The Intestinal Microbiota Modulates the Anticancer Immune Effects of Cyclophosphamide

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Cyclophosphamide is one of several clinically important cancer drugs whose therapeutic efficacy is due in part to their ability to stimulate antitumor immune responses. Studying mouse models, we demonstrate that cyclophosphamide alters the composition of microbiota in the small intestine and induces the translocation of selected species of Gram-positive bacteria into secondary lymphoid organs. There, these bacteria stimulate the generation of a specific subset of “pathogenic” Thelper 17 (pT_h17) cells and memory T_h17 immune responses. Tumor-bearing mice that were germ-free or that had been treated with antibiotics to kill Gram-positive bacteria showed a reduction in pT_h17 responses, and their tumors were resistant to cyclophosphamide. Adoptive transfer of pT_h17 cells partially restored the antitumor efficacy of cyclophosphamide. These results suggest that the gut microbiota help shape the anticancer immune response.

It is well established that gut commensal bacteria profoundly shape mammalian immunity (1). Intestinal dysbiosis, which constitutes a disequilibrium in the bacterial ecosystem, can lead to overrepresentation of some bacteria able to promote colon carcinogenesis by favoring chronic inflammation or local immunosuppression (2, 3). However, the effects of microbial dysbiosis on nongastrointestinal cancers are unknown. Anticancer chemotherapeutics often cause mucositis (a debilitating mucosal barrier injury associated with bacterial translocation) and neutropenia, two complications that require treatment with antibiotics, which in turn can result in dysbiosis (4, 5). Some antibiotic agents mediate part of their anticancer activity by stimulating anticancer immune responses (6). Cyclophosphamide (CTX), a prominent alkylating anticancer agent, induces immunogenic cancer cell death (7, 8), subverts immunosuppressive T cells (9), and promotes T_h11 and T_h17 cells controlling cancer outgrowth (10). Here, we investigated the impact of CTX on the small intestine microbiota and its ensuing effects on the anticancer immune response.

We characterized the inflammatory status of the gut epithelial barrier 48 hours after therapy with nonmyeloablatative doses of CTX or the anthracycline doxorubicin in naïve mice. Both drugs caused shortening of small intestinal villi, discontinuities of the epithelial barrier, interstitial edema, and focal accumulation of mononuclear cells in the lamina propria (LP) (Fig. 1A and B). After chemotherapy, the numbers of goblet cells and Paneth cells were increased in vili (Fig. 1C) and crypts (Fig. 1D), respectively. The antibacterial enzyme lysozyme (but not the microbiocide peptide RegIIIγ) was up-regulated in the duodenum of CTX-treated mice (Fig. 1E). Orally administered fluorescein isothiocyanate (FITC)-dextran became detectable in the blood (II) 18 hours after CTX treatment, confirming an increase in intestinal permeability (Fig. 1F). Disruption of the intestinal barrier was accompanied by a significant translocation of commensal bacteria in >50% mice into mesenteric lymph nodes and spleens that was readily detectable 48 hours after CTX treatment, and less so after doxorubicin treatment (Fig. 2A). Several Gram-positive bacterial species, including Lactobacillus johnsonii (growing in >40% cases), Lactobacillus murinus, and Enterococcus hirae, could be cultured from these lymphoid organs (Fig. 2B).

Next, we analyzed the overall composition of the gut microbiota by high-throughput 454 pyrosequencing, followed by quantitative polymerase chain reaction (qPCR) targeting the domain bacteria and specific bacterial groups. Although CTX failed to cause a major dysbiosis at early time points (24 to 48 hours, fig. S1), CTX significantly altered the microbial composition of the small intestine (but not of the caecum) in mice bearing subcutaneous cancers (namely, metastasizing B16F10 melanomas and nonmetastasizing MCA205 sarcomas) 1 week after its administration (Fig. 2C and fig. S2). Consistent with previous reports on fecal samples from patients (12), CTX induced a reduction in bacterial species of the Firmicutes phylum (fig. S2) distributed within four genera and groups (Clostridium cluster XIIIa, Roseburia, unclassified Lachnospiraceae, Coprococcus; table S1) in the mucosa of CTX-treated animals. qPCR was applied to determine the relative abundance (as compared to all bacteria) of targeted groups of bacteria (Lactobacillus, Enterococcus, cluster IV of the Clostridium leptum group) in the small intestine mucosa from CTX-versus vehicle-treated naïve and tumor-bearing mice. In tumor bearers, the total bacterial load of the small intestine at 7 days after CTX treatment, as well as the bacterial counts of the Clostridium leptum, was not affected (Fig. 2D). However, CTX treatment led to a reduction in the abundance of lactobacilli and enterococci (Fig. 2D). Together, these data reveal the capacity of CTX to provoke the selective translocation of distinct Gram-positive bacterial species followed by notable changes in the small intestinal microbiome.

Coinciding with dysbiosis 7 days after CTX administration, the frequencies of CD103+CD11b+ dendritic cells (fig. S3A) and T cell receptor αβ (TCRαβ)+CD3+ T cells expressing the transcription factor RORγt (fig. S3B) were significantly decreased in the LP of the small intestine (but not the colon), as revealed by flow cytometry of dissociated tissues (fig. S3B) and in situ immunofluorescence staining (fig. S3C). RORγt is required for the generation of T_h17 cells [which produce

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interleukin-17 (IL-17)), and strong links between gut-resident and systemic Th17 responses have been established in the context of autoimmune diseases affecting joints, the brain, or the pancreas (13–15). Confirming previous work (9, 10), CTX induced the polarization of splenic CD4+ T cells toward a Th11 [interferon-γ (IFN-γ)-producing] and Th17 pattern (Fig. 3A and fig. S3D). This effect was specific for CTX and was not found for doxorubicin (fig. S4). The gut microbiota was indispensable for driving the conversion of naïve CD4+ T cells into IL-17 producers in response to CTX. Indeed, the ex vivo IL-17 release by TCR-stimulated splenocytes increased in response to CTX. Indeed, the ex vivo IL-17 release by TCR-stimulated splenocytes increased upon CTX treatment of specific-pathogen-free (SPF) mice, yet failed to do so in germ-free (GF) mice (Fig. 3A, left panel). Sterilization of the gut by broad-spectrum antibiotics (ATB, a combination of colistin, ampicillin, and streptomycin; fig. S5) also suppressed the CTX-stimulated secretion of IL-17 (Fig. 3A, right panel) and IFNγ by TCR-stimulated splenocytes (fig. S3D). Treatment of mice with vancomycin, an antibiotic specific for Gram-positive bacteria (16), also reduced the CTX-induced Th17 conversion (Fig. 3A, right panel). In conventional SPF mice, the counts of lactobacilli and SFB measured in small intestine mucosa (Fig. 2D) positively correlated with the Th11 and Th17 polarization of splenocytes (Fig. 3B and fig. S3E), whereas that of Clostridium group IV did not (Fig. 3B). Together, these results point to a specific association between particular microbial components present in the gut lumen (and occasionally in lymphoid organs) and the polarity of Th17 responses induced by CTX treatment.

CTX increased the frequency of “pathogenic” Th17 (pTh17) cells, which share hallmarks of IL-17-producing and TH17 pattern (Fig. 3A and fig. S3F), whereas that of Th11 and Th17 (Fig. 3A and fig. S3E), whereas that of Clostridium group IV did not (Fig. 3B). Together, these results point to a specific association between particular microbial components present in the gut lumen (and occasionally in lymphoid organs) and the polarity of Th17 responses induced by CTX treatment.

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myeloid differentiation primary response gene 88 (MyD88), which signals downstream of toll-like receptors in several tumor models (19). In contrast, the two pattern recognition receptors, nucleotide-binding oligomerization domain-containing 1 (Nod1) and Nod2, were dispensable for the CTX-induced raise in splenic pTh17 cells and for the tumor growth-retarding effects of CTX (fig. S6B). These results establish the capacity of CTX to stimulate pTh17 cells through a complex circuitry that involves intestinal bacteria and MyD88, correlating with its anticancer effects. Beyond its general effect on the frequency of pTh17 cells, CTX induced TCR-restricted, antigen-specific immune responses against commensal bacteria (fig. S7). Hence, we addressed whether Gram-positive bacterial species that translocated into secondary lymphoid organs in response to CTX (Fig. 2A) could polarize naïve CD4+ T cells toward a Th11 or Th17 pattern. Both L. johnsonii and E. hirae stimulated the differentiation of naïve CD4+ T cells into Th11
Because commensal bacteria modulate intestinal and systemic immunity after CTX treatment, we further investigated the effect of antibiotics on CTX-mediated tumor growth inhibition. Long-term treatment with broad-spectrum ATB reduced the capacity of CTX to cure P815 mastocytomas established in syngeneic DBA2 mice (Fig. 4A and fig. S9A). Moreover, the antitumor effects mediated by CTX against MCA205 sarcomas were reduced in GF compared with SPF mice (Fig. 4B, left and middle panels). Driven by the observations that CTX mostly induced the translocation of Gram-positive bacteria and that Gram-positive bacteria correlated with splenic T<sub>H1</sub>/T<sub>H17</sub> polarization, we compared the capacity of several ATB regimens: namely, vancomycin (depleting Gram-positive bacteria) and colistin (depleting most Gram-negative bacteria) to interfere with the tumor growth-inhibitory effects of CTX. Vancomycin, and to a lesser extent colistin, compromised the antitumor efficacy of CTX against MCA205 sarcoma (Fig. 4C and fig. S9B). Using a transgenic tumor model of autochthonous lung carcinogenesis driven by oncogenic K-Ras coupled to conditional p53 deletion (20), we confirmed the inhibitory role of vancomycin on the anticancer efficacy of a CTX-based chemotherapeutic regimen (Fig. 4D). Vancomycin also prevented the CTX-induced accumulation of pT<sub>H17</sub> in the spleen (Fig. 4E) and reduced the frequencies of tumor-infiltrating CD3<sup>+</sup> T cells and T<sub>H1</sub> cells (Fig. 4F).

Although the feces of most SPF mice treated with ATB usually were free of cultivable bacteria (fig. S5), some mice occasionally experienced the outgrowth of Parabacteroides distasonis, a species reported to maintain part of the intestinal regulatory T cell repertoire and to mediate local anti-inflammatory effects (21–23). This bacterial contamination was associated with the failure of an immunogenic chemotherapy (doxorubicin) against established MCA205 sarcomas (fig. S10A). Moreover, experimental recolonization of ATB-sterilized mice with <i>P. distasonis</i> compromised the anticancer effects of doxorubicin (fig. S10B), demonstrating that gut microbial dysbiosis abrogates anticancer therapy. Finally, monoassociation of tumor-bearing GF mice with SFB, which promotes microbiota (each circle represents one mouse) depending on the treatment (NaCl: Co, gray circles; CTX-treated, black circles). A Monte Carlo rank test was applied to assess the significance of these clusterings. (D) QPCR analyses of various bacterial groups associated with small intestine mucosa were performed on CTX- or NaCl (Co)-treated, naïve, or MCA205 tumor-bearing mice. Absolute values were calculated for total bacteria, Lactobacilli, Enterococci, and Clostridium group IV and normalized by the dilution and weight of the sample. Standard curves were generated from serial dilutions of a known concentration of genomic DNA from each bacterial group and by plotting threshold cycles (C<sub>T</sub>) versus bacterial quantity (colony-forming units). Points below the dotted lines were under the detection threshold. Data were analyzed with the linear model or generalized linear model. *P < 0.5, **P < 0.1, ***P < 0.001; ns, not significant.

![Fig. 2. Cyclophosphamide induces mucosa-associated microbial dysbiosis and bacterial translocation in secondary lymphoid organs.](http://science.sciencemag.org/content/342/6160/973/F2)

**A** At 48 hours after CTX or Doxo treatment, mesenteric lymph node (mLN) and spleen cells from naïve mice were cultivated in aerobic and anaerobic conditions, and colonies were enumerated (A) from each mouse treated with NaCl (Co) (n = 10 to 16 mice), CTX (n = 12 to 27 mice), or Doxo (n = 3 to 17 mice) (three to four experiments) and identified by mass spectrometry (B). In NaCl controls, attempts at bacterial identification mostly failed and yielded 67% <i>L. murinus</i> (not shown). Data were analyzed with the Student’s t test. (C) The microbial composition (genus level) was analyzed by 454 pyrosequencing of the 16S ribosomal RNA gene from ilea and caeca of naïve mice and B16F10 tumor bearers. Principal-component analyses highlighted specific clustering of mice.
Fig. 3. CTX-induced pT<sub>h</sub>17 effectors and memory T<sub>h</sub>1 responses depend on gut microbiota. (A) Splenocytes from CTX- versus NaCl-treated animals reared in germ-free (GF) or conventional specific-pathogen–free (SPF) conditions (left panel) and treated (+) or not treated (−) with ATB or vancomycin (Vanco) (right panel) were cross-linked with antibody against CD3<sup>+</sup>CD28 for 48 hours. IL-17 was measured by enzyme-linked immunosorbent assay (ELISA). Two to three experiments containing two to nine mice per group are presented, with each symbol representing one mouse. (B) Correlations between the quantity of specific mucosal bacterial groups and the spleen T<sub>h</sub>17 signature. Each symbol represents one mouse bearing no tumor (circles), a B16F10 melanoma (diamonds), or a MCA205 sarcoma (squares); open symbols denote NaCl-treated mice and filled symbols indicate CTX-treated animals. (C) Intracellular analyses of splenocytes harvested from non–tumor-bearing mice after 7 days of either NaCl or CTX treatment, under a regimen of ATB or with water as control. Means ± SEM of percentages of IFN-γ<sup>+</sup> T<sub>h</sub>17 cells, T-bet<sup>+</sup> cells among RORγt<sup>+</sup> CD4<sup>+</sup> T cells, and CXCR3<sup>+</sup> cells among CCR6<sup>+</sup> CD4<sup>+</sup> T cells in two to eight independent experiments, with each circle representing one mouse. (D) Intracellular staining of total splenocytes harvested 7 days after CTX treatment from naïve mice orally reconstituted with the indicated bacterial species after ATB treatment. (E) Seven days after CTX or NaCl (Co) treatment, splenic CD4<sup>+</sup> T cells were restimulated ex vivo with bone-marrow dendritic cells loaded with decreasing amounts of bacteria for 24 hours. IFN-γ release, monitored by ELISA, is shown. The numbers of responder mice (based on the NaCl baseline threshold) out of the total number of mice tested is indicated (n). Statistical comparisons were based on the paired t test. Data were analyzed with either beta regression or linear model and correlation analyses from modified Kendall tau. *P < 0.05, **P < 0.001; ns, not significant.
TH17 cell differentiation in the LP (1, 13, 14), also had a detrimental impact on the tumor growth–inhibitory effect of CTX (Fig. 4B, right panel).

The aforementioned results highlight the association between specific CTX-induced alterations in gut microbiota, the accumulation of pTH17 cells in the spleen, and the success of chemotherapy. To establish a direct causal link between these phenomena, we adoptively transferred TH17...
or pTε17 populations into vancomycin-treated mice and evaluated their capacity to reestablish the CTX-mediated tumor growth retardation. Ex vivo propagated pTε17 exhibited a pattern of gene expression similar to that expressed by CTX-induced splenic CD4+ T cells in vivo (fig. S11). Only pTε17, but not pTε17 cells, could rescue the negative impact of vancomycin on the CTX-mediated therapeutic effect (Fig. 4G). These results emphasize the importance of pTε17 cells for CTX-mediated anticancer immune responses.

Although much of the detailed molecular mechanisms governing the complex interplay between epithelial cells, gut microbiota, and intestinal immunity remain to be deciphered, the functional role of these interactions in cancer treatment remains to be elucidated. The potential of pTε17 cells for CTX-mediated anticancer immune responses is of great importance for the development of new therapeutic strategies. Further studies are required to investigate the potential of these cells in the treatment of cancer and other chronic diseases.

References and Notes


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Supplementary Materials

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Editor's Summary

The Microbiota Makes for Good Therapy

The gut microbiota has been implicated in the development of some cancers, such as colorectal cancer, but—given the important role our intestinal habitants play in metabolism—they may also modulate the efficacy of certain cancer therapeutics. Iida et al. (p. 967) evaluated the impact of the microbiota on the efficacy of an immunotherapy [CpG (the cytosine, guanosine, phosphodiester link) oligonucleotides] and oxaliplatin, a platinum compound used as a chemotherapeutic. Both therapies were reduced in efficacy in tumor-bearing mice that lacked microbiota, with the microbiota important for activating the innate immune response against the tumors. Viaud et al. (p. 971) found a similar effect of the microbiota on tumor-bearing mice treated with cyclophosphamide, but in this case it appeared that the microbiota promoted an adaptive immune response against the tumors.