OncoImmunology 3, e27574; February 2014; © 2014 Landes Bioscience

Why should we need the gut microbiota to respond to cancer therapies?

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Keywords: antibiotics, cancer, chemotherapy, Gram-positive bacteria, immunomodulatory regimen, microbiota, pTh17

Cyclophosphamide, one of the most efficient tumoricidal, antiangiogenic, and immunostimulatory drugs employed to date mediates part of its effects through intestinal bacteria, against which the host becomes immunized during treatment. Our recent work suggests that anti-commensal effector pT_H17 and memory T_H1 $CD4^+$ T-cell responses are indispensable for optimal anticancer effects as mediated by cyclophosphamide.

The critical importance of the gut microbiome and its immunological and metabolic interactions with the host in health and disease is being increasingly recognized. Imbalances in the gut microbiota (a condition referred to as dysbiosis) has been associated with a growing list of chronic disorders,[1] but whether the microbiota has a causative role in disease or whether dysbiosis is one of its by-products remains an open conundrum. Transplantation experiments in which the gut microbiome of a diseased mouse is grafted into a germ-free healthy recipient have highlighted that several conditions (including obesity, metabolic syndrome, colitis) can be transferred by the microbiota.[1] Some epidemiological studies suggest a positive association between the use of antibiotics and the risk of developing breast cancer.[2] In line with these findings, pioneering preclinical work demonstrated that a prolonged combination of metronidazole and ciprofloxacin increases by 3-fold the incidence of breast carcinomas in HER-2/*neu* transgenic mice.[3] More importantly, the intestinal microbiota has been suggested to play a role in the development and severity of mucositis/mucosal barrier injury as induced by many chemotherapeutic agents.[4] These premises prompted us to probe the role of the gut microbiota in the immunogenicity of cell death during chemotherapy.

Cyclophosphamide is somehow a paradigmatic cytotoxic compound in that it can be used at metronomic doses to exert anti-angiogenic and immunostimulatory effects (for instance in combination with anticancer vaccines or adoptive T-cell transfer),[5-7] or as a high-intensity regimen for tumor debulking and/or bone marrow ablation prior to stem cell transplantation. At low doses, cyclophosphamide indeed induces robust T_H1 and T_H17 immune responses in both tumor-bearing mice (treated with a single intraperitoneal injection of 100 mg/kg cyclophosphamide) and cancer patients (receiving 50 mg/day cyclophosphamide for 3 wk).[8] However, not all the cyclophosphamide-induced T cells responses found in the circulation or in lymphoid organs target tumor-associated antigens (TAAs). Indeed, we have recently demonstrated that effector and memory T cells recognizing distinct commensal bacteria are elicited in response to cyclophosphamide, a by-stander effect that de facto facilitates tumor rejection.[9]

We first analyzed how various antibiotic regimens could affect the antitumor efficacy of cyclophosphamide in specific pathogen-free (SPF) animals. Broad spectrum antibiotics such as vancomycin (which kills Gram-positive bacteria) and colistin (which eliminates Gram-negative bacteria) compromised to various degrees the antineoplastic activity of cyclophosphamide in vivo. This finding was obtained in different murine strains (i.e., DBA/2 and C57BL/6 mice), with different tumor models, including transplantable (i.e., P815 mastocytoma cells, MCA205 fibrosarcoma cells) as well as autochthonous (i.e., upon the expression of oncogenic *KRas* and *Trp53* deletion) systems, and across different animal facilities (i.e., at CGFL, Dijon; Gustave Roussy, Villejuif; Institut Pasteur, Paris; and Harvard Medical School, Boston). Moreover, when we compared the tumoricidal activity of cyclophosphamide in SPF vs. germ-free mice, we also concluded that microbiota plays a crucial role in this setting. Corroborating these data, we also demonstrated that the ability of cyclophosphamide to polarize splenocytes toward a T_H1 and T_H17 program upon TCR stimulation is blunted in antibiotic-treated and

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Submitted: 12/16/2013; Revised: 12/18/2013; Accepted: 12/18/2013; Published Online: 01/17/2014
Citation: Viaud S, Daillère R, Yamazaki T, Lepage P, Boneca I, Goldszmid R, Trinchieri G, Zitvogel L. Why should we need the gut microbiota to respond to cancer therapies?. OncoImmunology 2014; 3:e27574; http://dx.doi.org/10.4161/onci.27574

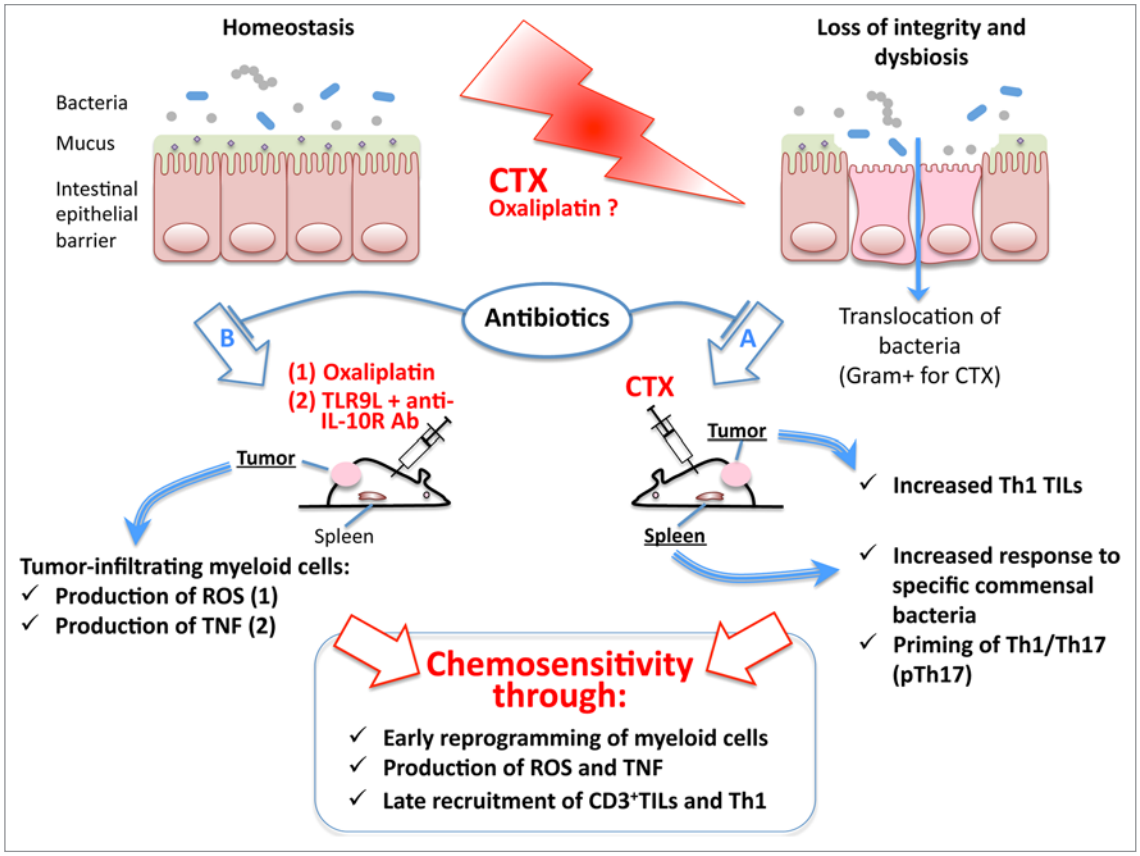

Figure 1. Mechanisms underlying the impact of the gut microbiota on the efficacy of anticancer therapy. (**A and B**) Some chemotherapeutics, presumably those affecting the integrity of the intestinal barrier like cyclophosphamide (CTX) and oxaliplatin, facilitate the translocation across the gut wall of distinct bacteria or bacterial products, causing a systemic immunological cascade that translates in the control of tumor growth. (**A**) Specific commensal Gram-positive bacteria play a prominent role in the elicitation of "pathogenic" T_H17 (pT_H17) responses associated with the accumulation of T_H1 cells in the tumor bed. (**B**) Reactive oxygen species (ROS)- and tumor necrosis factor (TNF)-producing myeloid cells conditioned by the presence of commensal gut bacteria influence the ability of oxaliplatin and immunomodulators, respectively, to mediate antineoplastic effects.

germ-free mice, suggesting that the gut microbiota is involved in the release of T cell-stimulatory cytokines triggered by cyclophosphamide.

We next analyzed the subsets of $CD4^+$ helper T cells elicited by cyclophosphamide in a microbiota-dependent manner by intracellular immunostaining and flow cytometry. In particular, we focused on the intracellular levels of cytokines such as IFNγ and interleukin (IL)-17, chemokine receptors such as chemokine (C-X-C motif) receptor 3 (CXCR3) and chemokine (C-C motif) receptor 6 (CCR6), and transcription factors like T-box 21 (Tbx21, best known as T-bet) and RAR-related orphan receptor γ (RORγ). We demonstrate that cyclophosphamide can promote the selective accumulation of "pathogenic" T_H17 (pT_H17) cells (co-expressing IFNγ/IL-17 and/or CXCR3/CCR6 and/or T-bet/RORγ) in a gut microbiota and myeloid differentiation primary response gene 88 (MYD88)-dependent manner. The therapeutic relevance of pT_H17 cells in tumor control by cyclophosphamide was further demonstrated by adoptively transferring splenic naïve $CD4^+$ T cells propagated ex vivo using anti-CD3/anti-CD28 antibodies and a cytokine cocktail that promote pT_H17 differentiation (i.e., IL-1β plus IL-6 plus IL-23). Such polyclonal pT_H17 cells, but not their regulatory counterparts differentiated in the presence of transforming growth factor β1 (TGFβ1) plus IL-6 and in the absence of IL-1β and IL-23, could indeed restore the sensitivity of tumor-bearing mice to cyclophosphamide despite the co-administration of vancomycin.

To further elucidate the mechanisms by which cyclophosphamide mobilizes the gut microbiota, we scrutinized the intestinal barrier and the repertoire of its 10^{14} inhabitants upon cyclophosphamide therapy (**Fig. 1**). First, cyclophosphamide compromised the integrity of the intestinal epithelium, enhanced its permeability and reduced the abundance of $CD103^+CD11b^+$ dendritic cells and T_H17 cells. Second, cyclophosphamide promoted the translocation of various Gram-positive bacteria (mainly *Lactobacillus johnsonii* and *Enterococcus hirae*) across the intestinal wall. These strains could indeed be propagated ex vivo by cultivating mesenteric lymph nodes and spleens in 50% of cyclophosphamide-treated mice and we demonstrated that cyclophosphamide induces memory T_H1 responses directed against *L. johnsonii* or *E. hirae*. Such responses were closely associated with the accumulation of T_H1 lymphocytes within neoplastic lesions upon the administration of cyclophosphamide, a process that was abrogated when mice

also received vancomycin. Third, cyclophosphamide eventually induced a profound dysbiosis in the small intestine of tumor-bearing mice, which was mainly characterized by a profound reduction in butyrate-producing bacteria (of the *Clostridium* cluster *XIVa*). The amount of lactobacilli in the ileal mucosa (monitored by quantitative PCR) highly correlated with the T_H17/T_H1 polarization as induced by cyclophosphamide. Finally, to demonstrate a causal relationship between the gut microbiota and systemic pT_H17 responses induced by cyclophosphamide, we performed an oral gavage of antibiotic-sterilized SPF animals with a cocktail of Gram-positive bacteria (*L. johnsonii* + *E. hirae)* and showed that this cocktail (but not *Lactobacillus reuteri* or *Lactobacillus plantarum*) promotes the accumulation of pT_H17 cells in the spleen of cyclophosphamide- (but not saline)-treated animals.

The crucial role of the intestinal microbiota in the response to malignant lesions to therapy has been reported in a companion paper.[10] In this work, the authors demonstrate that the very early tumor response to platinum salts or an immunomodulatory regimen comprising Toll-like receptor 9 (TLR9) agonists and anti-IL-10R antibodies) requires commensal gut bacteria that condition and prime tumor-infiltrating myeloid cells to produce inflammatory mediators.[10] These findings may have important implications for the management of cancer patients, supporting skepticism about the use of antibiotics in the course of chemotherapy (exception made for febrile neutropenia) and encouraging the search for appropriate probiotics that boost the immunostimulatory effects of the intestinal microbiota.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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