Cyclophosphamide induces differentiation of Th17 cells in cancer patients

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Running title: Th17 with CTX

Key Words: Th17, immunity, Th17 cells, immunotherapy

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Acknowledgements
We thank Dr. A. Rudensky as the source of the Foxp3DTR mice (Howard Hughes Medical Institute and Department of Immunology, University of Washington, Seattle, USA).
This work was supported by Ligue Nationale contre le Cancer (équipe labelisée by L. Zitvogel), Association pour la Recherche sur le Cancer (ARC), Fondation pour la Recherche Médicale en France (FRM), Institut National du Cancer (INCa), INFLA- CARE (7th Framework Program Health-2007-2.4.1-10), Novartis, Fondation de France contre la Leucémie and INSERM.

Disclosure of Conflicts of Interest
The authors declare no competing financial interests.

Abbreviations: CTL, Cytotoxic T lymphocytes; IFN, Interferon; IL, Interleukin; NK, natural killer cells; PBMC, peripheral blood mononuclear cell; Tconv, conventional CD4+ T cell; TCR, T cell receptor; TGF, transforming growth factor; Th1, Type 1 helper; TLR, Toll-like receptor; Treg, regulatory T cell.
Abstract
Low doses of the alkylating agent cyclophosphamide (CTX) mediate anti-angiogenic 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 cells (Th17 cells) that can be recovered in both blood and tumor beds. However, CTX does not convert T regulatory cells (Tregs) into Th17 cells. Since 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, as well as a potential need to optimize CTX-based immunotherapy in patients.

Precis
Results may impact clinical management of cancers treated with the alkylating agent cyclophosphamide, based on its effects on the differentiation of pro-inflammatory Th17 cells.
Introduction

Cyclophosphamide (CTX) at 100-150 mg/kg in mice is known for decades to suppress inhibitory T cell subsets promoting delayed type hypersensitivity responses (DTH), IFNγ-mediated antitumor T cell immunity synergizing with anti-cancer vaccines, TLR agonists or adoptive T cell transfer (1, 2). The CTX-induced IFNα production 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 Th1 responses and antitumor effects (4). Pioneering pilot studies indicated that CTX alone or combined with vaccines could restore NK and TCR-driven effector functions in end stage cancer patients (5), augment DTH responses (6), decrease the proportions of Tregs (5) and prolong survival of metastatic cancer patients (7).

Recent preclinical studies indicated a close association between IL-17 produced by tumor infiltrating lymphocytes and tumor destruction (8, 9, 10). IL-17A-deficient mice exhibited accelerated tumor progression correlating with decreased NK and tumor specific T cell IFNγ release (8) or enhanced establishment of lung metastases in a melanoma model (10). The adoptively transferred antigen–specific Th17 CD4+ T cells evolved into IFNγ-producing Th1-like lymphocytes capable of eliminating large tumor burdens (9) or behave as helper cells generating potent CTL responses (10).

Given the plasticity of Th17 cells (11) and the unstable phenotype of inducible Tregs (12), we addressed whether CTX could exert significant effects on these in transit-lymphocytes subsets. We found that at a low or lymphodepleting dosage in mice and metronomic in humans, CTX promoted the differentiation of Th17 cells in cancer patients that could be recovered in blood and tumor ascitis. Animal models suggested that CTX-induced Th17 differentiation did not result from the conversion of Tregs.
Material and methods

Mice. C57BL/6 (H-2b) mice were obtained from Harlan Laboratories (Europe). Foxp3DTR mice (DT receptor-enhanced GFP under the control of the Foxp3 promoter) (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 I clinical trial IMAIL-2. This clinical trial evaluated the safety of combination therapy with imatinib and interleukin-2 (Gleevec® and IL-2) after a 3 week-oral treatment with 50mg/day of CTX in patients with metastatic or locally advanced solid tumors (trial approved by the Kremlin Bicêtre Hospital Ethics Committee (n° 07-019) and the Agence Française de Sécurité Sanitaire des Produits de Santé (n°A70385-27) in 2007). Informed consent was obtained from each patient. Patient’s baseline characteristics are summarized in table 1. During this trial, serial blood sampling was performed for immunomonitoring but we focused here on pre-versus post-.CTX therapy. PBMCs were isolated on a Ficoll gradient and frozen in liquid nitrogen until analysis for phenotyping and functions.

Reagents. Cyclophosphamide (Endoxan®, Baxter) was provided by Institut de Cancérologie Gustave Roussy. Anti-mouse mAbs were purchased from BD Pharmingen (Le Pont de Claix, France) and eBioscience (San Diego, USA). LIVE/DEAD® fixable violet stain fluorescence for viability staining was purchased from Invitrogen/Molecular Probes. Anti-human mAbs were purchased from BD Pharmingen and beckman coulter (Marseille, France). All cells were analyzed on a Cyan flow cytometer (Beckman Coulter) with FloJo (Tree Star) software.
**Cytokine assays.** CD4\(^+\) and CD8\(α^+\) T cells were indirectly magnetically sorted (Miltenyi Biotec, France) from spleens. 2 × 10\(^5\) cells/well were incubated in MaxiSorp™ plates (Nunc) pre-coated with anti-CD3\(α\) mAb (0.5 µg/well, eBioscience) and/or anti-CD28 mAb (2 µg/ml, BD Pharmingen) and/or rIL-1\(β\) and rIL-23 (10 ng/ml, R&D Systems). The supernatants were assayed at 48 hrs by ELISA for mouse IL-17A (eBioscience) or IFN\(γ\) (BD OptEIA™). CD4\(^+\) CD25\(^+\) FoxP3 GFP\(^+\) T cells and CD4\(^+\) FoxP3 GFP\(^-\) T cells were isolated from 2 or 3 pooled spleens from Foxp3\(^{DTR}\) mice by MoFlo- or BD FACSVantage-mediated sorting. Human bulk PBMCs were cultured at 1×10\(^5\) CD3\(^+\) T cells/well in U bottom 96-well plates with 1×10\(^4\) anti-CD3/anti-CD28 coated microbeads (Invitrogen/Dynal) and 10 IU of rIL-2/ml (Proleukine®). CD4\(^+\) and CD8\(^+\) T cells were isolated by MoFlo-mediated sorting from PBMCs or ascitis fluids and incubated at a ratio of 10 cells for 1 anti-CD3/anti-CD28 microbead (with 10 IU of rIL-2/ml) The supernatants were assayed at day 3 by ELISA for human IL-17 (R&D Systems) or IFN\(γ\) (BD OptEIA™).

**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 of 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 γ\(δ\)T cell subsets (not shown). Seven days post-CTX, 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 IFN\(γ\) production was increased both in CD4\(^+\) and CD8\(^+\) T cells by CTX (Suppl. Fig. 2), CTX-driven IL-17 release rose specifically in CD4\(^+\) T cells (Fig. 1C). In similar conditions, CTX did not significantly induce IL-17 production by CD8\(^+\) (Fig. 1C). Interestingly, both low (10 mg/kg causing only a slight decrease of the B cell count) or high (200 mg/kg causing profound lymphopenia) dosing of CTX were associated with
such a Th17 pattern (Fig. 1D). Importantly, CTX also induced Th17 cells in naïve (tumor free) mice, suggesting that this drug does promote the conversion of naïve CD4+ into IL-17 polarized CD4+ T cells (Suppl. Fig. 1). Recent studies showed that regulatory Foxp3 expressing CD4+ T cells (Tregs) could secrete IL-17 (14, 15, 16, 17). Taking advantage of the Foxp3DTR mouse model, we clearly showed that Tregs failed to differentiate into IL-17-producing cells while conventional T cells (Foxp3 negative) did after CTX treatment (Fig. 2). Therefore, the major source of IL-17 at day 7 post-injection 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/day 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 of T cell absolute numbers, Fig. 3A). All except two patients exhibited a 2 to 10 fold increase in IL-17 production by T lymphocytes after TCR cross-linking, as measured in ELISA (Fig. 3B, left panel). Although the proportions of both CD4+ and CD8+ T cells producing IFNγ tend to increase following CTX (not shown), the amount of IFNγ produced by TCR-driven lymphocytes did not significantly augment with CTX after 72 hrs of stimulation (Fig. 3B, right panel). 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-17A (Fig. 3C, left panel). In contrast, CTX did not significantly modulate IFNγ secretion by CD4+ T cells after 72 hrs of stimulation (Fig. 3C, right panel). 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 ascitis and treated with metronomic CTX together with anti-VEGF antibodies for two months. The comparative analysis of TCR-driven IL-17 secretion by ascitis fluid-derived CD4+ T cells revealed that CTX significantly enhanced IL-17 at day 16 and 54 and IFNγ production at day 54 by these tumor infiltrating lymphocytes (TILs) thus...
induced also 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 pharmacological compound deriving from the pro-drug 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 sc established B16F10, the IL-17/IL-17R 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).
References


Table 1. Patients’ characteristics.

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Inclusion in a Phase I trial*

Number of patients 17

* : Approved by the Kremlin Bicêtre Hospital Ethics Committee (n° 07-019) and the Agence Française de Sécurité Sanitaire des Produits de Santé (n° A70385-27) in 2007, N° EudraCT : 2007-001699-35. All patients signed an informed consent for this study.

Abbreviations: ECOG, Eastern Cooperative Oncology Group; GIST, Gastrointestinal Stromal Tumor
Figure Legends

**Figure 1. CTX induced IL-17 production by CD4+ T cells in mice.** A. *Influence of CTX on the absolute numbers of the cell subsets within splenocytes.* Splenocytes were harvested 7 days post-CTX (100mg/kg intraperitoneally) and 12 days after 3x10^5 B16F10 cells inoculation and flow cytometry analyses were performed gating on live cells using anti-CD3ε, anti-CD4, anti-CD8α, and anti-CD19, mAbs. B. *CTX-driven cytokine production in mice.* Splenocytes were collected 7 days post-CTX in tumor bearing mice and stimulated with plastic bound anti-CD3ε and soluble anti-CD28. C. *IL-17 is mainly produced by CD4+ T cells during CTX therapy.* Id. as in B. After indirect magnetic sorting of CD3+CD4+ or CD3+CD8α+ T cells, lymphocytes were stimulated with plastic bound anti-CD3ε and/or soluble anti-CD28 or IL-23+IL-1β. The experiments from panel C were performed 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, C, D show IL-17 and/or IFNγ secretion levels in 48 hrs 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 one mouse. Statistical analyses using Mann Whitney test indicated significant differences at 95% confidence interval. Means and SEM are shown.* p<0.05, ** p<0.01, *** p<0.001 and ns: "non significant".

**Figure 2. CTX does not induce the differentiation of Tregs into Th17.** Tregs (CD4+ CD25+ FoxP3 GFP+) and Tconv cells (CD4+ FoxP3 GFP-) 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, or IL-23 + IL-1β. A representative experiment out of two is shown. The IL-17 secretion levels in 48 hrs supernatants were monitored by ELISA. nd: "non determined".
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/mm³ are depicted for 18 patients overtime. **B. Levels of IL-17A production by PBMCs pre-and post-oral CTX.** 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 panel). Each dot corresponds to one patient overtime. A Wilcoxon matched pairs test statistical analysis was performed on 17 patients. The right panel represents the 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 patients’ samples are shown with the means and SEM indicated on the graphs. Paired Student’s t test at 95% confidence interval 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 / day) and Avastin™ (7.5mg/kg at day 15 and every 21 days) until disease progression. At Day 0, 16 and 54 of ascitis fluid collection, CD3+CD4+ cells contained in the ascitis 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 performed twice with similar results. * indicates statistical significance at p<0.05, **: at p<0.01, ***: at p<0.001 and ns: “non significant”.
Figure 1. Viaud et al.

A. B cells (×10^6)

B. IL-17 ng/ml

C. CD8^+ T cells

D. CD3 CD28

** C. CD4^+ T cells

* D. IL-17 ng/ml
Figure 2. Viaud et al.

Author Manuscript Published OnlineFirst on December 9, 2010; DOI: 10.1158/0008-5472.CAN-10-1259
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3. Viaud et al.

**A.**

- **B cells** / mm³
  - Before CTX
  - After CTX
  - *p < 0.05

- **CD4⁺ T cells** / mm³
  - Before CTX
  - After CTX
  - *p < 0.05

**B.**

- **IL-17 ng/ml**
  - Before CTX
  - After CTX
  - *p < 0.05

- **IFN-γ ng/ml**
  - Before CTX
  - After CTX
  - *p < 0.05

**C.**

- **CD4⁺ T cells**
  - Before CTX
  - After CTX
  - *p < 0.05

- **CD8⁺ T cells**
  - Before CTX
  - After CTX
  - ns

**D.**

- **Cytokine secretion ng/ml**
  - IL-17
  - IL-6
  - IFN-γ
  - D0
  - D16
  - D54
  - ***p < 0.001
  - **p < 0.01
  - *p < 0.05
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Cancer Res  Published OnlineFirst December 9, 2010.

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Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-1259

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