Cyclophosphamide Induces Differentiation of Th17 Cells in Cancer Patients


Abstract

Low doses of the alkylating agent cyclophosphamide (CTX) mediate antiangiogenic and immunostimulatory effects, leading to potent tumoricidal activity in association with various immunotherapeutic strategies. Here, we show in rodents and cancer patients that CTX markedly promotes the differentiation of CD4+ T helper 17 (Th17) cells that can be recovered in both blood and tumor beds. However, CTX does not convert regulatory T cells into Th17 cells. Because Th17 are potent inducers of tissue inflammation and autoimmunity, these results suggest impact on the clinical management of various types of malignancies treated with alkylating agents and a potential need to optimize CTX-based immunotherapy in patients. Cancer Res; 71(3); 661–5. ©2011 AACR.

Introduction

Cyclophosphamide (CTX) at 100 to 150 mg/kg dose in mice is known for decades to suppress inhibitory T-cell subsets promoting delayed type hypersensitivity (DTH) responses, IFNγ-mediated antitumor T-cell immunity synergizing with anticancer vaccines, TLR agonists or adoptive T-cell transfer (1, 2). The production of CTX-induced IFNγ might account for the augmented antibody responses and the persistence of memory T cells (3). Interestingly, CTX can also ablate a subset of dendritic cells involved in peripheral tolerance, thereby contributing to enhanced T helper 17 (Th1) responses and antitumor effects (4). Pioneering pilot studies indicated that CTX alone or combined with vaccines could restore natural killer (NK) and T-cell receptor (TCR)-driven effector functions in end-stage cancer patients (5), augment DTH responses (6), decrease the proportions of regulatory T cells (Tregs; ref. 5), and prolong survival of metastatic cancer patients (7).

Material and Methods

Mice

C57BL/6 (H-2b) mice were obtained from Harlan Laboratories. Foxp3DTTP mice [DT receptor–enhanced green fluorescent protein (GFP) under the control of the Foxp3 promoter; ref. (13)] were bred in the local animal facility at our institution in pathogen-free conditions. Animals were used between 9 and 14 weeks of age. All animal experiments were carried out in compliance with French and European laws and regulations.

Phase 1 clinical trial IM1191.2

This clinical trial evaluated the safety of combination therapy with imatinib and IL-2 (Gleevec and IL-2) after a 3-week
Results and Discussion

At 100 mg/kg, CTX blocked the growth kinetics of subcutaneous B16F10 (not shown) in a T-cell–dependent manner (1, 2). We examined more closely the phenotype of T cells during CTX therapy. A single injection of 100 mg/kg of CTX resulted, by 7 days, in a 50% reduction in the spleen cellularity, including B, CD4+ T, and CD8+ T cells (Fig. 1A), but no significant modulations of the proportions of the CD4+CD8− and γδ T-cell subsets (not shown). Seven days post-CTX therapy, the TCR-driven cytokine release was markedly increased in melanoma-bearing mice (Fig. 1B) in spleens (but not in draining lymph nodes, not shown). While IFNγ production was increased both in CD4+ and CD8+ T cells by CTX (Supplementary Fig. 2), CTX-driven IL-17 release increased specifically in CD4+ T cells (Fig. 1C). In similar conditions, CTX did not significantly induce IL-17 production by CD8+ T cells (Fig. 1C). Interestingly, both low (10 mg/kg causing only a slight decrease in the B-cell count) and high (200 mg/kg causing profound lymphopenia) dosings of CTX were associated with such a Th17 pattern (Fig. 1D). Importantly, CTX also induced Th17 cells in naive (tumor-free) mice, suggesting that this drug does promote the conversion of naive CD4+ into IL-17–polarized CD4+ T cells (Supplementary Fig. 1). Recent studies showed that regulatory Foxp3-expressing CD4+ T cells (Tregs) could secrete IL-17 (14–17). Taking advantage of the Foxp3DTR mouse model, we clearly showed that Tregs failed to differentiate into IL-17–producing cells whereas conventional T cells (Foxp3 negative) did after CTX treatment (Fig. 2). Therefore, the major source of IL-17 at day 7 postinjection of CTX in spleens is the CD4+ T-cell pool.

To further elucidate whether metronomic dosage of the alkylating agent CTX may also convert CD4+ T cells into Th17 producers in humans, we analyzed the secretory pattern of T lymphocytes prior to and after a 21-day therapy with 50 mg/d of oral CTX in a cohort of 21 advanced cancer patients (Table 1). This metronomic dosage in cancer patients promoted a drop in B-cell counts (but no significant decrease in T-cell absolute numbers; Fig. 3A). All except 2 patients...
Splenocytes were collected 7 days post-CTX therapy in tumor-bearing mice and stimulated with plastic-bound anti-CD3 and/or soluble anti-CD28 or IL-23. CD4+ T cells, lymphocytes were stimulated with plastic-bound anti-CD3 and/or soluble anti-CD28 or IL-23 to IL-1β. The experiments from C were carried out on independent or pooled spleens (4 or 5 plots). D, CTX dose effect. Id. as in B, but using increasing dosages of intraperitoneal CTX. Experiments were carried out on 3 to 7 independent mice spleens. B–D, IL-17 and/or IFNγ secretion levels in 48-hour supernatants monitored using commercial ELISA kits. Graphs from A and B depict the data of 2 or 3 pooled experiments and each plot corresponds to 1 mouse. Statistical analyses using the Mann–Whitney test indicated significant differences at 95% CI. Means and SEM are shown. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Figure 2. CTX does not induce the differentiation of Tregs into Th17. Tregs (CD4+CD25+Foxp3GFP+) and Tconv cells (CD4+Foxp3GFP−) were sorted from the spleens of tumor-bearing mice 7 days post-CTX therapy and stimulated with plastic bound anti-CD3ε alone or in combination with soluble anti-CD28 with or without IL-23 + IL-1β. A representative experiment of 2 is shown. The IL-17 secretion levels in 48 hours supernatants were monitored by ELISA. nd, not determined.

exhibited a 2- to 10-fold increase in IL-17 production by T lymphocytes after TCR cross-linking, as measured by ELISA (Fig. 3B, left). Although the proportions of both CD4+ and CD8+ T cells producing IFNγ tended to increase following CTX (not shown), the amount of IFNγ produced by TCR-driven lymphocytes did not significantly augment with CTX after 72 hours of stimulation (Fig. 3B, right). Cell sorting allowing positive separation of various T-cell subsets, followed by TCR cross-linking, led to the identification of CD4+ T cells as the major source of IL-17 (Fig. 3C, left). In contrast, CTX did not significantly modulate IFNγ secretion by CD4+ T cells after 72 hours of stimulation (Fig. 3C, right). Interestingly, the pool of IL-17–positive CD4+ T cells failed to coproduce IFNγ (not shown). Here, we report a case of FIGO IIIc ovarian cancer presenting with ascites and treated with metronomic CTX together with anti-VEGF antibodies for 2 months. The comparative analysis of TCR-driven IL-17 secretion by ascites fluid–derived CD4+ T cells revealed that CTX significantly enhanced IL-17 at days 16 and 54 and IFNγ production at day 54 by these TILs, thus also induced Th1 cells at tumor site in patients. Interestingly, the increase of IL-17 was accompanied by a transient decrease in IL-6 secretion (Fig. 3D) known to favor tumor proliferation and survival (18).
Altogether, these data indicate that CTX can directly or indirectly increase the pool of Th17 cells in cancer-bearing hosts. It remains to be established whether mafosfamide, the active pharmacologic compound derived from the prodrug CTX, could directly promote the proliferation of pre-polarized RORγT+CD4+ T cells or act indirectly by favoring mechanisms involved in Th17 differentiation. Both the tumor microenvironment dominated by STAT3 signaling and inflammatory cytokines (such as TGF-β1, IL-6, and IL-23) and the TRAIL-mediated tumor cell death triggered by CTX (1) could induce Nlrp3 activation in inflammatory phagocytes leading to IL-1β release (19), hence contributing to the Th17 polarization of tumor-specific CD4+ TILs. In subcutaneous established B16F10, the IL-17/IL-17R

Figure 3. CTX induced IL-17 production by CD4+ T cells in cancer patients. A, B-cell counts selectively decrease during metronomic dosing of CTX in patients. Stainings by flow cytometry of PBMCs, using antibodies specific for each subsets of T cells and whole B cells. The absolute numbers of individual subsets per mm3 are depicted for 18 patients over time. B, levels of IL-17A production by PBMCs pre- and post-oral CTX therapy. PBMCs were stimulated with anti-CD3/anti-CD28 mAbs-coated microbeads for 3 days. The supernatants were monitored for hIL-17 production by ELISA (left). Each dot corresponds to 1 patient over time. A Wilcoxon matched-pairs test statistical analysis was carried out on 17 patients. Right, IFNγ production during the same incubation period measured in ELISA. C, CD4+ T cells are the major source of IL-17A post-CTX therapy. Id. as in B, but a MoFlo-sorting of PBMCs after thawing allowed the separation of CD4+ or CD8+ T cells that could be subjected to TCR-driven stimulation separately. The data from 7 patient samples are shown with the means and SEM indicated on the graphs. Paired Student’s t test at 95% CI was used to compare cytokine secretion before and after CTX treatment in CD4+ and CD8+ T cells. D, TCR-driven IL-17, IL-6, and IFNγ secretion from TILs in a case report. A FIGO IIIc ovarian cancer–bearing patient was treated with metronomic CTX (50 mg/d) and Avastin (7.5 mg/kg at day 15 and every 21 days) until disease progression. At days 0, 16, and 54 of ascites fluid collection, CD3+CD4+ cells contained in the ascites fluid were isolated by MoFlo-sorting after a Ficoll step and restimulated with anti-CD3/anti-CD28–coated microbeads and 10 IU of rIL-2/mL for 3 days. Cytokine release was monitored in EIA after incubation. This experiment was carried out twice with similar results. *, statistical significance at \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \); and ns, non significant.
pathway did not antagonize the CTX-mediated tumoricidal activity (data not shown). However, further studies will be needed to validate that CTX-driven Th17 producers play a beneficial or a deleterious role in tumor immunosurveillance, as suggested by previous reports (8, 9, 20). In such a case, CTX might be considered as a drug modulating the tumor microenvironment (decreasing Treg suppressors while favoring Th17 inflammatory cells and Th1 cells) without exerting a major, direct tumoricidal activity (18).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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