Cyclophosphamide induces a type I interferon-associated sterile inflammatory response signature in cancer patients’ blood cells: implications for cancer chemoimmunotherapy

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Previous studies highlighted the importance of the modalities of tumor cell death for the activation of antitumor immunity. The model stemming from the present data is that cyclophosphamide induces a proinflammatory cell death, along with danger signals (type I interferon), not only of tumor cells but also of PBMC, thus leading to an immunomodulation enhancing the antitumor efficacy of immunotherapy. The direct consequence of this new vision is that chemotherapy and immunotherapy can be successfully combined to boost antitumor immunity also in the absence of a detectable tumor burden (i.e. in tumor resected patients), thus adding a new tool for preventing cancer recurrences. The understanding of the core mechanisms underlying immunomodulation and of the short time window required for optimal combination of chemotherapy and immunotherapy may guide new clinical trial design. Moreover, this study identified novel potential biomarkers of response to cyclophosphamide that can be evaluated in forthcoming clinical trials for their role in predicting the efficacy of chemoimmunotherapy.
Abstract

Purpose: Certain chemotherapeutics, particularly cyclophosphamide, can enhance the anti-tumor 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 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), of death-related scavenger receptors, of antigen processing/presentation mediators, of T-cell activation markers and, noticeably, of a type I interferon (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, BAFF) were increased in patients’ plasma. Accordingly, cyclophosphamide induced the expansion/activation of CD14⁺CD16⁺ monocytes, of HLA-DR⁺, IL-8RA⁺, MARCO⁺ monocytes/dendritic cells and of CD69⁺, OX40⁺, 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 to blood cell death, through p53 and IFN-I-related mechanisms.
Introduction

Much interest has been recently gained by the possibility of manipulating the host milieu with chemotherapy and in particular with cyclophosphamide (CTX), in order to synergize with adoptively transferred immune cells for anti-tumor purposes (1, 2). Although historically regarded as immunosuppressive, CTX has been shown to act as a strong adjuvant for either adoptive or active immunotherapy when utilized with carefully defined dosages and combination modalities (1-4). Additionally, 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 anti-tumor efficacy of the combination of CTX 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, that 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 interferon (IFN-I) was identified as an important mediator of CTX immunomodulation (8). Subsequent studies showed that IFN-I was indeed induced in vivo by CTX and that this cytokine was responsible for the expansion of memory CD4+ and CD8+ T-cells (18). More recent data indicated that CTX can affect dendritic cell (DC) homeostasis (19) and can restore an activated polyfunctional helper phenotype in tumor-specific adoptively transferred CD4+ T-cells (20) through type I IFN-dependent mechanisms.

We also reported that the synergistic anti-cancer activity of chemotherapy and immunotherapy is associated to the induction of a “cytokine storm”, occurring primarily in the bone marrow of treated mice. Up-regulated factors included GM-CSF and interleukin (IL)-1β, cytokines regulating homeostatic expansion and T-cell survival (IL-7, IL-15, IL-2, IL-21) and involved in the polarization towards a T helper (Th)1 type of immune response (IFN-γ) (17). Genomic and
proteomic analyses in mouse models demonstrated that CTX modulates the expression of ~1000 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, inflammatory mediators, growth factors, cytokines, chemokines and chemokine receptors (21). The analysis of gene and protein expression kinetics and of the anti-tumor efficacy of different therapeutic schedules of combination demonstrated that the optimal timing for performing adoptive immunotherapy is 1 day after CTX treatment in mice (21).

The importance of the timing between chemotherapy and cell infusion has also been demonstrated 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 coworkers reported that a fully lymphodepleting chemotherapy (CTX 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 graft-versus-host disease (GVHD) compared to patients who received DLI alone (22). We reported that patients with acute leukemia, relapsed after an allogeneic SCT, undergoing DLI 2 days after a chemotherapeutic 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 (CTX 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 demonstrated that high-dose CTX 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 metastatic melanoma patients (24).
The present study aims at investigating for the first time the gene expression modulation after a single high-dose CTX administration in human PBMC and at identifying the mediators and mechanisms through which CTX conditions the host immune system to perceive and react to tumor antigens in chemoimmunotherapy strategies. Patients with hematologic malignancies, receiving high-dose CTX (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 (CTX) (Endoxan, Baxter) (3-4 g/m²) was administered as mobilization regimen before autologous haematopoietic stem cell transplant (SCT) to 1 plasma cell leukemia (PCL), 1 T-cell prolymphocytic leukemia (T-PLL) and 14 multiple myeloma (MM) patients (Supplementary Table S1). Patients were treated at the Department of Hematology, Azienda Policlinico Umberto I, Sapienza University of Rome according to the principles set out in the WMA Declaration of Helsinki.

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

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 Allyn MessageAmp™ II aRNA Amplification Kit (Ambion, Monza, Italy) 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 co-hybridized overnight in humidifying chambers at 50°C onto prehybridized microarray slides printed with 34,580 70mer probes, representing 24,650 genes and 37,123 transcripts (Human Genome Oligo Set Version 3.0, Operon) (LaRiM, ISS, Rome, Italy). The platform has been submitted to the Gene Expression Omnibus (GEO) database (GPL15718).
Microarray data analysis

Scanning and image file processing were performed 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) to exclude spots below a minimum intensity (200), flagged and with diameters <25 μm. 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 log2-tranformed ratios).

Statistically significant (P≤0.005) differentially expressed genes among post- and pre-treatment samples were identified with class comparison (paired T-test with random variance model, BRB-ArrayTools). Hierarchical clustering was performed 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-based and pathway-based analyses were performed by means of DAVID bioinformatic tool. Enriched biological processes and pathways were ranked according to the 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.

cDNA synthesis and real-time 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 performed 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 (Milan, Italy) (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 (ΔΔCT) method and were normalized by 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/TNFSF13B and CD163 in plasma was assessed with the ELISA-based assays Quantikine (R&D Systems). HB-EGF was analyzed with the Abnova ELISA kit (Abnova GmbH, Germany). Briefly, 50-100 μl 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, PBMC were stained with anti-CD14-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) IgG-IgM FITC), anti-CD217-Alexa Fluor 647 (Biolegend) and anti-CD181-PE (R&D Systems). Circulating DC 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 biotin conjugates were revealed with Streptavidin-APC-Cy7 (Becton Dickinson). DC were also stained with anti-HLA-DR-PerCP-Cy5.5, anti-MARCO-GAM-FITC, anti-DCIR-PE (R&D Systems), anti-CD217-Alexa Fluor 647 according to the manufacturers' instructions. To analyze the expression of T-cell markers, PBMC 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 FACSCanto flow cytometer (BD-Biosciences) and analysis was performed with DIVA (BD-Bioscience) and FlowJo (TreeStar) softwares. Statistical analysis of cytofluorimetric data was performed with IBM SPSS Statistic 20.
Results

Gene expression profiling of PBMC in response to CTX

To analyze the impact of a single high-dose CTX administration on human PBMC gene expression and to elucidate the mechanisms through which this drug may influence the immune system, the response to CTX was analyzed in one T-cell prolymphocytic leukemia, one plasma cell leukemia and eight multiple myeloma patients (Supplementary Table S1). Microarray analysis was performed before (pre) and at different times (1, 2 and 5 days) after chemotherapy. The data discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus 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. Fig. 1A shows that 1, 2 and 5 days after CTX administration the expression of, respectively, 145, 656 and 212 genes was significantly (P ≤ 0.005) modulated (Fig. 1A), thus showing that CTX has a strong impact on PBMC gene expression, particularly evident at day 2.

To analyze the kinetic of the transcriptional response to CTX, we pooled altogether the 890 genes differentially expressed post-CTX (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), pre-treatment samples (d0) and samples taken 5 days following CTX administration (d5) were more similar to each other than samples taken at day 1 or 2. Fig. 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 intra-patient variability. Moreover, the transcript levels of most of the genes modulated at day 1 and/or 2 reverted to pre-treatment levels by day 5 in 3 out 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 down-
regulated only at day 5. Supplementary Table S2 and S3 show the entire lists of genes up-regulated or down-regulated 1 and/or 2 days following CTX treatment.

**Functional classification of differentially expressed genes by gene ontology and pathway analyses**

To characterize the observed transcriptional profiles according to biological function and to interpret the data in the context of pathways and networks involved in CTX-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 (Database for Annotation, Visualization and Integrated Discovery) bioinformatic tool (25).

Remarkably, the most significant (P ≤ 0.05) biological process stimulated by CTX in patients’ PBMC included immune response-related genes. Other significantly enriched biological functions were: response to other organisms, response to stress, response to biotic stimulus, catabolic process and cell death (Table 1). The functional classification of down-regulated genes showed that the most significantly enriched biological 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 A, member 2), CD163L1 (scavenger receptor cysteine-rich type 1 protein M160), SCARB2/LIMP-2 (scavenger receptor class B, member 2) and C1R, a component of the multimolecular complex C1 comprising C1q and C1s, which were all shown to be involved in the recognition of stressed and dying cells (26-29) (Supplementary Table S2).

Moreover, 1-2 days post-chemotherapy 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 gene inducible gene, and several leukocyte Ig-like innate receptors (LIRs) (LILRA3, LILRB1, LILRB2, LILRB3, LILRB4) were up-regulated (Supplementary Table S2). At the
same time, the transcript levels of factors implicated in antigen processing and presentation were augmented, including *CIITA* (class II, major histocompatibility complex, transactivator), *CD68*, three cathepsins (*CTSC, CTSL1, CTSZ*), the alpha-galactosidase *GLA*, the alpha-glucosidase *GAA*, the serine protease *TPP1, NEU1, SLC11A1* (a late phagosomal protein) and *LAMP-2* (lysosomal-associated membrane protein 2).

Also genes belonging to the tumor necrosis factor (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 up-regulated. In addition, *CD97*, an activation-induced antigen expressed by lymphocytes was induced by CTX (Supplementary Table S2).

Of note, several of the CTX-induced genes are known to be induced by type I IFN, and are indicative of type I IFN signature in different settings (30, 31), among them *OAS1* (2′,5′-oligoadenylate synthetase 1), *BAFF, IFITM2, IFI6, IRF5, IRF7* (interferon regulatory factor 5 and 7), *STAT2, UBE2L6, UNC93B1, ISG20L1* and *TYK2*, a molecule required for optimal IFN-I-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 CTX 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 p53-independent apoptosis induced by DNA damage, *BAX* (Bcl2-associated X protein), *TRIAP1*, which prevents induction of apoptosis in response to low levels of DNA damage, *XRCC1*, 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 pro-apoptotic protein BAX or the anti-apoptotic 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 TPP1. The “p53 signaling pathway” induced by CTX 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 utilizing 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).

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

Altogether these results suggest that CTX 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 type I IFN-related and p53-related genes.

Validation of the microarray results by real-time PCR

To validate the microarray results, the expression of selected genes was assessed by real-time PCR on the same patients analyzed by microarray (Fig. 2A and 2B) as well as on a second cohort of 6 multiple myeloma patients (Supplementary Table S1 and Fig. 2C). In Fig. 2A are shown the mean fold changes of transcript levels at different times after CTX administration as compared to pre-
treatment levels. The scavenger receptor *MARCO* showed a trend to increase 2 and 5 days post-CTX, while *CLEC10A* levels were significantly decreased (Fig. 2A). Moreover, significantly increased expression of *IRF5* (involved in IFN-α transcriptional activation) and of *BAFF* was observed post-treatment (Fig. 2A). The correlation of microarray versus RT-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 type I IFN related genes and among them of *CXCL10* (IP-10), a proinflammatory chemokine identified as part of the "core" IFN signature observed in PBMC of IFNα-treated melanoma patients and healthy individuals (30). We therefore assessed by real-time PCR whether the expression of *CXCL10* and of another inflammatory IFN- and stress-induced chemokine, namely *CCL2* (MCP-1) (32), was modulated by CTX and not revealed by microarray analysis. Fig. 2A shows a trend to increased expression for both transcripts at day 2, even though statistical significance was not reached due to interpatient variability.

The analysis of the modulation of *MARCO, CLEC10A, IRF5, BAFF, CXCL10* and *CCL2* expression induced by CTX in a second set of patients further validated microarray and real-time PCR results (Fig. 2C).

**Rise of CXCL10, CCL2, IL-8 and BAFF plasma levels following CTX treatment**

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

As shown in Fig. 2B, CXCL10 and BAFF plasma levels were significantly (*P*≤0.05) raised 2 and 5 days following CTX administration, while 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 real-time PCR, but also further expanded this information pointing to the inflammatory milieu resulting from damage in self tissues induced by chemotherapy.

**Immunophenotype of monocytes, DCs and T lymphocytes**

In order to characterize the effect of CTX on numbers of circulating DCs, monocytes and T lymphocytes and on their expression of markers selected on the basis of the microarray results, a multiparametric flow cytometric analysis was carried out in all assessable patients.

The white blood cell (WBC) count before and after treatment showed, as expected, that high-dose CTX 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 pre-treatment levels (P=3.6x10⁻⁵), monocytes showed only a 1.5-fold decrease (P=0.002) (Fig. 3A). Accordingly, FACS analysis showed that the relative percentage of CD14⁺ monocytes in PBMC increased at day 1 and diminished at day 5 (P=0.05), while the relative percentage of CD3⁺ lymphocytes increased at day 5 (Fig. 3B and 3C), 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 DC resistance to CTX toxicity (19).

A deeper analysis of the effect of CTX on different cell subpopulations showed that while the percentage of conventional CD14⁺⁺CD16⁻ monocytes significantly decreased at day 2 (P=0.014) and 5 (P= 0.0012), the percentage of the more mature monocyte subset, CD14⁺⁺CD16⁺, increased at day 5 (P= 0.049) (Fig. 4A). Moreover, although the percentage of monocytes expressing HLA-DR did not change following CTX in either subset (Fig. 4B), an enhancement of its mean fluorescence
intensity (MFI) was observed at day 5, particularly in CD14++CD16- monocytes (P=0.05) (Fig. 4A and Supplementary Fig. S2B). In addition, the percentage of CD14++CD16- expressing either the scavenger receptor MARCO or the receptor for IL-8 (IL8RA) were significantly augmented 2 days following CTX administration (P=0.029) (Fig. 4B and Supplementary Fig. S2A). The percentage of CD14+CD16+ 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 CTX on the expression of selected markers in cDCs is shown in Fig. 4C. The percentage of MARCO-expressing cDCs was augmented 5 days post-CTX administration (P=0.05), while 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).

Fig. 4D shows the variations of CD3+CD8+ and CD3+CD8- T-cells Post-CTX. Interestingly, CD8+ T-cells declined more rapidly than CD8- T-cells. In addition, an increment in the percentage of CD3+CD8- 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+ subset, OX40+ cells showed a significant increase at day 5 (P=0.035), while IL-8RA+ 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 like CTX 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 chemotherapy and immunotherapy is crucial to improve synergisms between the two treatments and to turn weak immunotherapeutic interventions into potent anti-cancer tools.

Until recently, the efficacy of a chemotherapeutic treatment prior to 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 Tregs (15). We had shown in mouse models that CTX-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 CTX to potentiate immunotherapy in cancer patients. We report here that a single CTX 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 anti-cancer 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 type I IFN 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 anti-tumor 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 CTX 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, heat shock proteins, proinflammatory cytokines and IFN-α (37). The present microarray data showed that CTX exposure induces increased transcript levels of several
type I IFN-stimulated genes (ISGs) as well as genes regulating IFN expression (OASI, CXCL10, IFITM2, IFI6, IRF5, IRF7, STAT2, UBE2L6, UNC93B1, ISG20L1, TYK2). Type I IFNs 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 towards inducing anti-tumor immunity (1, 31).

Pathway analyses also showed that, upon CTX 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 up-regulation of IRF7 and IRF5 (39, 40), which were indeed augmented in response to CTX. 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 type I IFN signaling may cooperate to facilitate, on the one hand, CTX-mediated apoptosis and, on the other hand, a potent stimulation of the innate immunity. These results also suggest a possible positive interaction between type I IFN and CTX in potentiating anti-tumor immune responses, as previously shown in tumor models (18, 19).

Noticeably, ELISA showed that CXCL10 and BAFF (type I IFN-induced genes) were augmented in the patients’ serum 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 melanoma patients showing disease stabilization (42). CXCL10 was also identified as part of the "core" IFN signature observed in PBMC of IFN-α-treated melanoma patients 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 demonstrated that CTX potently induces the homeostatic proliferation of B cells, which correlated with elevated levels of tumor-specific serum antibodies (17).

Noteworthy, the induction of an IFN signature (including up-regulation of BAFF and CXCL10), was observed also in melanoma patients 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 DC maturation, increasing antigen presentation capacity, up-regulating costimulatory molecules and cytokine release and therefore activating T lymphocytes (37). Accordingly, following CTX 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 endocytotic 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 CTX cytotoxicity or that, upon CTX 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 raised in melanoma patients 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 pattern-recognition receptors (PRR) and, among them, scavenger receptors bind damaged or apoptotic/necrotic self cells (28, 46). We show here that CTX 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 up-regulated transcripts following phagocytic uptake of dead cells by DC (26). The same authors also showed that targeting MARCO expression can enhance both the trafficking and the anti-tumor efficacy of tumor lysate-pulsed-DC (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 CTX administration.

CTX treatment induces also the activation of the adaptive immune response, as shown by increased transcript levels of \textit{CD69}, \textit{OX40} and \textit{IL8RA}, 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 anti-tumor 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 CTX. 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 CTX 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 type I IFN signature) and activation of both innate and adaptive immune responses. Such a sterile inflammatory response may therefore produce an immunogenic milieu favouring immunotherapeutic interventions. Further studies are needed to clarify whether the immunomodulatory effects of low dose CTX, 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 CTX.
Of particular relevance, all the above described immunoadjuvant effects of CTX were shown to be early and transient. Accordingly, the anti-tumor effectiveness of chemotherapy 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 CTX-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.

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References


Table 1. Functional annotation charts of genes modulated 1-2 days after CTX administration

<table>
<thead>
<tr>
<th>Biological process(^*) of up-regulated genes</th>
<th>N.(^†)</th>
<th>P-value(^‡)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>25</td>
<td>7,0E-6</td>
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<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>
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<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>
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<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 biological quality</td>
<td>32</td>
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</tr>
<tr>
<td>Cell death</td>
<td>19</td>
<td>1,0E-2</td>
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<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>
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<tr>
<td>Alcohol metabolic process</td>
<td>13</td>
<td>1,6E-2</td>
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<tr>
<td>Positive regulation of immune system process</td>
<td>9</td>
<td>1,6E-2</td>
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<tr>
<td>Positive regulation of biological process</td>
<td>40</td>
<td>1,9E-2</td>
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<tr>
<td>Response to external stimulus</td>
<td>21</td>
<td>2,7E-2</td>
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<td>Multicellular organismal homeostasis</td>
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<td>3,9E-2</td>
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<td>Interspecies interaction between organisms</td>
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<tr>
<td>Oxidation reduction</td>
<td>15</td>
<td>4,7E-2</td>
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<tr>
<td>Positive regulation of cellular process</td>
<td>35</td>
<td>5,0E-2</td>
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<table>
<thead>
<tr>
<th>Biological process(^*) of down-regulated genes</th>
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<tbody>
<tr>
<td>Nitrogen compound metabolic process</td>
</tr>
<tr>
<td>Cellular metabolic process</td>
</tr>
<tr>
<td>Macromolecule metabolic process</td>
</tr>
<tr>
<td>Primary metabolic process</td>
</tr>
<tr>
<td>Cell cycle</td>
</tr>
<tr>
<td>Organelle organization</td>
</tr>
<tr>
<td>Ribonucleoprotein complex biogenesis</td>
</tr>
<tr>
<td>Cell cycle process</td>
</tr>
<tr>
<td>Biosynthetic process</td>
</tr>
<tr>
<td>Cellular macromolecular complex subunit Organization</td>
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<tr>
<td>Regulation of metabolic process</td>
</tr>
<tr>
<td>Establishment of RNA localization</td>
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<td>Interspecies interaction between organisms</td>
</tr>
</tbody>
</table>

*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 ≤ 0.05 were considered. The entire list of genes present on the array was used as background.
Table 2. Pathway analysis of genes modulated 1-2 days after CTX administration

<table>
<thead>
<tr>
<th>KEGG pathways* of up-regulated genes</th>
<th>N. †</th>
<th>P-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosome</td>
<td>CD68, CTSC, CTSL1, CTSZ, GLA, GAA, LAMP2, SCARB2, NEU1, SLC11A1, TPP1</td>
<td>11</td>
</tr>
<tr>
<td>p33 signaling pathway</td>
<td>BBC3, BAX, BID, CCND3, CDKN1A, DDB2, SESN2</td>
<td>7</td>
</tr>
<tr>
<td>Inflammation mediated by chemokine and cytokine signaling pathway</td>
<td>GNA15, IL10RB, NFKBIA, PLA2G4A, PLCG2, RHOC, RGS19, RRAS, ARPC3, TYK2</td>
<td>10</td>
</tr>
<tr>
<td>B cell activation</td>
<td>NFKBIA, PLCG2, RRAS, SYK</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KEGG pathways* of down-regulated genes</th>
<th>N. †</th>
<th>P-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spliceosome</td>
<td>RBM17, RP11-78J21.1, HNRNPA1, HNRPA3, HNRPM, SFRS2, SFRS5, SFRS6, TCERG1</td>
<td>10</td>
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<tr>
<td>DNA replication</td>
<td>MCM2, MCM3, MCM6, RPA1, RPA2</td>
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<tr>
<td>Cell cycle</td>
<td>DBF4, WEE1, CCND2, HDAC1, MCM2, MCM3, MCM6</td>
<td>7</td>
</tr>
<tr>
<td>Pyrimidine metabolism</td>
<td>AK3, CMPK, POLR3GL, RDH14, 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>
</tr>
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</table>

<table>
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<tr>
<th>Panther pathways* of down-regulated genes</th>
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<tbody>
<tr>
<td>Ubiquitin proteasome pathway</td>
<td>ATAD2, APPBP1, hCG_15200, PSMC2, PSMD11, UBR5, UBE2B, UBE2H</td>
</tr>
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</table>

*Pathway analysis by means of DAVID bioinformatic tool. †Number of genes in each pathway. ‡EASE Score, a modified Fisher Exact P-Value. The entire list of genes present on the array was used as background.
Figure legends

**Figure 1.** Gene expression profiles of PBMC following a single injection of CTX (3-4 mg/m²) in patients with lymphoproliferative disorders. Total RNA was isolated from PBMC either before (pre-CTX) (indicated as d0 in the dendrogram, n=10) or at day 1 (d1, n=10), day 2 (d2, n=8) and day 5 (d5, n=5) after CTX treatment (Post-CTX). After amplification, aRNAs were labelled with Cy5, mixed with Cy3-labeled “reference” aRNA (Universal Human Reference RNA, Stratagene) and hybridized to 34.5k oligonucleotide-based microarray. A) Class comparison (BRB-ArrayTools) was used to compare samples taken 1, 2 or 5 days following CTX treatment to pre-treatment samples by paired T-test. The expression of significantly (P≤0.005) modulated genes is shown for all samples. (B-C) Hierarchical Clustering analysis of all the 890 genes showing a statistically significant (P≤0.005) differential expression in post-treatment samples (Post-CTX) (1, 2 and 5 days after chemotherapy) with respect to pre-treatment samples (Pre-CTX), as assessed by paired T-test. B) Dendrogram showing differences and similarities of samples (unsupervised hierarchical clustering of samples). C) Unsupervised hierarchical clustering of genes. Hierarchical Clustering analysis was performed with Cluster Version 3.0. Treeview software was used for visualization of results following average correction.

**Figure 2.** CTX treatment modulates the transcript levels of MARCO, CLEC10A, IRF5, BAFF, CXCL10, CCL2 and the plasmatic levels of CXCL10, CCL2, IL-8 and BAFF. A), B), C) Time course analysis of the modulation of MARCO, CLEC10A, IRF-5, BAFF, CXCL10 and CCL2 gene expression at different times following CTX treatment. Relative mRNA levels were calculated by the comparative cycle threshold (C_T) method and were normalized to GAPDH expression. A) Each point represents the mean fold change of mRNA levels (±s.e.m.) in post-treatment samples relative to pre-treatment 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-CTX (d0), as assessed by microarray and real-time PCR, at the indicated time points following CTX treatment. C) Validation, by real-time
PCR, of the effect of CTX treatment on PBMC gene expression in a second cohort of 6 multiple myeloma patients. D) Before (day 0) and at the indicated time-points after CTX 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 (±s.e.m.) in post-treatment plasma relative to pre-treatment levels (d0 n=10, d1 n=10, d2 n=8, d5 n=5). Two independent experiments performed in duplicate. Statistical significance was determined by paired T-test comparing each time point to pre-treatment.

**Figure 3.** Effect of CTX administration on circulating leukocytes, monocytes, lymphocytes and dendritic cells (cDC). A) Box plot of median leukocyte, lymphocyte and monocyte counts assessed, before (0) and at the indicated times after CTX administration with an automated hematology analyzer. The boxes define the interquartile range and the thick line is the median. Open dots and asterisks are possible outliers. B) Box plot presentation showing the variation of the median percentages of CD14⁺ monocytes, CD3⁺ lymphocytes and cDC in patients’ PBMC following CTX injection, as assessed by flow cytometry (d0 n=10, d1 n=9, d2 n=8, d5 n=3). C) Dot plots showing variation of CD14⁺ and CD3⁺ cell percentages in one representative patient. Statistical significance was determined by paired T-test comparing each time point to pre-treatment.

**Figure 4.** Effect of CTX on monocyte/lymphocyte subsets and cDC and on their expression of selected markers. PBMC obtained before (0) and at the indicated times after CTX 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⁺HLA-DR⁺ and CD14⁺CD16⁻HLA-DR⁺ cells; B) the fold change of the same monocyte subsets expressing IL8RA, HLA-DR, IL-17RA, MARCO and the MFI of MARCO in CD14⁺CD16⁺HLA-DR⁺ cells; C) the fold change of percentages of


circulating DC (cDC) expressing HLA-DR, DCIR, IL-17RA and MARCO and the MFI of HLA-DR in cDC; D) the percentage of CD3⁺ lymphocytes expressing CD8 or not expressing it and 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 (±s.e.m.) (scatter plot), as indicated. Statistical significance was determined by paired T-test (reported in the Results section).
Figure 1

A  Day 1 vs. Pre (145 genes)

All genes modulated post-CTX (890 genes)
(day 1, day 2 and day 5 vs. Pre)

B

C  Day 5 vs. Pre (212 genes)
Figure 2

A. MARCO, CLEC10A, IRF-5, BAFF, CXCL10, and CCL2 mRNA levels relative to pre-CTX levels.

B. MARCO, CLEC10A, IRF-5, BAFF, CXCL10, and CCL2 mRNA levels relative to pre-CTX levels.

C. MARCO, CLEC10A, IRF-5, BAFF, CXCL10, and CCL2 mRNA levels relative to pre-CTX levels.

D. ELISA for CXCL10, CCL2, IL-8, BAFF, HB-EGF, and CD163 plasma levels relative to pre-CTX levels.

P-values indicated for statistical significance.
Figure 3

A. Mean number of cells x 10^6/ml

B. Mean nu

C. Mean nu

Days after CTX
Figure 4

A. 

Monocyte subsets

% of HLA-DR+CD14+

CD14++CD16- CD14++CD16-

HLA-DR MFI (x10^3) Monocyte subsets

B. 

CD14++CD16-

Fold Change

IL8RA+ HLA-DR+ IL17RA+ MARCO+

CD14+CD16+

Fold change

IL8RA+ HLA-DR+ IL17RA+ MARCO+

C. 

cDC

Fold change

HLA-DR MFI (x10^3)

IL8RA+ HLA-DR+ DCIR+ IL17RA+ MARCO+

D. 

Lymphocyte subsets

% of CD3+

CD8- CD8+

Fold change

OX40+ IL-8RA+ CD69+ IL-17RA+

CD3+CD8-

Fold change

OX40+ IL-8RA+ CD69+ IL-17RA+

CD3+CD8+

Fold change

OX40+ IL-8RA+ CD69+ IL-17RA+

Days after CTX
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