Cyclophosphamide Induces a Type I Interferon–Associated Sterile Inflammatory Response Signature in Cancer Patients' Blood Cells: Implications for Cancer Chemoimmunotherapy

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Abstract

**Purpose:** Certain chemotherapeutics, particularly cyclophosphamide, can enhance the antitumor efficacy of immunotherapy. A better understanding of the cellular and molecular basis of cyclophosphamide-mediated immunomodulation is needed to improve the efficacy of chemoimmunotherapy.

**Experimental Design:** Transcript profiling and flow cytometry were used to explore cyclophosphamide-induced immunoadjuvanticity in patients with hematologic malignancies.

**Results:** A single high-dose treatment rapidly (1–2 days) induced peripheral blood mononuclear cell (PBMC) transcriptional modulation, leading to reduction of cell-cycle and biosynthetic/metabolic processes and augmentation of DNA damage and cell death pathways (p53 signaling pathway), death-related scavenger receptors, antigen processing/presentation mediators, T-cell activation markers and, noticeably, a type I IFN (IFN-I) signature (OAS1, CXCL10, BAFF, IFITM2, IFI6, IRF5, IRF7, STAT2, UBE2L6, UNC93B1, ISG20L1, TYK2). Moreover, IFN-I–induced proinflammatory mediators (CXCL10, CCL2, IL-8, and BAFF) were increased in patients' plasma. Accordingly, cyclophosphamide induced the expansion/activation of CD14⁺CD16⁺ monocytes, of HLA-DR⁺, IL-8RA⁺, and MARCO⁺ monocytes/dendritic cells, and of CD69⁺, OX40⁺, and IL-8RA⁺ lymphocytes.

**Conclusions:** Altogether, these data identify the cyclophosphamide-induced immunomodulatory factors in humans and indicate that preconditioning chemotherapy may stimulate immunity as a consequence of danger perception associated with blood cell death, through p53 and IFN-I–related mechanisms. Clin Cancer Res; 19(15); 4249–61. ©2013 AACR.

Introduction

Much interest has been recently gained by the possibility of manipulating the host milieu with chemotherapy and in particular with cyclophosphamide, to synergize with adoptively transferred immune cells for antitumor purposes (1, 2). Although historically regarded as immunosuppressive, cyclophosphamide has been shown to act as a strong adjuvant for either adoptive or active immunotherapy when used with carefully defined dosages and combination modalities (1–4). In addition, it has been shown that the therapeutic outcome of conventional chemotherapy depends on the activation of the immune system as a consequence of immunogenic apoptosis of cancer cells that, in turn, depends on the coordinated emission of specific signals from dying cancer cells (5, 6).

The synergistic antitumor efficacy of the combination of cyclophosphamide and immunotherapy has long been studied in preclinical models (7–10), as well as in clinical trials (11–13), highlighting the multiple mechanisms underlying this paradoxical phenomenon, which include provision of “space” (14), suppression of regulatory T cells (15), augmentation of tumor infiltration by lymphocytes, functional activation of B and T cells, and homeostatic proliferation (8, 9, 16, 17).

In mouse models, type I IFN (IFN-I) was identified as an important mediator of cyclophosphamide immunomodulation (8). Subsequent studies showed that IFN-I was indeed induced in vivo by cyclophosphamide and that this cytokine was responsible for the expansion of memory CD4⁺ and CD8⁺ T cells (18). More recent data indicated that cyclophosphamide can affect dendritic cell homeostasis (19) and can restore an activated polyfunctional helper phenotype in tumor-specific adoptively transferred CD4⁺ T cells (20) through IFN-I–dependent mechanisms.

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We also reported that the synergistic anticancer activity of chemo- and immunotherapy is associated with the induction of a "cytokine storm," occurring primarily in the bone marrow of treated mice. Upregulated factors included granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-1β, cytokines regulating homeostatic expansion and T-cell survival (IL-7, IL-15, IL-2, and IL-21) and involved in the polarization toward a T-helper cell (Th) 1 type of immune response (IFN-γ; ref. 17). Genomic and proteomic analyses in mouse models showed that cyclophosphamide modulates the expression of approximately 1,000 genes in the bone marrow and spleen, and of a great number of cytokines in the plasma and bone marrow lysates of tumor-bearing mice, including danger signals, pattern-recognition receptors (PRR), inflammatory mediators, growth factors, cytokines, chemokines, and chemokine receptors (21). The analysis of gene and protein expression kinetics and of the antitumor efficacy of different therapeutic schedules of combination showed that the optimal timing for conducting adoptive immunotherapy is 1 day after cyclophosphamide treatment in mice (21).

The importance of the timing between chemotherapy and cell infusion has also been shown in clinical studies by our group and by others in the allogeneic stem cell transplant (SCT) setting for patients with hematologic malignancies (22, 23). Miller and colleagues reported that a fully lymphodepleting chemotherapy (cyclophosphamide 50 mg/kg/d on day 6 and fludarabine 25 mg/m²/d from day 6 to 2) followed by donor lymphocyte infusion (DLI) at day 0 leads to an in vivo lymphocyte expansion/activation and to an increment of high-grade GvHD compared with patients who received DLI alone (22). We reported that patients with acute leukemia, relapsed after an allogeneic SCT, undergoing DLI 2 days after a chemotherapy treatment, presented an overall increased production of IFN-γ, TNF-α, and IL-2 as well as an amplification of activated lymphocytes that correlated with a graft-versus-leukemia (GvL) effect and achievement of hematologic complete remission (23). In the context of solid tumors, it was shown that highly lymphodepleting regimens (cyclophosphamide 60 mg/kg/d for 2 days, fludarabine 25 mg/m²/d for 5 days, and 12-Gy total-body irradiation) followed by the adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) resulted in objective response rates of 70% in patients with metastatic melanoma refractory to standard therapies (13). More recent data showed that high-dose cyclophosphamide alone (4 g/m²), as conditioning before the infusion of melanoma-specific CD8+ T-cell clones, determined long-term T-cell persistence, their acquisition of a central memory phenotype and, noticeably, a clinical benefit in 50% of patients with metastatic melanoma (24).

The present study aims at investigating for the first time the gene expression modulation after a single high-dose cyclophosphamide administration in human peripheral blood mononuclear cell (PBMC) and at identifying the mediators and mechanisms through which cyclophosphamide conditions the host immune system to perceive and react to tumor antigens in chemoimmunotherapy strategies. Patients with hematologic malignancies, receiving high-dose cyclophosphamide (3–4 g/m²) for stem cell mobilization before SCT, were investigated.

Materials and Methods

Patients’ characteristics, treatment, and PBMC isolation

A single high dose of cyclophosphamide (Endoxan; Baxter; 3–4 g/m²) was administered as mobilization regimen before autologous hematopoietic SCT to 1 patient with plasma cell leukemia (PCL), 1 patient with T-cell prolymphocytic leukemia (T-PLL), and 14 patients with multiple myeloma (Supplementary Table S1). Patients were treated at the Department of Hematology, Azienda Policlinico Umberto I, Sapienza University, (Rome, Italy) according to the principles set out in the World Medical Association (WMA) Declaration of Helsinki.

Blood samples were obtained after written informed consent. Heparinized blood was collected pretherapy and 1, 2, and 5 days after cyclophosphamide administration by venipuncture. After separation of plasma, PBMCs were obtained using Lymphoprep density centrifugation (Nycomed AS). Part of the cells was cryopreserved and part was lysed in RLT buffer for RNA extraction (Qiagen).

RNA isolation, labeling, and hybridization

Total RNA was obtained by RNeasy purification according to the manufacturer’s instructions (Qiagen). Amino-allyl-modified antisense RNA (aRNA) was synthesized in two amplification rounds from 1 μg total RNA using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion), and its quality was assessed with the 2100 Bioanalyzer (Agilent Technologies). aRNAs were coupled to monoreactive Cy3 or
Microarray data analysis

Scanning and image file processing were conducted with GenePix 4200A instrument (Axon Instruments) and the obtained data were filtered with BRB-ArrayTools (developed by Dr. Richard Simon and BRB-ArrayTools Development Team, National Cancer Institute) to exclude spots below a minimum intensity (200), flagged and with diameters less than 25 \( \mu \)m. Data were normalized using Lowess Smoother. Only genes expressed in at least 70% of samples were analyzed in subsequent statistical analyses (all done using the log-transformed ratios).

Statistically significant \((P < 0.005)\) differentially expressed genes among post- and pretreatment samples were identified with class comparison (paired test with random variance model, BRB-ArrayTools). Hierarchical clustering was conducted using average linkage and uncentered correlation (Cluster Version 3.0). Following average correction, the results of clustering analyses were visualized with TreeView software.

Functional annotation- and pathway-based analyses were conducted by means of Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatic tool. Enriched biologic processes and pathways were ranked according to the EASE score, a modified Fisher Exact P-Value, 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.

cDNA synthesis and real-time reverse transcription PCR (qRT-PCR)

cDNA templates were obtained by reverse transcription of aRNA (Promega). Quantitative measurements of specific transcripts were acquired using an iCycler iQ real-time Thermocycler Detection System (Bio-Rad), and the amplifications were conducted with QuantiTect SYBR Green PCR reagents (Qiagen). The primers for GAPDH, BAFF, CLEC10A, IRF5, MARCO, CXCL10, and CCL2 were manually designed and synthetized by PRIMM (Supplementary Methods).

To verify the amplification of a single product, a melting curve was generated after every run. Relative expression levels were calculated by the comparative cycle threshold (\( \Delta \Delta C_t \)) method and were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as previously described (21).

Plasma protein levels determination by ELISA

The concentration of CXCL10/IP-10, CXCL8/IL-8, CCL2/MCP-1, BAFF/TNF\( \alpha \)F13B, and CD163 in plasma was assessed with the ELISA-based assays Quantikine (R&D Systems). Heparin-binding EGF-like growth factor (HB-EGF) was analyzed with the Abnova ELISA Kit (Abnova GmbH). Briefly, 50 to 100 \( \mu \)L of plasma was used in each assay, according to the manufacturers’ instructions, and the absorbance of the developed color was determined using a microplate reader set to 450 nm. Cytokine concentrations were extrapolated from the standard curves generated using recombinant human proteins.

Flow cytometry

PBMC phenotype was determined by six-color immunofluorescence staining. For monocyte characterization, PBMCs were stained with anti-CD14-phycocerythin (PE)-Cy7, anti-CD16-APC-H7, anti-HLA-DR-PerCP-Cy5.5 (Becton Dickinson), purified anti-MARCO [Pierce Antibodies; Thermo Scientific; revealed with goat anti-mouse (GAM) immunoglobulin G-immunoglobulin M (IgG-IgM) fluorescein isothiocyanate (FITC)], anti-CD217-Alexa Fluor 647 (BioLegend), and anti-CD181-PE (R&D Systems). Circulating dendritic cells (cDC) were identified as negative for staining with a cocktail of PE-Cy7-conjugated monoclonal antibodies directed against CD3, CD14, CD19 (lineage negative; Becton Dickinson) and positive for a mix of biotinylated antibodies: anti-CD11c, anti-CD141 (BDCA3), and anti-CD304 (BDCA4; Miltenyi). The B-1a subset was revealed with streptavidin–APC–Cy7 (Becton Dickinson). Dendritic cells were also stained with anti-HLA-DR-PerCP-Cy5.5, anti-MARCO-GAM-FITC, anti-DCIR-PE (R&D Systems), and anti-CD217-Alexa Fluor 647 according to the manufacturers’ instructions. To analyze the expression of T-cell markers, PBMCs were labeled with anti-CD3-APC-H7, anti-CD8-PE-Cy7, anti-CD134-FITC, anti-CD69-PerCP-Cy5.5 (Becton Dickinson), anti-CD217-Alexa Fluor 647, and anti-CD181-PE (R&D Systems).

Samples were acquired on a FACScan flow cytometer (BD Biosciences) and analysis was conducted with DIVA (BD Biosciences) and FlowJo (TreeStar) software. Statistical analysis of cyttofluorimetric data was conducted with IBM SPSS Statistic 20.

Results

Gene expression profiling of PBMC in response to cyclophosphamide

To analyze the impact of a single high-dose cyclophosphamide administration on human PBMC gene expression and to elucidate the mechanisms through which this drug may influence the immune system, the response to cyclophosphamide was analyzed in 1 patient with T-PLL, 1 patient with PCL, and 8 patients with multiple myeloma (Supplementary Table S1). Microarray analysis was conducted before (pre) and at different times (1, 2, and 5 days) after chemotherapy. The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s (NCBI) GEO and are accessible through GEO Series accession number GSE39324.

Statistically significant differentially expressed genes were identified by means of class comparison (BRB-ArrayTools) followed by hierarchical clustering analysis. Figure 1A shows

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that 1, 2, and 5 days after cyclophosphamide administration the expression of, respectively, 145, 656, and 212 genes was significantly \((P < 0.005)\) modulated (Fig. 1A), thus showing that cyclophosphamide has a strong impact on PBMC gene expression, particularly evident at day 2.

To analyze the kinetic of the transcriptional response to cyclophosphamide, we pooled altogether the 890 genes differentially expressed post-cyclophosphamide (118 genes were modulated at more than one time point) and subjected them to hierarchical clustering analyses. As shown in Fig. 1B (unsupervised clustering of the samples), pretreatment samples (d0) and samples taken 5 days following cyclophosphamide administration (d5) were more similar to each other than samples taken at day 1 or 2. Figure 1C (unsupervised clustering of the genes) shows that the genes modulated at day 2 have the tendency to show similar expression variations already at day 1 with a certain degree of intrapatient variability. Moreover, the transcript levels of most of the genes modulated at day 1 and/or 2 reverted to pretreatment levels by day 5 in 3 of 5 patients and remained differentially expressed in the remaining 2, showing that the kinetic of the gene expression modulation is variable among patients, being transient in some patients and more durable in others. A few genes were either up- or downregulated only at day 5. Supplementary Tables S2 and S3 show the entire lists of genes upregulated or downregulated 1 and/or 2 days following cyclophosphamide treatment.
Functional classification of differentially expressed genes by Gene Ontology and pathway analyses

To characterize the observed transcriptional profiles according to biologic function and to interpret the data in the context of pathways and networks involved in cyclophosphamide-mediated immunomodulation, the lists of genes modulated at day 1 and/or 2 were subjected to Gene Ontology–based and pathway-based annotation by means of DAVID bioinformatic tool (25).

Remarkably, the most significant \( P \leq 0.05 \) biologic process stimulated by cyclophosphamide in patients' PBMC included immune response–related genes. Other significantly enriched biologic functions were: response to other organisms, response to stress, response to biotic stimulus, catabolic process, and cell death (Table 1). The functional classification of downregulated genes showed that the most significantly enriched biologic classes included genes regulating metabolic processes, cell cycle, organelle organization, ribonucleoprotein complex biogenesis, and biosynthetic processes (Table 1).

Immune-related increased transcripts included scavenger receptors, such as CD68, MARCO (scavenger receptor class

Table 1. Functional annotation charts of genes modulated 1 to 2 days after cyclophosphamide administration

<table>
<thead>
<tr>
<th>Biologic processa of upregulated genes</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>25</td>
<td>7.0E-6</td>
</tr>
<tr>
<td>Response to other organism</td>
<td>13</td>
<td>2.6E-4</td>
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<tr>
<td>Response to stress</td>
<td>39</td>
<td>9.2E-4</td>
</tr>
<tr>
<td>Response to biotic stimulus</td>
<td>14</td>
<td>1.1E-3</td>
</tr>
<tr>
<td>Catabolic process</td>
<td>31</td>
<td>1.6E-3</td>
</tr>
<tr>
<td>Cellular response to stimulus</td>
<td>23</td>
<td>2.3E-3</td>
</tr>
<tr>
<td>Regulation of molecular function</td>
<td>24</td>
<td>7.9E-3</td>
</tr>
<tr>
<td>Regulation of biologic quality</td>
<td>32</td>
<td>1.0E-2</td>
</tr>
<tr>
<td>Cell death</td>
<td>19</td>
<td>1.0E-2</td>
</tr>
<tr>
<td>Cellular homeostasis</td>
<td>14</td>
<td>1.1E-2</td>
</tr>
<tr>
<td>Regulation of response to stimulus</td>
<td>14</td>
<td>1.2E-2</td>
</tr>
<tr>
<td>Positive regulation of response to stimulus</td>
<td>9</td>
<td>1.6E-2</td>
</tr>
<tr>
<td>Alcohol metabolic process</td>
<td>13</td>
<td>1.6E-2</td>
</tr>
<tr>
<td>Positive regulation of immune system process</td>
<td>9</td>
<td>1.6E-2</td>
</tr>
<tr>
<td>Positive regulation of biologic process</td>
<td>40</td>
<td>1.9E-2</td>
</tr>
<tr>
<td>Response to external stimulus</td>
<td>21</td>
<td>2.7E-2</td>
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<tr>
<td>Multicellular organism homeostasis</td>
<td>5</td>
<td>2.8E-2</td>
</tr>
<tr>
<td>Regulation of immune system process</td>
<td>11</td>
<td>3.9E-2</td>
</tr>
<tr>
<td>Interspecies interaction between organisms</td>
<td>9</td>
<td>4.4E-2</td>
</tr>
<tr>
<td>Oxidation reduction</td>
<td>15</td>
<td>4.7E-2</td>
</tr>
<tr>
<td>Positive regulation of cellular process</td>
<td>35</td>
<td>5.0E-2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biologic processa of downregulated genes</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen compound metabolic process</td>
<td>128</td>
<td>1.3E-9</td>
</tr>
<tr>
<td>Cellular metabolic process</td>
<td>186</td>
<td>6.0E-8</td>
</tr>
<tr>
<td>Macromolecule metabolic process</td>
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<tr>
<td>Primary metabolic process</td>
<td>187</td>
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<tr>
<td>Cell cycle</td>
<td>38</td>
<td>4.7E-6</td>
</tr>
<tr>
<td>Organelle organization</td>
<td>53</td>
<td>1.3E-5</td>
</tr>
<tr>
<td>Ribonucleoprotein complex biogenesis</td>
<td>13</td>
<td>5.2E-4</td>
</tr>
<tr>
<td>Cell-cycle process</td>
<td>26</td>
<td>6.4E-4</td>
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<tr>
<td>Biosynthetic process</td>
<td>99</td>
<td>1.2E-3</td>
</tr>
<tr>
<td>Cellular macromolecular complex subunit organization</td>
<td>17</td>
<td>3.1E-3</td>
</tr>
<tr>
<td>Regulation of metabolic process</td>
<td>96</td>
<td>7.9E-3</td>
</tr>
<tr>
<td>Establishment of RNA localization</td>
<td>7</td>
<td>1.7E-2</td>
</tr>
<tr>
<td>Interspecies interaction between organisms</td>
<td>13</td>
<td>2.2E-2</td>
</tr>
</tbody>
</table>

Table 1. Functional annotation charts of genes modulated 1 to 2 days after cyclophosphamide administration

\( ^a \)Functional annotation charts by means of DAVID bioinformatic tool (Biologic process, level 2).

\( ^b \)Number of genes in each class.

\( ^c \)EASE score, a modified Fisher exact \( P \) value, only \( P \) values 0.05 or less were considered. The entire list of genes present on the array was used as background.
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A, member 2), CD163L1 (scavenger receptor cysteine-rich type I protein M160), SCARB2/LIMP-2 (scavenger receptor class B, member 2), and C1R, a component of the multi-molecular complex C1 comprising C1q and C1s, which were all shown to be involved in the recognition of stressed and dying cells (refs. 26–29; Supplementary Table S2).

Moreover, 1 to 2 days postchemotherapy, the receptors of inflammatory cytokines IL-8 (IL8RA) and IL-17 (IL17R), as well as the receptor of IL-10 (IL10RB), LST1 (leukocyte specific transcript 1), an LPS and IFN-γ-inducible gene, and several leukocyte Ig-like innate receptors (LIR; LILRB1, LILRB2, LILRB3, and LILRB4) were upregulated (Supplementary Table S2). At the same time, the transcript levels of factors implicated in antigen processing and presentation were augmented, including CITA (class II, MHC, transactivator), CD68, three cathepsins (CTSC, CTSL1, and CTSZ), the α-galactosidase GLA, the α-glucosidase GAA, the serine protease TTP1, NEU1, SLC11A1 (a late phagosomal protein), and LAMP-2 (lysosomal-associated membrane protein 2).

Also genes belonging to the TNF superfamily, such as TNFRSF1A (TNF-α-receptor 1), TNFSF13B (BAFF), playing a critical role in B-cell expansion/migration, and TNFRSF4 (OX40), important for antigen-specific T-cell expansion/survival, were upregulated. In addition, CD97, an activation-induced antigen expressed by lymphocytes was induced by cyclophosphamide (Supplementary Table S2).

Of note, several of the cyclophosphamide-induced genes are known to be induced by IFN-γ, and are indicative of IFN-γ signature in different settings (30, 31), among them are: OAS1 (2′,5′-oligoadenylate synthetase 1), BAFF, IFITM2, IFI16, IRF5, IRF7 (IFN regulatory factor 5 and 7), STAT2, UBE2L16, UNCG93B1, ISG20L1, and TYK2, a molecule required for optimal IFN-γ-induced signaling (Supplementary Table S2).

The expression of numerous genes associated with stress, cell death, DNA repair, autophagy, and chemotherapy resistance was increased in patients’ PBMC early after cyclophosphamide treatment. Among them, BCL6, which controls DNA-damage response, survival, cell cycle, and cytokine-signaling, DRAM (damage-regulated autophagy modulator), BBC3 (Bcl2-binding component 3), which is an essential mediator of p53-dependent and -independent apoptosis induced by DNA damage, BAX (Bcl2-associated X protein), TRAP1, which prevents induction of apoptosis in response to low levels of DNA damage, XORC1, which corrects defective DNA strand-break repair and sister chromatid exchange following treatment with ionizing radiation and alkylating agents, BID (BH3-interacting domain death agonist), which is phosphorylated upon DNA damage and forms heterodimers either with the proapoptotic protein BAX or the antiapoptotic protein Bcl-2, and PYCARD, which mediates the activation of caspase-1 and the subsequent secretion of IL-1β and IL-18.

Pathway analyses confirmed and expanded previous results. In fact, the most significantly represented KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were “lysosome” and “p53 signaling.” Lysosomal genes included CD68, CTSC, CTS1, CTSZ, GLA, GAA, LAMP2, SCARB2, NEU1, SLC11A1, and TTP1. The “p53 signaling pathway” induced by cyclophosphamide included CDKN1A (cyclin-dependent kinase inhibitor 1A), CCND3 (cyclin D3), BAX, BBC3 (PUMA), BID, DDB2 (damage-specific DNA-binding protein 2), and SESN2 (sestrin 2), which are all involved in DNA damage repair, cell-cycle arrest, and apoptosis following different stress signals (Table 2 and Supplementary Fig. S1). Pathway analysis using Panther pathway database showed that most augmented transcripts encode for genes involved in the “Inflammation mediated by chemokine and cytokine signaling” pathway (GNA15, IL10RB, NFkBIA, PLA2G4A, PLCG2, RHOC, RGS19, RRAS, ARPC3, and TYK2) and “B cell activation pathway” (NFkBIA, PLCG2, RRAS, and SYK; Table 2).

Downregulated pathways included “Spliceosome,” “DNA replication,” “Cell cycle,” “Pyrimidine metabolism,” “Aminoacyl-tRNA biosynthesis,” and “Ubiquitin proteasome” (Table 2).

Altogether these results suggest that cyclophosphamide induces biosynthetic/metabolic process and cell-cycle arrest, as well as cell death as a consequence of its cytotoxic activity on PBMC, and that, at the same time, it stimulates the expression of genes involved in the perception of stressed and dead cells and of immunomodulatory genes, including IFN-γ-related and p53-related genes.

Validation of the microarray results by qRT-PCR

To validate the microarray results, the expression of selected genes was assessed by qRT-PCR on the same patients analyzed by microarray (Fig. 2A and B) as well as on a second cohort of 6 patients with multiple myeloma (Supplementary Table S1 and Fig. 2C). In Fig. 2A are shown the mean fold changes of transcript levels at different times after cyclophosphamide administration as compared with pretreatment levels. The scavenger receptor MARCO showed a trend to increase 2 and 5 days post-cyclophosphamide, whereas CLEC10A levels were significantly decreased (Fig. 2A). Moreover, significantly increased expression of IRF5 (involved in IFN-α transcriptional activation) and of BAFF was observed posttreatment (Fig. 2A). The correlation of microarray versus qRT-PCR results is shown in Fig. 2B for the significant time points.

Previous gene expression analyses showed that also another alkylating agent, namely dacarbazine, induces the expression of IFN-γ-related genes and among them CXL10 (IP-10), a proinflammatory chemokine identified as part of the “core” IFN signature observed in PBMC of patients with IFN-γ–treated melanoma and healthy individuals (30). We therefore assessed by qRT-PCR whether the expression of CXL10 and of another inflammatory IFN-γ and stress-induced chemokine, namely CCL2 (MCP-1; ref. 32), was modulated by cyclophosphamide and not revealed by microarray analysis. Figure 2A shows a trend to increased expression for both transcripts at day 2, even though statistical significance was not reached because of interpatient variability.
The analysis of the modulation of MARCO, CLEC10A, IRF5, BAFF, CXCL10, and CCL2 expression induced by cyclophosphamide in a second set of patients further validated microarray and qRT-PCR results (Fig. 2C).

Increase of CXCL10, CCL2, IL-8, and BAFF plasma levels following cyclophosphamide treatment

To further characterize the inflammatory response to cyclophosphamide, the variations of CXCL10, CCL2, and IL-8 plasma levels were assessed at different times after cyclophosphamide administration. Moreover, we analyzed the modulation of BAFF, HB-EGF, and the soluble form of CD163.

As shown in Fig. 2D, CXCL10 and BAFF plasma levels were significantly \( P \leq 0.05 \) raised 2 and 5 days following cyclophosphamide administration, whereas CCL2 was significantly increased 5 days after chemotherapy and IL-8 plasma levels were augmented at day 2 and returned to baseline levels by day 5. On the contrary, HB-EGF and soluble CD163 levels were not affected by treatment.

These data not only validate at the protein levels some of the changes observed in gene expression both by microarray analysis and/or by qRT-PCR, but also further expanded this information pointing to the inflammatory milieu resulting from damage in self tissues induced by chemotherapy.

**Immunophenotype of monocytes, dendritic cells, and T lymphocytes**

To characterize the effect of cyclophosphamide on numbers of cDCs, monocytes, and T lymphocytes and on their expression of markers selected on the basis of the microarray results, a multiparametric flow-cytometric analysis was conducted in all assessable patients.

The white blood cell (WBC) count before and after treatment showed, as expected, that high-dose cyclophosphamide was indeed strongly leukotoxic, inducing a significant \( P = 0.009 \) WBC decline already at day 2 (Fig. 3A). Remarkably, lymphocytes and monocytes showed different kinetics of depletion, with lymphocytes declining more rapidly (starting at day 1; \( P = 0.008 \)) than monocytes. At day 2, when lymphocytes were 3.5 times less than pretreatment levels \( (P = 3.6 \times 10^{-5}) \), monocytes showed only a 1.5-fold decrease \( (P = 0.002; \) Fig. 3A). Accordingly, fluorescence-activated cell sorting (FACS) analysis showed that the relative percentage of CD14\(^+\) monocytes in PBMC increased at day 1 and diminished at day 5 \( (P = 0.05) \), whereas the relative percentage of CD3\(^+\) lymphocytes increased at day 5 (Fig. 3B).

### Table 2. Pathway analysis of genes modulated 1 to 2 days after cyclophosphamide administration

<table>
<thead>
<tr>
<th>KEGG pathways(^a) of upregulated genes</th>
<th>( N^b )</th>
<th>( P^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosome</td>
<td>CD68, CTSC, CTSL1, CTSZ, GLA, GAA, LAMP2, SCARB2, NEU1, SLC11A1, TFF1</td>
<td>11</td>
</tr>
<tr>
<td>p53 signaling pathway</td>
<td>BCC3, BAX, BID, CCND3, CDKN1A, DDB2, SESN2</td>
<td>7</td>
</tr>
<tr>
<td>Panther pathways(^a) of upregulated genes</td>
<td>GNA15, IL10RB, NFKBIA, PLA2G4A, PLGC2, RHOC, RGS19, RRRAS, ARPC3, TYK2</td>
<td>10</td>
</tr>
<tr>
<td>B cell activation</td>
<td>NFKBIA, PLCG2, RRAS, SYK</td>
<td>4</td>
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<tr>
<th>KEGG pathways(^a) of downregulated genes</th>
<th>( N^b )</th>
<th>( P^c )</th>
</tr>
</thead>
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<tr>
<td>Splicesosome</td>
<td>RBM17, RP11-78J21.1, HNRNPA1, HNRPA3, HNRPM, SFRS2, SFRS5, SFRS6, TCERG1</td>
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<td>DNA replication</td>
<td>MCM2, MCM3, MCM6, RPA1, RPA2</td>
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<td>Cell cycle</td>
<td>DBF4, WEE1, CCND2, HDAC1, MCM2, MCM3, MCM6</td>
<td>7</td>
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<tr>
<td>Pyrimidine metabolism</td>
<td>AK3, CMPK, POLR3GL, RH14, RRM2, TYMS</td>
<td>6</td>
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<tr>
<td>Aminoacyl-tRNA biosynthesis</td>
<td>DTD1, IARS2, FARS2, YARS2</td>
<td>4</td>
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<tr>
<td>Panther pathways(^a) of downregulated genes</td>
<td>ATAD2, APPBP1, hCG, 15200, PSMC2, PSMD11, UBR5, UBE2B, UBE2H</td>
<td>8</td>
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</table>

\(^a\)Pathway analysis by means of DAVID bioinformatic tool.
\(^b\)Number of genes in each pathway.
\(^c\)EASE score, a modified Fisher exact \( P \) value. The entire list of genes present on the array was used as background.
Cyclophosphamide (CTX) treatment modulates the transcript levels of MARCO, CLEC10A, IRF5, BAFF, CXCL10, and CCL2 and the plasmatic levels of CXCL10, CCL2, IL-8, and BAFF. A–C, time course analysis of the modulation of MARCO, CLEC10A, IRF-5, BAFF, CXCL10, and CCL2 gene expression at different times following cyclophosphamide treatment. Relative mRNA levels were calculated by the comparative cycle threshold (CT) method and were normalized to GAPDH expression. A, each point represents the mean fold change of mRNA levels (±SEM) in posttreatment samples relative to pretreatment levels (d0, n = 10; d1, n = 10; d2, n = 8; d5, n = 5). B, fold change of transcript levels of MARCO, CLEC10A, IRF-5, and BAFF relative to pre-cyclophosphamide (d0), as assessed by microarray and qRT-PCR, at the indicated time points following cyclophosphamide treatment. C, validation, by qRT-PCR, of the effect of cyclophosphamide treatment on PBMC gene expression in a second cohort of 6 patients with multiple myeloma. D, before (day 0) and at the indicated time-points after cyclophosphamide administration, blood samples were drawn, plasma was isolated and immediately frozen at −80°C. The variation of the protein levels of CXCL10/IP10, CCL2/MCP-1, CXCL8/IL-8, TNFSF13B/BAFF, and CD163 in plasma was assessed with the ELISA-based assays Quantikine. The levels of HB-EGF (DTR) were analyzed with the Abnova ELISA Kit. Data represent the mean fold-change of the protein concentrations (±SEM) in posttreatment plasma relative to pretreatment levels (d0, n = 10; d1, n = 10; d2, n = 8; d5, n = 5). Two independent experiments carried out in duplicate. Statistical significance was determined by paired t test comparing each time point with pretreatment.

and C), suggesting that some of the observed modulations in PBMC transcript levels may represent the signature of a cell type whose relative percentage in PBMC is changing. The proportion of cDC in PBMC did not change following treatment (Fig. 3B), confirming previous data of dendritic cell resistance to cyclophosphamide toxicity (19).
A deeper analysis of the effect of cyclophosphamide on different cell subpopulations showed that while the percentage of conventional CD14<sup>+</sup>CD16<sup>-</sup> monocytes significantly decreased at day 2 ($P = 0.014$) and 5 ($P = 0.0012$), the percentage of the more mature monocyte subset, CD14<sup>-</sup>CD16<sup>+</sup>, increased at day 5 ($P = 0.049$; Fig. 4A). Moreover, although the percentage of monocytes expressing HLA-DR did not change following cyclophosphamide in either subset (Fig. 4B), an enhancement of its mean fluorescence intensity (MFI) was observed at day 5, particularly in CD14<sup>+</sup>CD16<sup>-</sup> monocytes ($P = 0.05$; Fig. 4A and Supplementary Fig. S2B). In addition, the percentage of CD14<sup>+</sup>CD16<sup>-</sup> expressing either the scavenger receptor MARCO or the receptor for IL-8 (IL8RA) were significantly augmented 2 days following cyclophosphamide administration ($P = 0.029$; Fig. 4B and Supplementary Fig. S2A). The percentage of CD14<sup>-</sup>CD16<sup>+</sup> monocytes expressing the same markers showed a tendency to increase at day 5 (Fig. 4B and Supplementary Fig. S2A). The expression of the IL-17 receptor (IL17RA) did not change at any time point.

The effect of cyclophosphamide on the expression of selected markers in cDCs is shown in Fig. 4C. The percentage of MARCO-expressing cDCs was augmented 5 days post-cyclophosphamide administration ($P = 0.05$), whereas the proportion of cDCs expressing DCIR, HLA-DR, and IL17RA was not influenced by treatment. As for monocytes, a trend to enhanced MFI of HLA-DR was observed in cDC at day 5 (Fig. 4C).

Figure 4D shows the variations of CD3<sup>+</sup>CD8<sup>-</sup> and CD3<sup>+</sup>CD8<sup>-</sup> T cells post-cyclophosphamide. Interestingly, CD8<sup>-</sup> T cells declined more rapidly than CD8<sup>+</sup> T cells. In addition, an increment in the percentage of CD3<sup>+</sup>CD8<sup>-</sup> T cells expressing the costimulatory molecule OX40 ($P = 0.048$), the activation marker CD69 ($P = 0.017$) and IL8RA was observed at day 5 (Fig. 4D). In the CD8<sup>-</sup> subset, OX40<sup>+</sup> cells showed a significant increase at day 5 ($P = 0.035$), whereas IL8RA<sup>+</sup> cells were raised at day 1 ($P = 0.014$; Fig. 4D and Supplementary Fig. S2C). The frequency of cells expressing IL17RA did not change at any time point.

Taken together, these data show that the transcriptional profile induced by a drug such as cyclophosphamide on whole PBMC may be the result of both the variation in the relative percentage of different leukocyte subpopulations and signatures of given markers, whose expression is modulated in a given subpopulation, opening therefore novel unknown mechanistic hypotheses that will be discussed hereafter.

Discussion

Understanding the complex mechanisms responsible for the positive interactions between chemo- and immunotherapy is crucial to improve synergisms between the two treatments and to turn weak immunotherapeutic interventions into potent anticancer tools.

Until recently, the efficacy of a chemotherapeutic treatment before the adoptive transfer of cells of the immune system was believed to mainly rely on the induction of lymphopenia, that creates “room” for tumor-specific lymphocytes (14), or on the selective depletion of regulatory T cells (Treg; ref. 15). We had shown in mouse models that cyclophosphamide-mediated immunomodulation is
multifactorial and that high throughput technologies may help the comprehension of such a complex phenomenon (21). Consistently with such view, the present study was designed to unravel the multiple factors underlying the ability of high-dose cyclophosphamide to potentiate immunotherapy in patients with cancer. We report here that a single cyclophosphamide injection rapidly (1–2 days) induces an extensive transcriptional modulation in PBMC of patients with hematologic malignancies, leading on the one hand to the reduction of cell-cycle and biosynthetic/metabolic processes, as expected by an anticancer agent, and, on the other hand, to augmented transcript levels of cell death-, DNA damage-, stress-, and immune system–related genes, including those related to IFN-I response. Moreover, microarray analysis pointed out a signature of apoptotic cell death. Although apoptosis was previously described as an immunologically silent cell-death modality, it is now recognized that the antitumor efficacy of certain chemotherapeutics depends on the induction of immunogenic apoptosis of tumor cells, through calreticulin exposure (33) and HMGB1 release (34). Studies on mouse models from our group had shown that cyclophosphamide induces immunogenic apoptosis of tumor cells (19).

Several reports have shown that stress-induced cell death may produce exposure or release of danger signals and that the perception of them may alert the host, leading to a sterile inflammatory response (35, 36). According to Matzinger’s “danger model,” endogenous non-foreign alarm signals include DNA, RNA, HSPs, proinflammatory cytokines, and IFN-α (37). The present microarray data showed that cyclophosphamide exposure induces increased transcript levels of several IFN-I–stimulated genes (ISG) as well as genes regulating IFN expression (OAS1, CXCL10, IFI16M2, IFI6, IRF5,

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**Figure 4.** Effect of cyclophosphamide on monocyte/lymphocyte subsets and cDC and on their expression of selected markers. PBMC obtained before (0) and at the indicated days after cyclophosphamide administration were analyzed by flow cytometry for: (A) the percentage of HLA-DR+ cells expressing CD16 and/or CD14 and the MFI of HLA-DR in CD14+/CD16−, CD14+/CD16+, and CD14−/CD16− cells; (B) the fold change of the same monocyte subsets expressing IL8RA, HLA-DR, IL-17RA, and MARCO, and the MFI of MARCO in CD14+/CD16− HLA-DR+ cells; (C) the fold change of percentages of cDC expressing HLA-DR, DCIR, IL-17RA, and MARCO and the MFI of HLA-DR in cDC; (D) the fold change of CD3+/CD8+ T cells expressing OX40, IL8RA, CD69, and IL-17RA.

Results are shown as median percentage (box plot), median MFI (box plot), or mean fold-change (±SEM; scatter plot), as indicated. Statistical significance was determined by paired t test (reported in the Results).
Mechanisms of Immunoactivation by Cyclophosphamide

IRF7, STAT2, UBE2L6, UNC93B1, ISG20L1, and TYK2). IFN-αs are cytokines known to be induced by viral infection, but whose essential role in cell survival, differentiation, and immunomodulation has been recently underscored, pointing to an effective immune adjuvant role of these cytokines toward inducing antitumor immunity (1, 31).

Pathway analyses also showed that, upon cyclophosphamide treatment, several transcripts of the p53 signaling pathway (known to be involved in DNA damage repair, cell-cycle arrest, and apoptosis) are augmented. Remarkably, it has been shown that IFN-α/β signaling contributes to boosting p53 responses to stress signals (38), suggesting that IFN renders cells more susceptible to p53-dependent apoptosis in response to DNA-damaging agents. More recently, it was shown that p53, in turn, can enhance IFN signaling, thus contributing to innate immunity, most likely through the upregulation of IRF7 and IRF5 (39, 40), which were indeed augmented in response to cyclophosphamide. Interestingly, IRF5 has been indicated as the master regulator of macrophage commitment to the M1 lineage, characterized by proinflammatory cytokine production (41). Therefore, it is possible to speculate that p53 activation and IFN-α signaling may cooperate to facilitate, on the one hand, cyclophosphamide-mediated apoptosis and, on the other hand, a potent stimulation of the innate immunity. These results also suggest a possible positive interaction between IFN-α and cyclophosphamide in potentiating antitumor immune responses, as previously shown in tumor models (18, 19).

Noticeably, ELISA showed that CXCL10 and BAFF (IFN-α-induced genes) were augmented in the patients’ plasma along with the inflammatory mediators CCL2 and IL-8. CXCL10, indeed, was shown to be induced by the combination of IFN-α and peptide-based vaccination in monocytes of patients with melanoma showing disease stabilization (42). CXCL10 was also identified as part of the “core” IFN signature observed in PBMC of patients with IFN-α-treated melanoma and healthy individuals (30). CCL2 (MCP-1), on the other hand, is one of the key chemokines regulating migration of monocytes/macrophages to areas of inflammation (32). Furthermore, along with IL-8, CCL2 has been shown to be secreted in response to apoptotic bodies in the course of sterile inflammatory responses (43). BAFF is an IFN-induced, B cell–activating molecule expressed by innate immune cells. In this context, mouse model studies showed that cyclophosphamide potently induces the homoeostatic proliferation of B cells, which correlated with elevated levels of tumor-specific serum antibodies (17).

Noteworthy, the induction of an IFN signature (including upregulation of BAFF and CXCL10), was observed also in patients with melanoma treated with the combination of dacarbazine and a peptide-based vaccination (44), suggesting that this signature could represent the hallmark of in vivo cell exposure to IFN as well as to alkylating agents, thus representing a potential biomarker of immune system activation by chemotherapy.

Danger signals were shown to act by stimulating dendritic cell maturation, increasing antigen presentation capacity, upregulating costimulatory molecules, and cytokine release, and therefore activating T lymphocytes (37). Accordingly, following cyclophosphamide treatment, we observed an increased signature of antigen-processing mediators and increased percentages and activation of a monocyte subset (CD14⁺CD16⁺) characterized by macrophage-like morphology, potent endocytic activity, high antigen presentation capacity and high production of proinflammatory cytokines (45). The increased percentages of this subset may be interpreted either as an indication that these cells are less responsive than other subsets to cyclophosphamide cytotoxicity or that, upon cyclophosphamide treatment, monocytes acquire a more phagocytic/activated phenotype as a consequence of phagocytosis of neighboring dead cells (46). Noticeably, it has been observed that the percentage of CD14⁺CD16⁺ monocytes was also increased in patients with melanoma treated with IFN-α in combination with peptide-based vaccination (42). Moreover, monocytes, as well as cDCs, showed an increase of HLA-DR MFI, thus suggesting enhanced antigen presentation capabilities of both subsets.

Recognition of apoptotic cells by phagocytes is mediated by several PRR and, among them, scavenger receptors bind damaged or apoptotic/necrotic self cells (28, 46). We show here that cyclophosphamide treatment induces increased transcript levels of the scavenger receptors CD68, CD163L1, MARCO, and SCARB2. Of particular interest, MARCO was identified as one of the most upregulated transcripts following phagocytic uptake of dead cells by dendritic cell (26). The same authors also showed that targeting MARCO expression can enhance both the trafficking and the antitumor efficacy of tumor lysate-pulsed dendritic cell (47). FACS analysis confirmed that the percentages of MARCO-expressing cDC as well as of MARCO-positive CD16⁺CD14⁺ and CD14⁺CD16⁺ monocytes are increased following cyclophosphamide administration.

Cyclophosphamide treatment induces also the activation of the adaptive immune response, as shown by increased transcript levels of CD69, OX40, and IL18R, confirmed by increased frequency of T lymphocytes (CD8⁺ and CD8⁻) expressing these markers. CD69 is an early membrane receptor transiently expressed upon lymphocyte activation and modulating inflammatory responses. OX40 and its ligand, OX40L, are TNF family members that augment expansion, cytokine production, and survival of CD4⁺ and CD8⁺ T cells, whose signaling has been shown to enhance antitumor immunity and inhibit suppression by Tregs (48).

Noticeably, the observed augmented expression of IL-8 receptor in T cells was accompanied by an early increase in IL-8 plasma levels, which may therefore amplify the inflammatory response to cyclophosphamide. The IL-8 receptor has been shown to be mobilized to the surface of CD4⁺ T cells upon activation and its expression identifies a CD8⁺ subset showing a high cytotoxic potential (49).

Altogether these data suggest that the ability of cyclophosphamide to boost immunity stems directly from its cytotoxicity on patients’ blood cells that produces DNA damage, cell-cycle arrest, activation of the p53 signaling
pathway, recognition of damaged self in the context of danger signals (including IFN-1 signature), and activation of both innate and adaptive immune responses. Such a sterile inflammatory response may therefore produce an immunogenic milieu favoring immunotherapeutic interventions. Further studies are needed to clarify whether the immunomodulatory effects of low-dose cyclophosphamide, which has been used in combination with cancer vaccines in several clinical trials (4), can have similar mechanistic basis to that described here for high-dose cyclophosphamide.

Of particular relevance, all the immunoadjuvant effects of cyclophosphamide described herein were shown to be early and transient. Accordingly, the antitumor effectiveness of chemo- and immunotherapy combination has been shown to depend on the rapid sequential administration of the two treatments in mouse models (21). On the basis of the results reported herein, new therapeutic strategies may be hypothesized for the treatment of aggressive diseases, such as resistant solid tumors as well as hematologic malignancies relapsed after an allogeneic SCT, for which DLI alone is successful in a minority of patients. A cyclophosphamide-based chemotherapy followed by adoptive immunotherapy or DLI with a precise timing and combination modalities may therefore represent the treatment of choice for these patients.

References


Disclosure of Potential Conflicts of Interest

M.T. Pennuci has honoraria from Speakers Bureau of Janssen-Cilag and Celgene. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Moschella, G.F. Torelli, M. Valentini, F. Urbani, C. Buccione, M.T. Pennuci, F. Natalini
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Moschella, M. Valentini, F. Urbani
Writing, review, and/or revision of the manuscript: F. Moschella, G.F. Torelli, F. Urbani, F. Belardelli, R. Foà, E. Proietti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Urbani, C. Buccione
Study supervision: F. Belardelli, E. Proietti

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Mechanisms of Immunoactivation by Cyclophosphamide

Cyclophosphamide Induces a Type I Interferon–Associated Sterile Inflammatory Response Signature in Cancer Patients' Blood Cells: Implications for Cancer Chemoimmunotherapy

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