Published OnlineFirst December 9, 2010; DOI: 10.1158/0008-5472.CAN-10-1259

Priority Report

Cancer Research

Cyclophosphamide Induces Differentiation of Th17 Cells in Cancer Patients

Sophie Viaud[1–3], Caroline Flament[2–4], Mustapha Zoubir[1–4], Patricia Pautier[3,5], Axel LeCesne[3,6], Vincent Ribrag[3,5], Jean-Charles Soria[1,3,5], Virginie Marty[3,7], Philippe Vielh[3,7], Caroline Robert[3,8], Nathalie Chaput[1,3,4,9], and Laurence Zitvogel[1–4]

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 IFNα 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).

Authors' Affiliations: [1]Faculté de Médecine de l'Université Paris-Sud XI, Le Kremlin-Bicêtre, France; [2]Institut National de la Santé et de la Recherche Médicale, U1015; [3]Institut de Cancérologie Gustave Roussy; [4]Center of Clinical Investigations in Biotherapies of Cancer CICBT 507; [5]Service Innovations Thérapeutiques Essais Précoces (SITEP), [6]Department of Medicine, [7]Laboratory of Translational Research, [8]Department of Dermatology, and [9]Cell Therapy Unit, Institut de Cancérologie Gustave Roussy, Villejuif, France

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Sophie Viaud and Caroline Flament share first authorship. Nathalie Chaput and Laurence Zitvogel share senior authorship.

Corresponding Authors: Nathalie Chaput and Laurence Zitvogel, Centre d'Investigation Clinique Biothérapie 507, Institut de Cancérologie Gustave Roussy, 114 rue Edouard Vaillant, 94805 Villejuif, France. Phone: 33142116616; Fax: 33142116094. E-mail: chaput@igr.fr, zitvogel@igr.fr

doi: 10.1158/0008-5472.CAN-10-1259

Recent preclinical studies indicated a close association between interleukin (IL)-17 produced by tumor-infiltrating lymphocytes (TIL) and tumor destruction (8–10). 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 ascites. Animal models suggested that CTX-induced Th17 differentiation did not result from the conversion of Tregs.

Material and Methods

Mice

C57BL/6 ($H\text{-}2^b$) mice were obtained from Harlan Laboratories. $Foxp3^{DTR}$ 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 I clinical trial IMAIL-2

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

Published OnlineFirst December 9, 2010; DOI: 10.1158/0008-5472.CAN-10-1259

Table 1. Patients' characteristics

Characteristics	*n*	%
Sex		
Male	9	43
Female	12	57
Age, y		
Median (range)	51 (27–74)	
ECOG performance status		
0	13	62
1	8	38
Previous lines of systemic therapies		
Median (range)	3 (0–5)	
Tumor type		
Melanoma	11	
Ovarian cancer	4	
Gastrointestinal stromal tumor	2	
Breast cancer	1	
Colon cancer	1	
Merkel cell carcinoma	1	
Inclusion in a phase I trial[a]		
Number of parents	17	

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
[a]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.

oral treatment with 50 mg/d 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. Patients' baseline characteristics are summarized in Table 1. During this trial, serial blood sampling was carried out 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 monoclonal antibodies (mAb) were purchased from BD Pharmingen and eBioscience. 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. All cells were analyzed on a Cyan flow cytometer (Beckman Coulter) with FloJo (Tree Star) software.

Cytokine assays

$CD4^+$ and $CD8\alpha^+$ T cells were indirectly magnetically sorted (Miltenyi Biotec) from spleens. A total of 2×10^5 cells per well were incubated in MaxiSorp plates (Nunc) precoated with anti-CD3α mAb (0.5 μg per well; eBioscience) and/or anti-CD28 mAb (2 μg/mL; BD Pharmingen) and/or rIL-1β and rIL-23 (10 ng/mL; R&D Systems). The supernatants were assayed at 48 hours by ELISA for mouse IL-17A (eBioscience) or IFNγ ((BD OptEIA) . $CD4^+CD25^+Foxp3GFP^+$ T cells and $CD4^+Foxp3GFP^-$ T cells were isolated from 2 or 3 pooled spleens from $Foxp3^{DTR}$ mice by MoFlo- or BD FACSVantage–mediated sorting. Human bulk peripheral blood mononuclear cells (PBMC) were cultured at 1×10^5 $CD3^+$ T cells per 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 ascites 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 IFNγ (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 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 $CD8^+$ (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 $Foxp3^{DTR}$ 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

Published OnlineFirst December 9, 2010; DOI: 10.1158/0008-5472.CAN-10-1259

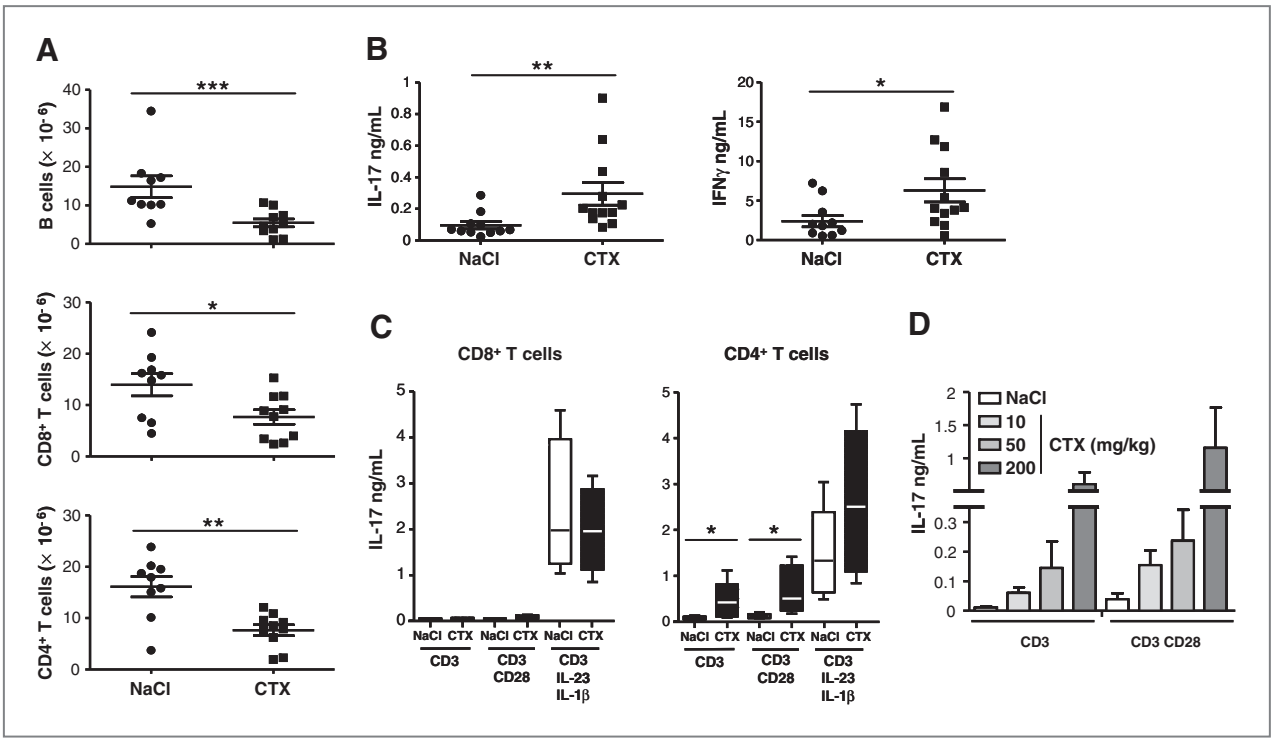

Figure 1. CTX induced IL-17 production by $CD4^+$ T cells in mice. A, influence of CTX on the absolute numbers of the cell subsets within splenocytes. Splenocytes were harvested 7 days post-CTX therapy (100 mg/kg intraperitoneally) and 12 days after 3×10^5 B16F10 cells inoculation, and flow cytometric analyses were gated on live cells by 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 therapy in tumor-bearing mice and stimulated with plastic-bound anti-CD3ε and soluble anti-CD28 mAbs. 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 $CD3^+CD8\alpha^+$ T cells, lymphocytes were stimulated with plastic-bound anti-CD3ε and/or soluble anti-CD28 or IL-23 + 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 ***, $P < 0.001$.

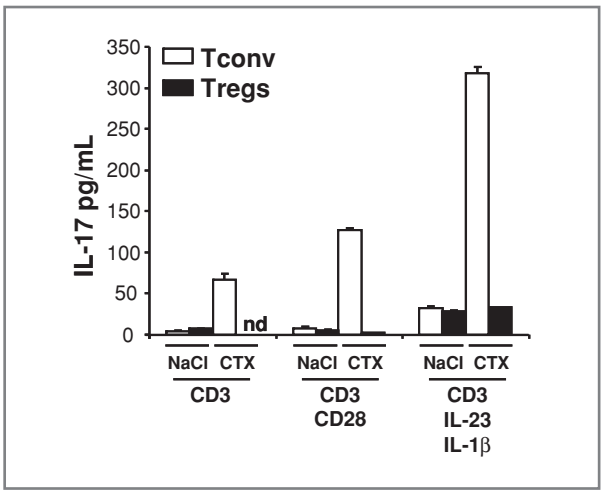

Figure 2. CTX does not induce the differentiation of Tregs into Th17. Tregs ($CD4^+CD25^+Foxp3GFP^+$) and Tconv cells ($CD4^+Foxp3GFP^-$) were sorted from the spleens of tumor-bearing mice 7 days post-CTX therapy and stimulated with plastic bound anti-CD3ε alone or in combination with soluble anti-CD28 with or 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 tend 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-17A (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).

Published OnlineFirst December 9, 2010; DOI: 10.1158/0008-5472.CAN-10-1259

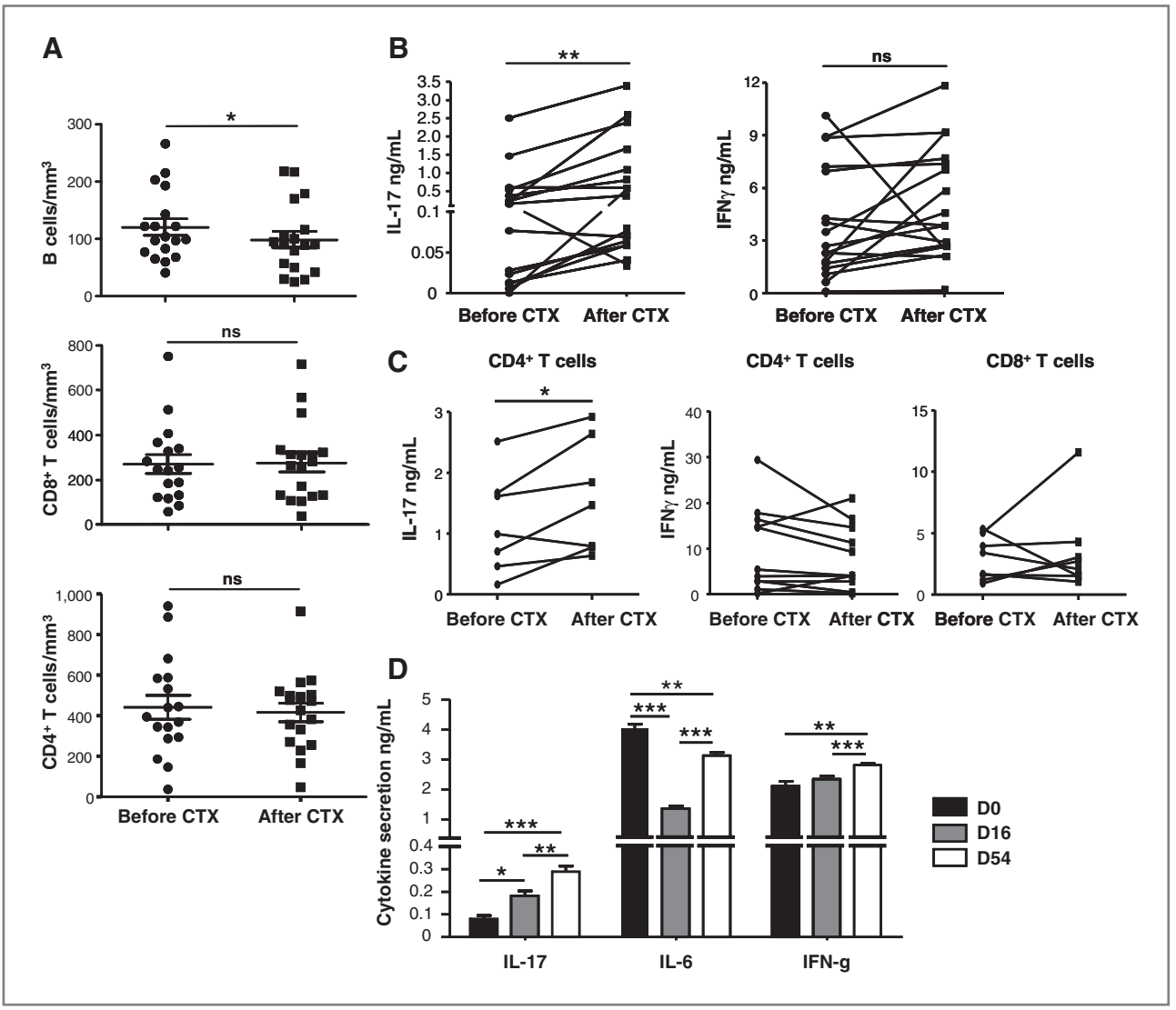

Figure 3. CTX induced IL-17 production by $CD4^+$ T cells in cancer patients. A, B-cell counts selectively decrease during metronomic dosing of CTX in patients. Stainings by flow cytometry of PBMCs, using antibodies specific for each subsets of T cells and whole B cells. The absolute numbers of individual subsets per mm^3 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.

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 $ROR\gamma T^+$ $CD4^+$ 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

Published OnlineFirst December 9, 2010; DOI: 10.1158/0008-5472.CAN-10-1259

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.

Acknowledgments

We thank Dr. A. Rudensky as the source of the Foxp3^{DTR} mice (Howard Hughes Medical Institute and Department of Immunology, University of Washington, Seattle, WA).

Grant Support

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.

Received April 14, 2010; revised October 4, 2010; accepted October 19, 2010; published OnlineFirst January 25, 2011.

References

1. Van Der Most RG, Currie AJ, Cleaver AL, Salmons J, Nowak AK, Mahendran S, et al. Cyclophosphamide chemotherapy sensitizes tumor cells to TRAIL-dependent CD8 T cell-mediated immune attack resulting in suppression of tumor growth. PLoS One 2009;4:e6982.
2. Taieb J, Chaput N, Schartz N, Roux S, Novault S, Menard C, et al. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. J Immunol 2006;176:2722–9.
3. Schiavoni G, Mattei F, Di Pucchio T, Santini SM, Bracci L, Belardelli F, et al. Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. Blood 2000;95:2024–30.
4. Nakahara T, Uchi H, Lesokhin AM, Avogadri F, Rizzuto GA, Hirschhorn-Cymerman D, et al. Cyclophosphamide enhances immunity by modulating the balance of dendritic cell subsets in lymphoid organs. Blood 2010;115:4384–92.
5. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, et al. Metronomic cyclophosphamide regimen selectively depletes CD4$^+$CD25$^+$ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007;56:641–8.
6. Berd D, Maguire HC Jr, Mastrangelo MJ. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. Cancer Res 1986;46:2572–7.
7. MacLean GD, Miles DW, Rubens RD, Reddish MA, Longenecker BM. Enhancing the effect of THERATOPE STn-KLH cancer vaccine in patients with metastatic breast cancer by pretreatment with low-dose intravenous cyclophosphamide. J Immunother Emphasis Tumor Immunol 1996;19:309–16.
8. Kryczek I, Wei S, Szeliga W, Vatan L, Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. Blood 2009;114:357–9.
9. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood 2008;112:362–73.
10. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 2009;31:787–98.
11. Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4$^+$ T cells. Immunity 2009;30:155–67.
12. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity 2008;29:44–56.
13. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol 2007;8:191–7.
14. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25^{high}Foxp3^{pos} regulatory T cells differentiate into IL-17-producing cells. Blood 2008;112:2340–52.
15. Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, et al. Human memory FOXP3$^+$ Tregs secrete IL-17 *ex vivo* and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. Proc Natl Acad Sci U S A 2009;106:8635–40.
16. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009;113:4240–9.
17. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-producing FOXP3$^+$ regulatory T cells in humans. Proc Natl Acad Sci U S A 2009;106:4793–8.
18. Lawrence T, Hageman T, Balkwill F. Cancer. Sex, cytokines, and cancer. Science 2007;317:51–2.
19. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat Med 2009;15:1170–8.
20. Hinrichs CS, Kaiser A, Paulos CM, Cassard L, Sanchez-Perez L, Heemskerk B, et al. Type 17 CD8$^+$ T cells display enhanced antitumor immunity. Blood 2009;114:596–9.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Cyclophosphamide Induces Differentiation of Th17 Cells in Cancer Patients

Sophie Viaud, Caroline Flament, Mustapha Zoubir, et al.

Cancer Res 2011;71:661-665. Published OnlineFirst December 9, 2010.