Unraveling Cancer Chemoimmunotherapy Mechanisms by Gene and Protein Expression Profiling of Responses to Cyclophosphamide

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Abstract

Certain chemotherapeutic drugs, such as cyclophosphamide (CTX), can enhance the antitumor efficacy of immunotherapy because of their capacity to modulate innate and adaptive immunity. Indeed, it has been argued that this capacity may be more significant to chemotherapeutic efficacy in general than is currently appreciated. To gain insights into the core mechanisms of chemoimmunotherapy, we methodically profiled the effects of CTX on gene expression in bone marrow, spleen, and peripheral blood, and on cytokine expression in plasma and bone marrow of tumor-bearing mice. Gene and protein expression were modulated early and transiently by CTX, leading to upregulation of a variety of immunomodulatory factors, including danger signals, pattern recognition receptors, inflammatory mediators, growth factors, cytokines, chemokines, and chemokine receptors. These factors are involved in sensing CTX myelotoxicity and activating repair mechanisms, which, in turn, stimulate immunoactivation events that promote efficacy. In particular, CTX induced a T-helper 17 (Th17)-related gene signature associated with an increase in Th17, Th1, and activated CD25+CD4+Foxp3− T lymphocytes and a slight recovery of regulatory T cells. By analyzing gene and protein expression kinetics and their relationship to the antitumor efficacy of different therapeutic schedules of combination, we determined that optimal timing for performing adoptive immunotherapy is approximately 1 day after CTX treatment. Together, our findings highlight factors that may propel the efficacy of chemoimmunotherapy, offering a mechanistic glimpse of the important immune modulatory effects of CTX.

Introduction

Although immunotherapy represents a promising nontoxic anticancer strategy, the different treatment modalities employed so far in the clinical setting resulted in limited and sporadic successes, highlighting the need for the identification of effective strategies to enhance its clinical efficacy (1). Mounting evidence suggests that combination therapies may produce synergistic antitumor responses and that certain chemotherapeutic agents, rather than being immunosuppressive can, under defined conditions, act as strong adjuvants for either adoptive or active immunotherapy (2, 3). Currently, cyclophosphamide (CTX) represents the gold standard immunomodulatory chemotherapeutic drug, and the antitumor efficacy of its combination with immunotherapy has long been studied in preclinical models (4–7), as well as in clinical trials (8–11). Several mechanisms have been considered to explain this paradoxical phenomenon, among them great relevance was attributed to the reduction of regulatory T-cell (Treg) number and function, observed in both the mouse model (12, 13) and cancer patients (14). Additional CTX-mediated immunoactivating effects include increased tumor infiltration by lymphocytes, functional activation of B and T cells, and homeostatic proliferation, occurring during the recovery phase that follows chemotherapy-mediated myelolymphodepletion and that, as a bystander effect, can promote antitumor immunity (5, 6, 15, 16). Notably, preconditioning the host with CTX also induces the expression of type I IFN, leading to the expansion of CD4+ and CD8− T cells exhibiting a memory phenotype (17). More recent data indicate that CTX can restore an activated polyfunctional helper phenotype in tumor-specific adoptively transferred CD4+ T cells through an IFN-α/β-dependent mechanism (18). Further studies underscore the effects of this drug not only on the adaptive but also on the innate immune system. In this regard, it was shown that CTX-induced expansion of vaccine-specific CD8+ T cells is associated with increased number and activation status of dendritic cells (DC; refs. 19, 20).

In a previous study, we had shown that CTX acts through the production of hitherto unidentified soluble factors...
sustaining the proliferation, survival, and activity of transferred lymphocytes (5). Subsequently, we reported that the synergistic anticancer activity of chemotherapy and immunotherapy was associated with the induction of a "cytokine storm," occurring primarily in the bone marrow of treated mice (16). Upregulated factors included hematopoietic growth factors [granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-1β], cytokines regulating homeostatic expansion and memory CD8+ T-cell survival (IL-7, IL-15, IL-2, and IL-21) and cytokines involved in the polarization toward a T-helper (Th)1 type of immune response (IFN-γ). The present study aims at investigating the mechanisms underlying the immunomodulatory activity of CTX by means of genomic and proteomic approaches. In particular, we evaluated the effect of CTX on the gene expression of bone marrow, spleen, and peripheral blood mononuclear cells (PBMC) and on the protein levels of 32 cytokines in plasma and bone marrow lysates of tumor-bearing mice at different times after a single chemotherapeutic treatment. The overall results provide new insights into the mechanisms by which CTX can render immune lymphocytes capable of eradicating metastatic tumors and provide a scientific rationale for performing adoptive immunotherapy early after chemotherapy.

Materials and Methods

Animals, tumor cells, and chemoimmunotherapy

Six- to 7-week-old DBA/2 mice (Harlan) were implanted with 2 × 10^6 IFN-α/β-resistant, in vivo passaged, highly metastatic 3CI-8 Friend leukemia cells (3CI-8 FLC; ref. 21). CTX (Sigma-Aldrich) was administered intraperitoneally 4 days after tumor injection. Five hours later, some mice received the adoptive transfer of 40 × 10^7 lymphomonocytes derived from spleens of tumor-immunized mice, prepared as in ref. 16. Vaccination was carried out with IFN-α-producing tumor cells (IFN-α-CL-1; ref. 22). Mice were vaccinated with either irradiated (100 Gy) cells or a tumor lysate obtained from 3 freeze-thaw cycles. Vaccination was carried out twice (3 times for lysates) at a time interval of 2 weeks. The experimental protocols were approved by the Istituto Superiore di Sanità Review Board.

RNA isolation, labeling, and hybridization

Total RNA was obtained by TRIZOL reagent extraction (Invitrogen) for spleens and by RNeasy (Qiagen) purification for bone marrow cells (femurs and tibias) and PBMC (obtained by gradient centrifugation with Lympholyte-Mammas-Cederlane).

Amino-allyl modified antisense RNA (aRNA) was synthesized in 1 amplification round from 2 μg total RNA by using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion), in accordance with manufacturer's instructions, and its quality was assessed with the 2100 Bioanalyzer (Agilent Technologies). aRNAs were coupled to monoreactive Cy3 or Cy5 dyes (GE Healthcare), fragmented (RNA Fragmentation Reagents, Ambion), mixed, and cohybridized overnight in humidifying chambers at 42°C onto prehybridized microarray slides, and printed with 13,443 70mer oligonucleotides (Operon version 1.1; CRIBI Microarray Service, University of Padua, Italy). The platform has been submitted to the Gene Expression Omnibus (GEO) database (accession GPL5098).

Data analysis

Scanning and image file processing were carried out with the GenePix 4200A instrument (Axon Instruments), and the obtained data were filtered with BRB ArrayTool (23) to exclude spots below a minimum intensity (200), flagged and with diameters less than 25 μm and normalized by using Lowess Smoother. Only genes expressed in 70% of samples were analyzed in subsequent statistical analyses (all done by using the log2-tranformed ratios).

Average linkage unsupervised hierarchical clustering, with Pearson correlation distance measure, was carried out with genes whose expression differed by at least 1.5-fold from the median in at least 20% of samples. Following median centering, results were visualized with Treeview software (24).

Statistically significant (P < 0.001) differentially expressed genes between posttherapy and pretreatment samples were identified with supervised class comparison (parametric t-test) and further analyzed by using Cluster and TreeView softwares.

Gene annotation and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses were carried out by means of DAVID (Database for Annotation, Visualization, and Integrated Discovery) bioinformatic tool (25). Enriched biological processes and pathways were ranked according to EASE score, indicating the abundance of genes fitting each class in proportion to the number of genes expected to be in each class by chance, calculated on the global composition of the array.

Intracellular cytokine staining and flow cytometry

Splenocytes were stratified over Lympholyte-M Gradient (Cederlane) for lymphocyte enrichment. For Treg and activated CD4+ lymphocyte analyses, cells were stained with anti-CD4-FITC and anti-CD25-PE (BD Biosciences), and fixed, permeabilized, and stained with anti-Foxp3-APC according to the manufacturer’s instructions (eBioscience).

For Th1 and Th17 analyses, cells were stimulated overnight with 100 ng/mL PMA and 1 μg/mL Ionomycin, in the presence of 1 μg/mL Brefeldin-A and 0.7 μg/mL Monensin (GolgiPlug, GolgiStop, BD-Bioscience) to prevent cytokine secretion. Following staining with anti-CD4-FITC, cells were fixed and permeabilized, and intracellular staining was carried out by using anti-IFN-γ-PE-Cy7 and anti-IL-17-APC (e-Bioscience). Acquisition and analysis were carried out with FACS-Calibur flow cytometer (BD-Bioscience) and FlowJo software. Production of IFN-γ and IL-17 was evaluated in CD4+ in both unstimulated (data not shown) and PMA/1 stimulated samples.

Multiplex proteomic analysis of cytokines, chemokines, and growth factors

Bone marrow cells were obtained from femurs and tibias. After lysing erythrocytes through a 3-minute incubation in 0.16 mol/L Tris-buffered NH4Cl (pH 7.2), cells were lysed in
Bio-Plex cell lysis buffer. The mouse group I (23-plex) and group II (9-plex) Bio-Plex cytokine assays were carried out according to the manufacturer’s instructions (Bio-Rad). Beads were read on the Bio-Plex 200 suspension array system, and data were analyzed by using Bio-Plex Manager software.

Results

The combination of CTX and adoptive immunotherapy results in complete tumor regression

A highly metastatic tumor model, 3Cl-8 FLC (21), was used to assess the antitumor effectiveness of the combination of a single CTX injection and adoptive cell transfer (ACT) of lymphomonoocytes from tumor-vaccinated syngeneic mice, according to previously published protocols (5). Donor mice were vaccinated with either irradiated (Fig. 1A) or lysed (Fig. 1B) IFN-α-producing tumor cells (IFN-α1-CI-11), based on the fact that these cells were shown to be effective cancer vaccines (22).

ACT alone had no significant effect on tumor growth (Fig. 1) and mouse survival (data not shown), whereas CTX alone induced a transient delay in tumor growth but ultimately tumors developed in all mice. In contrast, the sequential treatment with CTX and ACT from mice vaccinated with irradiated IFN-α1-CI-11 resulted in complete tumor regression in 100% of mice with no recurrences or metastases (Fig. 1A). ACT from nonvaccinated mice had no effect either alone or in combination with CTX (data not shown). Noteworthy, when chemotherapy was followed by ACT from tumor lysate-vaccinated mice, tumor growth was delayed (more than with CTX alone), but no tumor regressed (Fig. 1B). These results, along with previously published data (5, 16), show that the therapeutic effectiveness of this chemoimmunotherapy approach depends on CTX-mediated immunomodulatory mechanisms potentiating adoptive immunotherapy and that also the quality of the antitumor immunity induced in donor mice has an impact on the outcome. Irradiated tumor cells may, in fact, still produce IFN-α, which influences the quality of the vaccine-induced immune response (26). In addition, irradiation has been shown to induce an immunogenic cell death leading to optimal immunization of mice against subsequent tumor challenge (27).

Global gene expression analysis of the effects of CTX on bone marrow, spleen, and PBMC

To gain insights into the CTX-mediated effects responsible for the antitumor efficacy of chemoimmunotherapy, we evaluated the impact of a single CTX treatment on the gene expression of primary (bone marrow) and secondary (spleen) lymphoid organs and of PBMC. Microarray analysis, evaluating the expression of 13,443 genes, was carried out before (ctr) and at different times (1, 2, and 5 days) after chemotherapy. The experiment was carried out in tumor-bearing mice to take into account the possible immunomodulation induced by the tumor implant itself. The data discussed in this article have been deposited in National Center for Biotechnology Information’s GEO and are accessible through GEO Series accession number GSE27440 (28).

To get an overall picture of the transcriptional modulation by CTX and of its kinetics, data were preliminarily subjected to unsupervised hierarchical clustering analysis (Fig. 2A). In both lymphoid organs and PBMC, this analysis segregated samples into 2 principal clusters reflecting distinct gene expression patterns, one including ctr and samples taken 5 days post-treatment and the other containing samples from day 1 and 2, showing that CTX has a strong impact on gene expression, inducing at early time points (1–2 days) the modulation of a large number of genes, that, however, return to baseline by day 5.

To identify genes undergoing a statistically significant differential expression, microarray data were reanalyzed by using a supervised approach (class comparison), followed by clustering analysis. Class comparison was conducted gathering in one class samples from day 1 and 2 and comparing them with ctr samples, based on the fact that, on separate comparisons between day 1, day 2, and day 5 versus ctr, most genes undergoing a statistically significant modulation of expression at day 1 showed a similar profile at day 2 (Supplementary Fig. S1).

In the bone marrow, 1 to 2 days after CTX administration, the expression of 1,123 genes was significantly (P ≤ 0.001) modulated with respect to ctr (580 overexpressed and 543 downregulated; Fig. 2B and Supplementary Table S1). Most transcripts returned to baseline levels at day 5.

Figure 1. Antitumor efficacy of the combination of CTX and adoptive immunotherapy. DBA/2 mice were implanted s.c. with 2 × 10⁸ 3Cl-8 FLC. After 4 days, some mice (n = 5) were injected with either CTX (83 mg/kg) or saline (CTR) and others received the adoptive transfer of 40 × 10⁵ spleen lymphomonocytes from tumor-vaccinated donor mice (ACT). In mice treated with chemoimmunotherapy, ACT was carried out 5 hours after CTX injection (CTX + ACT). Donor mice were vaccinated with either irradiated (100 Gy) or lysed (B), IFNα1-CI-11 tumor cells. Data are reported as the average tumor diameter of 5 mice per group ± SE. Representative experiments out of 5 are shown.
Day 5

Day 5

Day 1

OF4

modulation, the lists of upregulated and downregulated genes and molecular pathways involved in CTX-mediated immune-related genes. KEGG pathway analysis indicated that upregulated genes comprised mostly apoptotic pathways, cytokine-cytokine receptor interaction, and apoptosis (Fig. 3A and Supplementary Figs. S2 and S3). Both analyses indicated that upregulated genes comprised mostly immune-related genes.

Functional classification of genes differentially expressed in bone marrow by gene ontology and KEGG pathway analysis

To characterize the observed transcriptional profiles according to biological function and to identify functional and molecular pathways involved in CTX-mediated immunomodulation, the lists of upregulated and downregulated genes were subjected to gene ontology–based and KEGG pathway-based annotation by means of the DAVID bioinformatic tool (29). Interestingly, the biological processes found to be more significantly (P ≤ 0.05) stimulated by CTX in the bone marrow included genes regulating defense response, developmental processes, and response to external/chemical stimuli (see Table 1 and Supplementary Table S4, for a detailed list of upregulated genes). The most significantly represented KEGG pathways included chemokine and B-cell-receptor (BCR) signaling pathways, cytokine–cytokine receptor interaction, and apoptosis (Fig. 3A and Supplementary Figs. S2 and S3). Both analyses indicated that upregulated genes comprised mostly immune-related genes.

Increased transcripts included several chemokines, that is, CCL21C, CCL21B (ligands for CCR7), CXCL2 (MIP-2, chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells, HSC), CXCL7 (proplatelet basic protein) and
Mechanisms of Antitumor Efficacy of Chemoimmunotherapy

Table 1. Functional annotation charts of genes modulated 1–2 days after CTX administration in bone marrow and spleen

<table>
<thead>
<tr>
<th>Genes upregulated in bone marrow</th>
<th>N</th>
<th>P</th>
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<tr>
<td>Defense response</td>
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<td>Pattern specification process</td>
<td>17</td>
<td>0.020</td>
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<tr>
<td>Response to external stimulus</td>
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<td>0.028</td>
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<tr>
<td>Behavior</td>
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<tr>
<td>Response to chemical stimulus</td>
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<td>Embryonic development</td>
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<td>0.045</td>
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<th>P</th>
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<tr>
<td>Cell division</td>
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<tr>
<td>RNA processing</td>
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<td>Establishment of protein localization</td>
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<td>Chromosome segregation</td>
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<th>P</th>
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</thead>
<tbody>
<tr>
<td>Regulation of immune system process</td>
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<tr>
<td>Immune response</td>
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<td>Cellular developmental process</td>
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<td>Death</td>
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<tr>
<td>Leukocyte activation</td>
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<td>0.0178</td>
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<tr>
<td>Macromolecule localization</td>
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<td>0.0249</td>
</tr>
<tr>
<td>Regulation of multicellular organismal process</td>
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<td>Cell proliferation</td>
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<td>Cellular localization</td>
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<table>
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<th>Genes downregulated in the spleen</th>
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<th>P</th>
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<tbody>
<tr>
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<tr>
<td>Cell division</td>
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<td>Chromosome segregation</td>
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<td>Secondary metabolic process</td>
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<tr>
<td>Cellular metabolic process</td>
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<td>0.0406</td>
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*Functional annotation charts by means of DAVID bioinformatic tool (biological process, level 2).
*Number of genes in each class.
*EASE score, a modified Fisher exact P-value; only P-values \( \leq 0.05 \) were considered. The entire list of genes present on the array was used as background.

CXCL12 (chemoattractant for CD133+ HSC), Upregulated chemokine receptors included CCR6, CCR9, and IL8RB (Supplementary Fig. S2 and Tables S1 and S4). Of note, CTX treatment also induced increased expression of genes involved in cytokine/chemokine signaling pathways (JAK3, GRK6, PIK3CG, IAV2, IKBKG, and NFκB; Supplementary Fig. S3A). Accordingly, IL1B (IL-1β) and NOS2 (nitric oxide synthase 2), important mediators of inflammation, were upregulated (Supplementary Tables S1 and S4).

In addition, the receptors for granulocyte colony-stimulating factor (G-CSF; CSF3R), for VEGF (FLT1, KDR), and for TNF-related apoptosis-inducing ligand (TRAIL; TNFRSF10B) were upregulated at day 1–2. Noticeably, the upregulation of IL17B and of its receptor IL17R was accompanied by increased expression of IL1B, of the receptor for TGF-β (TGFBR2), and of FOXH1 and GREM1 (TGF-β signaling pathway), which are essential for Th17 differentiation (30; Supplementary Tables S1 and S4).

Among the immune-related genes that CTX induced in the bone marrow, we also found immunoglobulin heavy chain-2 (IGH-2), C20orf14 (ficolin A), and complement component C1q subunit A (C1QA), whose exposure is required for the immunogenicity of cell death, 2 alarmins (DEFB2 and DEFCR4), the stress response factor (HSF2), and several soluble and cell-associated pattern recognition receptors (PRR) responsible for the recognition of apoptotic/necrotic cells, such as mannose binding lectin (MBL1), FCN1 (ficolin A), PFC (complement protein properdin), and complement component C1q subunits (C1QA and C1QB), were upregulated 1 to 2 days after treatment (Supplementary Tables S1 and S4).

The functional classification of downregulated genes showed that the most significantly enriched biological classes included genes regulating cell division, RNA processing, metabolic processes, establishment of cellular localization, and chromosome segregation (Table 1). These results suggest that CTX induces the reduction of biosynthetic/metabolic processes and cell cycle presumably as a consequence of its cytotoxic activity and that, at the same time, it stimulates the expression of a plethora of immune-related genes.

Functional classification of genes differentially expressed in spleen and PBMC by gene ontology and KEGG pathway analysis

As is the case with bone marrow, the most significantly enriched biological classes of genes upregulated in the spleen were related to the regulation of immune response, whereas cell cycle, chromosome segregation, and metabolic process classes were enriched for downregulated genes (Table 1 and Supplementary Table S4). Also, KEGG pathway enrichment analysis showed a great similarity between bone marrow and spleen, because enriched pathways included chemokine and BCR signaling pathways, cytokine–cytokine receptor interaction, and apoptosis. In addition, in the spleen lysosome and TGF-β signaling pathways were also enriched (Fig. 3B and Supplementary Figs. S3 and S4).

Within immune-related transcripts, some were increased also in bone marrow (CXCL12, CXCL17, CXCL12, IL1B, C20orf14, TGFBR2), whereas others were peculiarly overexpressed in the...
spleen, such as the C-type lectin receptors (CLR) CLEC4A2 (DCIR), CD209B (SIGNR-1), CLEC4N (Dectin-2), and CLEC7A (Dectin-1), involved in the uptake of apoptotic cells by DC, and genes involved in CLR signal-transduction pathway (STK, BCL-10). Noteworthy, PTX3, which inhibits the capture of apoptotic cells by DC, was downregulated after treatment. Moreover, we observed the induction of genes associated with various processes of DNA repair, cell death, autophagy, and drug resistance (DDIT4, HIF1A, CLU, ATG7, HSP70–2; Supplementary Tables S2 and S4).

Additional upregulated genes comprised AHR (regulator of Treg and Th17 cell differentiation), factors regulating inflammation (IL-1 family members IL1B, IL1R1, IL1RAP) and cell proliferation (TNFSF13-APRIL, TNFSF13B-BAFF), and growth factors acting on different cell populations (FGF1, HBEGF, IGFI, PDGFB, VEGFA; Supplementary Fig. S4 and Tables S2 and S4). Noticeably, IL2RG (IL-2 receptor γ chain), which is involved in the response to homeostatic cytokines IL-2, IL-7, IL-9, IL-15, and IL-21, was overexpressed following treatment, corroborating previous findings indicating that CTX increases the expression of homeostatic cytokines (16).

Although in PBMC no significant enrichment of any biological process was found within upregulated genes, we could identify some cytokine-encoding transcripts (IL12A, IL12B, IL12R, IL15, IL15R, IL17A). As is the case with the spleen, CTX induced the expression of CD164 (expressed by primitive HSC), of the CLR CD209B and of HIF1A (Supplementary Table S3). Only one biological process (establishment of cellular localization) was enriched within downregulated genes (data not shown). Moreover, the results of KEGG pathway analysis were not similar to those for bone marrow or spleen (Fig. 3C).

To directly compare the correspondence of bone marrow, spleen, and PBMC transcriptional profiles, the overlap of the gene lists was examined in Venn diagrams. Interestingly, the spleen displayed a profile intermediate between bone marrow and PBMC, which were the least similar to each other (Fig. 3D and E); this could be related to the fact that, in mice, the spleen is an active hematopoietic organ and, at the same time, comprises fully mature cells. The most significantly enriched biological classes of genes simultaneously upregulated in bone marrow and spleen were defense response and response to stress and to external stimuli (data not shown). Altogether these results suggest that hematopoietic organs (spleen and bone marrow) are the most responsive organs to the damage mediated by CTX and react to it by activating immune-related genes.

To validate the results obtained by transcriptional profiling, the expression of selected genes was assessed by real-time...

Figure 3. Plots illustrating KEGG pathways significantly enriched within genes upregulated by CTX and Venn diagrams indicating the degree of overlap between gene expression profiles of bone marrow (BM), spleen, and PBMC. (A, B, C) The genes upmodulated 1–2 days after CTX treatment were clustered in different KEGG pathways by using DAVID and the significantly enriched pathways (P ≤ 0.05, EASE score) are shown for BM (A), spleen (B), and PBMC (C). The lists of genes significantly up- (D) and downregulated (E) 1–2 days following CTX treatment were used to create the Venn diagrams. The number of genes exhibiting common (or uncommon) modulation of expression in each tissue is shown.
PCR. In accordance with the results obtained by microarray, CARL and CLU transcript levels were augmented early after treatment in bone marrow and spleen (but not in PBMC). Moreover, although the expression of IL1B was increased at day 2 and decreased at day 5 in all tissues, CDCA8 was downregulated at day 2 and returned to baseline by day 5 (Supplementary Fig. S7 and Supplementary Methods).

**Effect of CTX on T-helper subpopulations**

Because Th17 cells were shown to exert a protective role against murine cancer by providing a more significant help than Th1 cells for tumor-specific CD8+ T cells (31), the novel observation of a Th17-related gene signature induced by CTX prompted us to evaluate, in the spleen, the modulation of this T-cell subtype at different times following CTX injection. The modulation of Th1, Th17, Tregs (CD4+ /Foxp3+), and activated CD4+ T cells (CD4+/CD25+/Foxp3+) was examined simultaneously by intracellular staining followed by FACS analysis. Interestingly, the absolute numbers of IFN-γ-producing CD4+ T-helper cells and Treg cells (CD4+ /Foxp3+ Foxp3+) T-helper cells and Treg cells (CD4+ /Foxp3+ Foxp3+) lymphocytes underwent a transient decrease 7 days following chemotherapy and were significantly (P < 0.05) augmented above the levels of untreated mice at day 10 and 14 (Fig. 4A). Th1, as expected, showed the most pronounced expansion, reaching a 6-fold increase compared with untreated mice, and Th17 and activated CD4+ T cells underwent a 2.5 and a 3.8 increment, respectively. We did not find CD4+ T cells coexpressing IL-17 and IFN-γ (data not shown). In contrast, Treg cell numbers, although showing kinetics of depletion similar to those of the other CD4+ subpopulations, exhibited a lower rebound, evident at a later time (significant only at day 14; Fig. 4A). These results indicate that CTX induces an imbalance in CD4+ T-cell subtypes, favoring effective immunity and disadvantaging tolerance, and corroborate recent findings suggesting that CTX induces Th17 cells in cancer patients (32). However, it should be noted that Tregs are only held back with respect to other CD4+ populations, because their number is anyway restored to pretreatment level, indicating that this is a transient imbalance and not a permanent depletion. In Fig. 4B, the effect of CTX on lymphocytes and CD4+ T cells is shown. CTX injection induced a transient depletion of lymphocytes followed by a rebound of their number above baseline without significantly altering the proportion of CD4+ T cells (Fig. 4B).

**CTX treatment stimulates a “storm” of cytokines, chemokines, and growth factors**

To characterize the cytokine response to CTX at the protein level, plasma and bone marrow lysates from tumor-bearing mice were subjected to Bio-Plex cytokine assays, which allowed the simultaneous analysis of 32 analytes.

Three days (7 for IL-9) after chemotherapy, the levels of IL-3, IL-5, IL-9, CCL2, CCL4, CCL11, G-CSF, and GM-CSF were significantly (P < 0.05) increased in plasma with respect to untreated mice, whereas IL-12, CXCL2, and macrophage colony-stimulating factor (M-CSF) were reduced (Fig. 5A, B, and C).

Regarding the bone marrow, CTX induced a transient depletion of cell numbers (and accordingly of their total protein content) at day 3, and a rebound, overcoming baseline,
Figure 5. CTX modulates the protein levels of cytokines, chemokines, and growth factors in plasma and bone marrow (BM). Before (day 0) and at the indicated time points after CTX, plasma and BM cells were collected from tumor-bearing mice and BM was lysed in Bio-Plex cell lysis buffer. The variation of the protein levels of cytokines (A, D), chemokines (B, E), and growth factors (C, F) was analyzed by Bio-Plex cytokine assay. Data represent the mean ± SE of the protein levels from 3 individual mice tested in duplicate. *, P ≤ 0.05, unpaired t-test.
Nevertheless, the analysis of cytokine expression per cell, that is, after normalizing the concentration of each cytokine to the number of cells at each time point, showed that the amount of several cytokines (IL-1β, IL-9, IL-12, IL-15, IL-18, and IFN-γ) increased significantly (P < 0.05) at day 3 and went back to baseline or below baseline by day 7 (Fig. 5D). Noticeably, the chemokines CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL9, and LIF and the growth factors platelet-derived growth factor (PDGF)-basic, fibroblast growth factor (FGF)-2, M-CSF, G-CSF, and GM-CSF followed the same kinetics (Fig. 5E-F). Of note, in both plasma and bone marrow, the majority of protein expression changes took place early (day 3), immediately following gene expression modulation (day 1 and 2) and preceding the recovery of cell numbers and, as for transcript, the protein expression modulation was transient.

These data not only represented the validation at the protein levels of some of the changes in gene expression assessed either by microarray analysis (i.e., IL-1β, CXCL2, PDGF, FGF) or previously by real-time PCR (IL-15, IFN-γ, GM-CSF; ref. 16), but also further expanded this information pointing out the many cytokines, chemokines, and growth factors regulating the recovery of bone marrow cells after the myelosuppressive stress exerted by CTX and constituting the microenvironment capable of supporting HSC proliferation, differentiation, and mobilization to the circulation.

The antitumor efficacy of the combined therapy depends on the timing of combination

The kinetics of gene and protein expression modulation showed that CTX-mediated immunomodulation is induced early after treatment and rapidly turned off. On the basis of these results, we investigated whether the timing of optimal combination of CTX and ACT was congruent, and possibly dependent, on the kinetics. As shown in Fig. 6, the antitumor efficacy of chemoimmunotherapy was maximal when ACT was carried out either 5 hours or 1 day after chemotherapy (i.e., at the time of the majority of gene expression changes), resulting in the cure of 5 out of 6 and 4 out of 6 tumor-bearing mice, respectively, and was completely lost if donor cells were infused 3 days after chemotherapy, indicating that transferred cells should be infused as early as possible to enable the benefit of the CTX-mediated cytokine milieu.

Discussion

Different studies have addressed systematically the effects of CTX on several of the multiple cells and factors driving tumor-induced tolerance and antitumor immunity (reviewed in ref. 2). However, such hypothesis-driven approaches, although deeply clarifying the contribution of each single actor, do not take into account the complexity of CTX-mediated immunomodulation. High-throughput analyses, such as genomics and proteomics, can efficiently complement...
these type of studies, giving a comprehensive picture of a complex situation, uncovering unknown factors and driving new hypotheses. Previously, the antitumor efficacy of chemoinmunotherapy was believed to mainly rely on the induction of lymphopenia by chemotherapy, which creates "room" for the adoptive transfer of tumor-specific lymphocytes (10, 11), or on the selective depletion of Tregs (12, 14). Although CTX indeed depletes Tregs in vivo, we and others have shown that these cells can repopulate the periphery (Fig. 4; ref. 33), highlighting the transient and possibly limiting nature of this hypothesis. We argued that the tolerogenic milieu induced by tumors is multifactorial and includes Tregs as well as other factors and that CTX conditioning can not only mitigate tolerogenic mechanisms but also produce a multifactorial immunogenic milieu favoring immunotherapeutic interventions. Consistent with such a view, the present study was designed to unravel the multiple factors underlying the ability of CTX to potentiate cancer immunotherapy. Taken together, microarray analyses show that CTX modulates the expression of an unexpectedly high number of genes in lymphoid organs, leading on one hand to the downregulation of genes involved in cell cycle and metabolic processes, which is in accordance with the inherent toxicity of an anticancer agent, and, on the other hand, to the upmodulation of immunomodulatory genes. An important finding, stemming from both genomic and proteomic analyses, is that CTX-mediated immunomodulation is early and transient, as previously shown in a different tumor model (RBL-5 lymphoma), by real-time PCR analyses of a limited panel of cytokines (GM-CSF, IL-1β, IL-7, IL-15, IL-2, IL-21, IL-13, and IFN-γ); ref. 16). These data point out that, to be effective, chemotheraphy and immunotherapy should be combined within a short time window. Remarkably, this hypothesis was confirmed by tumor growth experiments showing that the synergistic antitumor effectiveness of CTX and ACT strictly depends on the timing of combination and is progressively lost as days pass between 1 treatment and the other (Fig. 6).

Recent data suggest that the antitumor efficacy of chemotherapy (especially anthracyclines) depends on the induction of immunogenic apoptosis of tumor cells that stimulate antitumor immunity (34). Key elements dictating the immunogenicity of anthracycline-mediated cell death are the surface exposure of CARL and the release of HMGB1, which, in turn, activate TLR-4 (27). Studies from our group showed that CTX induces CARL exposure and release of DC-activating soluble factors by apoptotic tumor cells, resulting in increased cross-presentation of tumor antigens to CD8⁺ T cells (35). Here we show for the first time that CTX can augment, in bone marrow cells, the simultaneous expression of CARL and of 2 PRRs (MBL1 and C1Q) involved in its recognition and in apoptotic cell clearance (36). In addition, CTX augmented the transcript levels of other danger signals, that is, the alarmins DEFCR4 (defensin-α-4) and DEFB2 (defensin-β-2), the first involved in macrophage and T-cell chemotraction and the second able to induce DC maturation via TLR-4 (the same receptor of HMGB1; ref. 37).

Interestingly, additional DC-specific CLRIs were upregulated in the spleen, namely SIGNR1 and DCIR, involved in CD8⁺ T-cell cross-priming (38) and Dectin-1 and Dectin-2, whose role in the clearance of apoptotic cells has been recently underscored (39), and that, after ligand engagement, are able to induce DC activation and Th1/Th17 differentiation via a SYK-dependent signaling pathway that leads to the activation of NFKB (40–42). Strikingly, the upregulation of Dectins was associated with increased expression of SYK and of the adaptor BCL-10. Of note, it is possible to speculate that the Th17 signature induced by CTX and the expansion of Th1 and Th17 cells observed in the rebound phase after chemotherapy depends on the activation of this signaling pathway after "sensing" the damage of chemotherapy by PRR. In this regard, early after CTX injection, we observed the induction of genes associated with DNA repair, cell death, autophagy, and drug resistance (DDIT4, HIF1A, CLU, ATG7, and HSP70–2; ref. 43–45).

In physiologic settings, the engulfment of apoptotic cells by phagocytes inhibits the release of inflammatory cytokines and leads to T-cell tolerance. The perception of danger signals may have a role in tipping the balance from tolerance to inflammation and effective T-cell activation (46). Accordingly, we show here that after CTX treatment several IL-1 family members are upregulated in the spleen and both transcript and protein levels of proinflammatory cytokines (IL-1β, IL-17, IL-18, IFN-γ, LIF) and chemokines (CCL2, CCL3, CCL4, CCL5; ref. 47) are augmented in bone marrow. Remarkably, IL-1β and IL-18, the most important mediators of inflammation, control innate and adaptive (including Th1-Th17) responses (48). Moreover, CTX increased the expression of NOS2, an enzyme responsible for the generation of free radical NO and involved in inflammation, the expression of which has been shown to be triggered by IL-17 via the transcription factor NFBK (49). The proinflammatory action of IL-17 induces the expression of target genes such as CXCL1, CXCL2, and defensins (50), which were indeed augmented in bone marrow and plasma. IL-17 is also an important modulator of hematopoiesis through the induction of GM-CSF, G-CSF, and CCL2 (51), which were augmented in bone marrow lysates and plasma. Moreover, HIF1A and its target gene VEGFA levels were increased in the spleen, which is in accordance with data showing that CTX induces hypoxia along with HIF1A and VEGFA upregulation, playing a key role in inflammation and hematopoiesis (52).

The hypothesis stemming from these data is that CTX induces an immunogenic apoptosis not only of tumor cells but also of host bone marrow and spleen cells and, at the same time, activates inflammatory mediators, leading to the promotion of hematopoiesis and immune response. In accordance with this hypothesis, the apoptosis of self T cells was shown to elicit self-reactive CD8⁺ T cells, thus inducing a systemic immunoactivation during HIV infection (53). This premise also suggests that CTX may be suitable for enhancing the efficacy of immunotherapy in adjuvant settings, in which mounting an effective vaccine-induced antitumor immunity in the absence of tumor burden is fundamental to prevent recurrences. In this regard, we have recently shown that dacarbazine, an alkylating agent similar to CTX, can enhance CD8⁺ memory T-cell responses to peptide-based vaccination in resected melanoma patients (54).
We have previously shown that the antitumor effectiveness of chemoimmunotherapy depends on adoptively transferred CD4+ T cells (16) and, among them, Th17 was proved to exhibit a stronger therapeutic efficacy than Th1 (31). Several authors reported differential effects of CTX on CD4+ T cells, including a Th2 to Th1 shift in cytokine production (16, 55). The present data reveal for the first time that CTX induces an environment simultaneously favoring Th1, Th17, and activated CD4+ T cells, suggesting that this milieu may condition not only host cells but also transferred tumor-specific CD4+ T cells, thus leading to improved tumor control.

Taken together, our data sustain the fascinating hypothesis that CTX-mediated immunomodulation stems directly from its cytotoxicity that produces DNA damage, cell cycle arrest, and cell homeostasis perturbation not only of tumor cells but also of host highly proliferating cells, such as bone marrow and splenocytes. DNA-damage responses are complex and involve "sensor" proteins perceiving danger and transmitting signals to "transducer" proteins, which, in turn, convey signals to "effector" factors (56). Cells damaged or killed by CTX within lymphoid organs would therefore expose/release danger signals that in turn, through PRR signaling, would transiently activate proinflammatory pathways and create a beneficial environment aimed at repairing the damage and able, as a bystander effect, to promote innate and adaptive immunity, including Th1 and Th17 responses. When an immune intervention (vaccination or ACT) is carried out during the recovery phase, also tumor-specific immune cells can take advantage of this drug-mediated immunomodulation. Although with the present experimental design we could not ascertain in which cell subtype the expression of a certain gene or protein was modulated, this study describes for the first time all the sensor, transducer, and effector factors involved in the host response to CTX, opens novel hypotheses that may guide further experimentation, and, lastly, supplies a clear-cut indication of the short time window in which chemotherapy and ACT should be combined to maximize chemoimmunotherapy effectiveness against cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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