the effects of CTX on the immune system by DNA microarray analyses highlighted that CTX upregulates the transcription of several genes encoding chemokines, factors involved in inflammatory, and innate immune responses (21) in primary and secondary lymphoid organs. Hence, CTX resets dendritic cells (DC) homeostasis, activates both cellular and humoral immunity (22), induces Th1 and Th17 polarization in secondary lymphoid organs (23) while selectively reducing regulatory T-cell numbers and functions (24, 25). All these effects culminate in blunting neoangiogenesis and enhancing tumor infiltration by lymphocytes and DC (26).

Sterilization of gut environment (or ecosystem) by prolonged, broad-spectrum ATB therapy or skewing gut microbiota with 3-week vancomycin therapy severely impinged on the CTX-induced, T-cell receptor (TCR)–dependent Th1 and Th17 polarization of splenocytes. Flow cytometry analyses of T-cell subsets contained in CTX-treated spleens revealed that ATB significantly reduced the induction of T-bet$^+$RORγt$^+$ CD4$^+$ T lymphocytes, also defined as "pathogenic Th17 cells"(27), comprising IFNγ^+IL17$^+$CD4$^+$ T cells as well as CXCR3$^+$

CCR6^{+} CD4^{+} T cells (18). In germ-free mice, the CTX-mediated induction of Th17 splenocytes was not significant either, consistent with a role of gut microbiota in the differentiation of pTh17/Th17 lymphocytes. In accordance with a role of commensal microbes in the immunomodulatory effects of CTX, the proportions of pTh17 recovered in Myd88-deficient mice treated with CTX were dramatically reduced compared with that observed in wild-type animals.

What are the immunosuppressive effects induced by ATB on the tumor microenvironment?

Metronomic CTX induces T-cell–dependent antitumor effects in many transplantable tumor models, including MCA205 sarcoma and P815 mastocytoma (28). CTX modulates the contexture of the tumor microenvironment by inducing the recruitment and accumulation of CD3^{+} T cells, most specifically of Th1 cells by day 8 after therapy in mice(18) and Th17 in ascitis fluids of a patient with ovarian cancer (23).

In sharp contrast with tumors implanted in SPF mice, experimental neoplasia expanding in germ-free mice or animals treated with vancomycin failed to recruit TCRαβ CD4^{+} T lymphocytes secreting IFNγ as well as TCRγδ lymphocytes releasing IL17 into tumor beds (unpublished data; ref. 18). In the autochthonous lung cancer model mimicking human tumorigenesis, the efficacy of CTX-based chemotherapy correlated with a raise in the intratumoral CD8^{+} T effector/Foxp3^{+} regulatory T-cell ratio, which was no longer observed during concomitant eradication of Gram-positive bacteria by vancomycin (unpublished data). Not only the adaptive arm of intratumoral immunity was influenced by ATB but also innate components of local immunity fluctuated in two independent therapeutic settings.

First, Iida and colleagues analyzed the biologic effects of a broad-spectrum ATBx regimen (imipenem, vancomycin, and neomycin) on the gene expression profile in two transplantable tumor models. ATBx downregulated several gene products involved in inflammation, phagocytosis, and antigen presentation while upregulating those promoting cancer metabolism and development. ATBx decreased the recruitment of monocyte derived–MHC class II[+]Ly6C^{+} and Ly6G^{high} cells into tumors and diminished their expression of proinflammatory cytokine genes such as *Il1α, Il1β, Il12β,* and *Cxcl10* as well as the necrosis-inducer TNF, all indispensable for tumor regression. Supporting a role for bacterial products, *Tlr4^{−/−}* mice failed to respond to CpG+anti-IL10R combinatorial regimen, whereas lipopolysaccharide could restore, to some extent, *Tnf* mRNA expression in tumors of ATBx-treated mice receiving CpG+ anti-IL10R therapy (19).

Second, Iida and colleagues extended the role of microbiota in changing the intratumoral myeloid phenotype of MC38 and EL4 tumors, occurring rapidly (by 18 hours) after therapy with oxaliplatin or cisplatin. In this setting, ATBx attenuated the oxaliplatin-induced expression of Cybb encoding reactive oxygen species (ROS)–generating NADPH oxidase 2. This enzymatic activity resided in intratumoral neutrophils and macrophages of tumors treated with platinum salts, all components being indispensable for proper tumoricidal activity of these cytotoxic compounds. Indeed, inhibition of ROS by genetic (*Cybb^{−/−}*mice) or pharmacologic (N-acetyl cysteine) maneuvers and depletion of Gr-1^{+} cells impaired oxaliplatin-mediated antitumor effects (19).

Altogether, this work unraveled the unsuspected role of commensal bacteria in affecting the inflammatory microenvironment required for a TNF- or ROS-dependent therapeutic effect in the settings of immunomodulators or cytotoxics.

Are there causative links between commensal bacteria and anticancer effects of effective therapies?

We addressed whether CTX therapy might have exposed the host to bacteria or bacterial products. Hence, we analyzed how CTX modulated the polarization of adoptively transferred CBir (flagellin epitope of a *Clostridium*; ref. 29)-specific TCR transgenic (Tg) CD4+ T cells. CTX boosted the accumulation and differentiation of CBir-specific TCR Tg lymphocytes into effector Th17 cells and memory Th1 cells (18). To investigate which bacteria could be preferentially recognized by the immune system after CTX and whether CTX could facilitate the translocation of distinct commensal species through the intestinal barrier, we attempted to propagate on blood agar plates (in both aerobic and anaerobic conditions) mesenteric lymph nodes and spleens of CTX-treated mice. This maneuver revealed the specific outgrowth of high numbers of colonies consisting in *Lactobacillus johnsonii* and *Enterococcus hirae* (18). However, in normal conditions (without CTX), spontaneous bacterial translocation events were very rare and mass spectrometry analyses revealed the presence of *L. murinus* or *L. intestinalis* (and not *E. hirae*). Next, we addressed whether enforced colonization of ATB-treated SPF mice with such Gram-positive translocating bacterial isolates could restore pTh17 responses in the spleen. Indeed, the cocktail of *L. johnsonii* + *E. hirae* restored the pTh17 splenic pool generated after CTX, whereas *L. plantarum* or *L. reuteri* failed to do so (18). Finally, we analyzed memory T-cell responses directed against a variety of Gram-positive bacteria, including the translocating and non translocating bacterial species after a therapy with metronomic CTX. In 50% and 30% cases, CTX could elicit memory Th1 responses against *L. johnsonii* and *E. hirae*, respectively (18). In contrast, Th1 memory responses directed against most commensal bacteria that failed to translocate in secondary lymphoid organs (such as *Escherichia coli* or *L. plantarum* or *L. reuteri*) were undetectable(18).

To bridge a pathway between pTh17-inducing commensals and CTX-induced anticancer activity, we performed an adoptive transfer of *ex vivo*–propagated pTh17 (generated with a cocktail of IL1, IL6, and IL23) in vancomycin-treated tumor bearers that failed to respond to CTX. These IFNg/IL17 coproducing polyclonal CD4+ T cells restored the antitumor activity of CTX, whereas Th17 generated *ex vivo* in TGFβ+IL6 failed to do so (18). In conclusion, CTX facilitates the priming of effector pTh17 and the differentiation of memory Th1 cell responses directed against distinct commensals, which are associated with tumor regression.

To link TNF-producing myeloid cells to the success of CpG oligonucleotides/anti-IL10R Ab, principal component analyses of the fecal microbiota composition versus TNF production in tumors were performed by Iida and colleagues and showed a codependence. *Alistipes* and *Ruminococcus* genera, on the one hand, and *Lactobaccilli*, on the other hand, positively and negatively correlated with TNF intratumoral accumulation, respectively. Moreover, reconstitution of ATBx-treated mice with *Alistipes shahii* restored the capacity of intratumoral myeloid cells to secrete TNF, whereas *L. fermentum* failed to do so (19).

Discussion and Future Prospects

These novel findings support the concept that distinct commensal isolates (*L. johnsonii* and *A. shahii*) or pathobionts (*E. hirae*) nesting in the small intestine of tumor bearers could modulate the functions of antigen-presenting cells on the one hand and elicit pTh17 cells in the spleen after translocation on the other hand, giving rise to memory Th1 cells eventually accumulating in inflammatory tumors.

Several questions remain unsolved. The molecular mechanisms, underlying how cytotoxic drugs can compromise intestinal integrity, create bacterial translocation, and local dysbiosis have to be deciphered. Indeed, CTX could augment gut permeability, generate a loss of CD103$^+$ DC and Th17 cells in the lamina propria, and finally decreased Tr1 cells in the spleen, all participating in breaking tolerance to commensals (unpublished data; ref. 18). Investigating cell autonomous stresses (endoplasmic reticulum stress, autophagy, apoptosis, necroptosis, NF-κB, and/or inflammasome activation…) at the level of intestinal epithelial cells, and/or mucosal alterations as primary events (such as IL22-producing innate lymphoid cells, Tregs, Tr1, GM-CSF–producing macrophages…) will be of utmost importance to pinpoint the very target of CTX (listed and discussed in ref. 30; Fig. 1). Once solved, these issues will explain why not any cytotoxic compound may generate a favorable microbial context. Indeed, anthracyclines could not induce pTh17 (even in the enforced absence of Treg, using a depleting anti-CD25 Ab) and therefore mediated a tumoricidal activity independently of the gut microbiome (18).

At a metronomic dosing, CTX does not induce a rapid dysbiosis of the small intestine microbiota. We analyzed the overall gut bacterial composition and diversity by targeting the 16S rRNA gene and applying high-throughput 454 pyrosequencing, followed by qPCR targeting the domain Bacteria and specific bacterial groups, at various time points. CTX marginally affected the microbiota of the small intestine at all early time points but significantly altered the microbial composition at day 7. In particular, the bacterial species *Lactobacillus reuteri* did increase and *Clostridium leptum* clone 40 did decrease at day 7 after CTX. Moreover, qPCR results corroborated that *Lactobacilli* started decreasing at day 7 and not at earlier time points. Similarly, *Clostridium* group IV proportions only decreased at day 7 (18). Hence, these findings indicate that dysbiosis detected in the small intestine mucosa established late when pTh17 are already primed. Could specific bacterial species be incriminated? Indeed, we observed by serendipity an outgrowth of *Parabacteroides distasonis* (known to promote intestinal Tregs; ref. 31) isolated in the feces of mice treated with a prolonged ATB therapy combining ampicillin, colistin, and streptomycin. We could show that *P. distasonis* severely compromised the efficacy of an immunogenic cell death–mediating cytotoxic compound, doxorubicin (18). In addition, monocolonization of germ-free mice with segmented filamentous bacterium aggravated the lack of efficacy of CTX in these gnotobiotic conditions (18).

These observations underscore the importance of bacterial translocation in the modulation of chemotherapeutics effects. Not only bacterial translocation but also circulating microbial products can provide adjuvant effects (32). Hence, total body irradiation enhanced the

efficacy of adoptive CD8^{+} T-cell transfer in tumor bearers by promoting elevation of lipopolysaccharide serum levels necessary and sufficient to break tolerance to tumors (32).

Are these observations made in rodent models relevant for patients? Our preliminary data indicate that, indeed, some patients develop Th1 immune responses against distinct *Enterococci* and *Lactobacilli* isolates after CTX-based therapeutic regimen, whereas others failed to do so or mount Tr1 immunity (unpublished data). Therefore, taking into account the dysbiosis observed in cancer-bearing patients, secondary to their malignancy or their morbidity in general (diet perturbations, neutropenia, recurrent cycles of ATB), novel medical needs are emerging about (i) establishing a diagnosis of microbial dysbiosis at the level of the small intestine and/or the colon, which should be informative to predict the potential resistance of a patient to a therapy, (ii) proposing a compensatory therapy using pre- or probiotics known to influence gut microbial ecosystem, its metagenome, transcriptome, and metabolome (11, 30).

Acknowledgments

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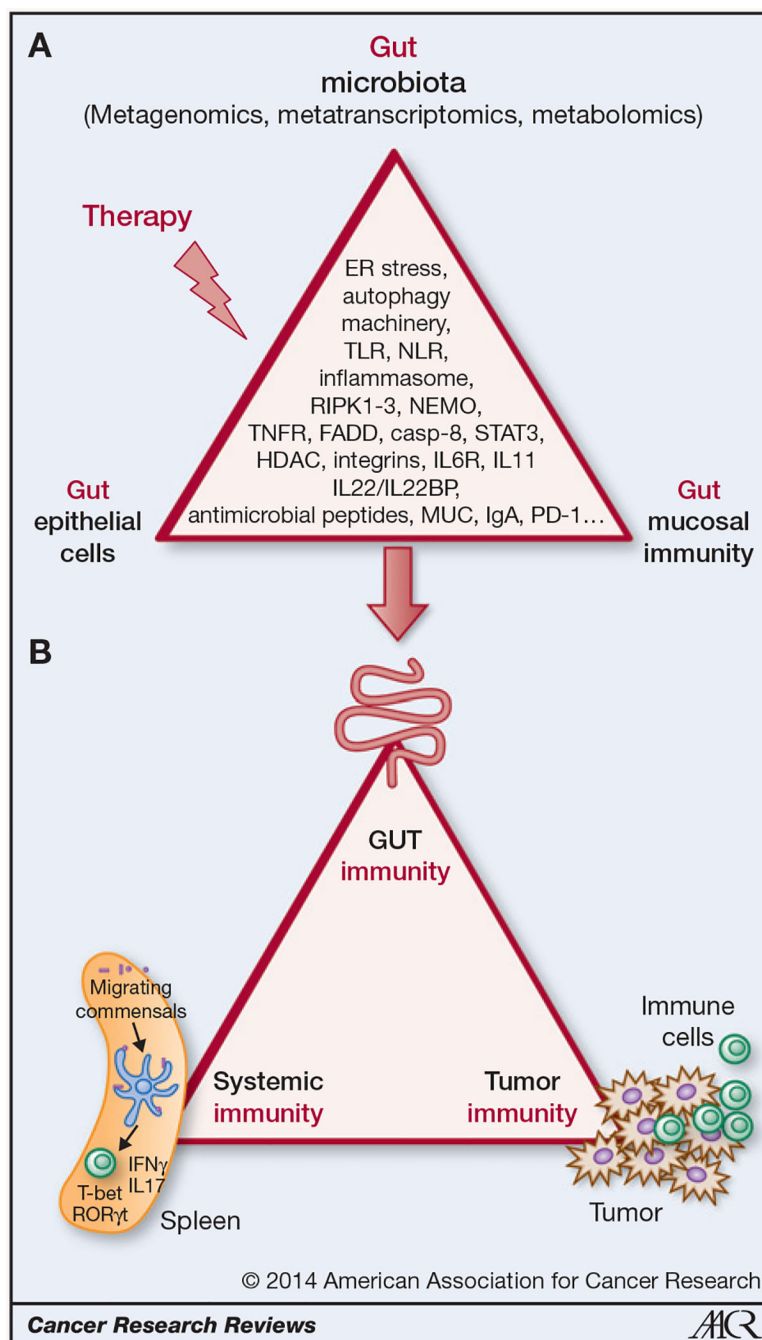
Figure 1.
Interactions between inner and outer compartments leading to "transversal" immunity. A, the intestinal epithelial cell functions as a crucial cell lineage that integrates microbial signals to regulate gene expression and intestinal homeostasis. Increasing evidence has emerged that supports an important intersection between four fundamental cell biologic pathways in the pathogenesis of inflammatory bowel disease: ER stress response, autophagy, cell death, and microbial sensing [toll-like receptor (TLR), NOD-like receptor (NLR), inflammasome, NF-κB, histone deacetylase (HDAC3)…]. Moreover, genome-wide analyses

implicate a central role of transcriptional pathways emanating from specialized cells (Paneth, goblet) as well as innate leukocytes [innate lymphoid cells (ILC), macrophages, Treg, Th17, B cells, and IgA] in the regulation of multiple cellular processes. These cells integrate signals emanating from gut dysbiosis and will alert several actors of the mucosal immune system. B, in turn, when gut homeostasis is compromised, the activation of the intestinal immune system has a major impact at distant sites (sites of priming such as secondary lymphoid organs, and sites of inflammation such as tumor microenvironment, inflammation in the brain/pancreas/joints…).