Cyclophosphamide induces type I interferon and augments the number of CD44<sup>hi</sup> T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer

Giovanna Schiavoni, Fabrizio Mattei, Tiziana Di Pucchio, Stefano M. Santini, Laura Bracci, Filippo Belardelli, and Enrico Proietti

Introduction

Cyclophosphamide (CTX) is a widely used chemotherapeutic agent in cancer therapy<sup>1</sup> and in some autoimmune diseases.<sup>2</sup> Combined regimens with CTX and immunotherapy are used in clinical trials with cancer patients.<sup>3-6</sup> However, the mechanisms of CTX action are not fully understood. On the one hand, CTX can act as a conventional anticancer drug by affecting the in vivo proliferation of tumor cells.<sup>1</sup> On the other hand, CTX can exhibit immunomodulatory effects, which may play an important role in the antitumor response.<sup>7-20</sup> In this regard, many studies had reported that CTX can increase the efficacy of immunotherapeutic agents by removing tumor-induced suppressor T cells.<sup>21-25</sup> However, the nature of these suppressor cells is still a matter of debate.<sup>26</sup>

By using transplantable mouse tumor models, we have recently reported that CTX can induce marked effects on T cells, which are important for a successful tumor eradication in response to adoptive immunotherapy.<sup>27</sup> In particular, the results of an ensemble of experiments aimed at understanding the mechanisms underlying the synergistic antitumor response observed in tumor-bearing mice injected with CTX and tumor-sensitized lymphocytes have suggested that CTX acts by means of bystander effects (possibly through production of T-cell growth factors occurring during the rebound events after drug administration) that may sustain the proliferation, survival, and activity of the transferred lymphocytes.<sup>27</sup> In considering which CTX-induced factor(s) can be important for explaining the effects on T cells and the antitumor response observed in tumor models, we focused our attention on type I interferon (IFN). In fact, the working hypothesis that this cytokine could somehow induced in our experimental system with tumor-bearing mice. (2) Recent reports have clearly indicated that type I IFN is the major factor responsible for the in vivo proliferation and long-term survival of certain subsets of T cells (especially CD4<sup>+</sup>CD8<sup>+</sup> T lymphocytes) in response to viruses or other stimuli.<sup>28,29</sup>

All of this prompted us to investigate whether the injection of CTX in normal mice could result in any induction of type I IFN and to characterize the effects of CTX on T cells.

The results reported in this article demonstrate that CTX can induce type I IFN expression, which may represent an important mediator of the immunomodulatory effects of CTX, especially with regard to the expansion and persistence of memory T cells.
Materials and methods

Mice and in vivo treatments

Six- to 8-week-old male DBA/2 mice were obtained from Charles River Breeding Laboratories (Calco, Italy). All mice were treated in accordance with the European Community Guidelines. For bromodeoxyuridine (BrdU)-incorporation studies, mice were given drinking water containing BrdU (Sigma Chemical, St Louis, MO) at 0.8 mg/mL, which was made fresh and changed daily. CTX (Sigma) was dissolved in 0.15 mol/L NaCl (saline) and filter sterilized, and 0.5 mL of freshly prepared solution was injected intraperitoneally (CTX-treated mice). Polyinosinic-polycytidylic acid (poly (I:C)) was injected intraperitoneally in a volume of 0.15 mL of saline solution. Control preparations consisted of saline unless otherwise stated.

Antibody to mouse interferon α/β

Sheep antibodies to mouse interferon α/β and normal sheep immunoglobulin were a generous gift from Dr Ion Gresser (Villejuif, France). The origin of sheep antibodies to mouse interferon α/β (sheep no. 1) and normal sheep immunoglobulin, their purification, and their assay have been previously described in detail. Antibody titer was 1.6 × 10^9 neutralizing units against 8 interferon units; mice were injected with 0.2 mL of a 1:10 dilution against 8 interferon units; mice were injected with 0.2 mL of a 1:10 dilution.

Preparation of spleen and lymph node cells suspensions

Mice were killed and spleen and lymph nodes were removed aseptically. Lymph node cells were pooled from cervical, axillary, inguinal, and mesenteric nodes. Tissues were gently disaggregated with a tissue homogenizer and processed for a standard IFN assay on L929 mouse cell monolayers.

Flow cytometry

Monoclonal antibodies (mAbs) used to stain cell surface antigens were the following: biotinylated anti-Ly-6C (104-2.1, mouse immunoglobulin IgG2a) (PharMingen, San Diego, CA), biotinylated anti-CD44 (IM7, rat IgG) (PharMingen), phycoerythrin (PE)–conjugated anti-CD4 (GIBCO-BRL, Gaithersburg, MD), PE–conjugated anti-CD8 (GIBCO-BRL). Bound biotinylated L antibodies were detected with red 670-streptavidin (GIBCO-BRL). After surface staining by conventional techniques, cells were washed, resuspended in cold saline, and fixed by dropwise addition of cold 95% ethanol for 30 minutes on ice. For BrdU-incorporation studies, an intracellular staining method was used. Briefly, the cells were washed with PBS, then incubated with PBS containing 1% paraformaldehyde and 0.01% Tween 20 for 30 minutes. Cells were pelleted, then incubated with 50 Kunitz units DNAse I (Sigma) in 0.15 mol/L NaCl, 4.2 mmol/L MgCl₂, pH 5.0, for 10 minutes. After washing, cells were incubated with fluorescein isothiocyanate–conjugated anti-BrdU mAb (Becton Dickinson, Mountain View, CA) and analyzed on FACSsort flow cytometer (Becton Dickinson). A total of 10 000 events per sample were collected. Erythrocytes, dead cells, and tissue debris were excluded according to forward- and side-scatter properties in order to gate only live lymphocyte populations.

Interferon titration

Detection of murine α and β interferon messenger RNAs in the spleen by reverse transcriptase (RT)–polymerase chain reaction (PCR)

At different times after treatments, mice were killed and their spleens were immediately removed and directly homogenized with a tissue homogenizer in 2 mL RNAzol B (Bioteck, Houston, TX) in an ice bath. Total RNA was then subjected to chloroform extraction and isopropanol precipitation. RNA was resuspended and treated with ribonuclease-free DNAse (Boehringer Mannheim, Germany), further purified, and then quantitated by UV absorbance at 260 nm. One microgram of total RNA was incubated for 5 minutes with oligo-(dt) 12-18 (Pharmacia, Uppsala, Sweden) at 75°C, cooled at room temperature, and reverse-transcribed by 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) for 1 hour at 37°C in a final volume of 20 μL. We amplified 2 μL of complementary DNA in a final volume of 20 μL (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% gelatin, 200 μmol/L deoxyribonucleoside triphosphate (dNTP), and 10 pmol of each primer) using a Perkin Elmer Thermal Cycler (Perkin Elmer, Mannheim, Germany), further purified, and then quantitated by UV absorbance at 260 nm. One microgram of total RNA was incubated for 5 minutes with oligo-(dt) 12-18 (Pharmacia, Uppsala, Sweden) at 75°C, cooled at room temperature, and reverse-transcribed by 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) for 1 hour at 37°C in a final volume of 20 μL. We amplified 2 μL of complementary DNA in a final volume of 20 μL (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% gelatin, 200 μmol/L deoxyribonucleoside triphosphate (dNTP), and 10 pmol of each primer) using a Perkin Elmer Thermal Cycler (Perkin Elmer, Norwalk, CT). Samples were heated at 94°C for 5 minutes, and each cycle was performed as follows: 40 seconds denaturation at 94°C, 40 seconds annealing at 72°C, and 1 minute extension at 72°C. At the end, samples were further incubated at 72°C for an additional 10 minutes. Table 1 reports the cytokine primer sequences, the number of amplification cycles, and the size of the fragment amplified in this study. For reaction product visualization, 10 μL of each PCR product was run on 1% agarose gel in 0.5 × tris borate dectrophoresis (TBE) buffer. As molecular weight markers, 1 μg of 132 bp DNA ladder (GIBCO-BRL) were run in parallel. As positive controls for IFN-α or IFN-β RT-PCR, we used messenger RNA (mRNA)
Detection of interferon activity in sera of mice treated with CTX

In a first set of experiments, mice were injected intraperitoneally with 2 doses of CTX (83 or 150 mg/kg body weight), with saline or poly (I:C) (positive control for IFN production). Sera were collected at different times after injection and tested for IFN activity by a standard biological assay on L929 cells. Table 2 shows the IFN activity detected in sera from individual mice at 12, 24, and 48 hours after treatment.

Figure 1. RT-PCR analysis of IFN-α and IFN-β mRNA levels in the spleen of mice treated with CTX or poly (I:C). Seven-week-old DBA/2 mice were treated intraperitoneally with CTX (83 mg/kg, D; and 150 mg/kg, h); poly (I:C) (0.15 mg/mouse), s; or saline, d. After 6, 12, 24, and 48 hours, mice were killed, and total spleen RNA was isolated and assayed for the presence of IFN-α and IFN-β mRNAs by RT-PCR as described in “Materials and methods.” Reaction products were run on 1% agarose gel in the presence of molecular markers (not shown). Values represent the mean ± SE of 3 mice per group. (A) Each band corresponds to a single mouse spleen. Densitometric values of ethidium bromide–stained bands, expressed as absorbance units (OD) and normalized to the control values, are reported, (B) for IFN-α mRNAs and (C) for IFN-β mRNAs. (D) Spleen weight of mice treated as above was measured 6, 12, 24, and 48 hours after treatment.

Figure 2. Effects of CTX injection on the expression of Ly-6C antigen and on BrdU incorporation in spleen lymphocytes at different times after treatment. Seven- to 8-week-old DBA/2 mice were placed on BrdU water from day 0 to sacrifice. Mice were injected intraperitoneally with CTX (83 mg/kg) (dotted bars) or saline (white bars) at day 0. At different time intervals, spleens were taken and disaggregated. Cells were stained for Ly-6C; this was followed, after fixation, by nuclear staining for BrdU incorporation. Cell fluorescence was evaluated by fluorescence-activated cell sorter (FACS). There were 3 mice per group. The data show the mean (% ± SE) of the percentage of Ly-6C<sup>hi</sup> cells (upper panel) or BrdU<sup>hi</sup> cells (lower panel) with respect to the total number of spleen lymphocytes.

Statistical analyses

Data were analyzed by Student t test.

Results

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Statistical analyses

Data were analyzed by Student t test.
Kinetics of accumulation of IFN-α and IFN-β messenger RNAs in the spleen of mice treated with cyclophosphamide

We then evaluated the levels of type I IFN mRNA expression in splenocytes harvested at different times after CTX injection. Thus, total RNA was extracted from spleen cells of mice treated with saline, CTX (83 or 150 mg/kg), or poly (I:C) and processed for RT-PCR by using 2 sets of primers specific for mouse IFN-α1,2 and IFN-β, and for β actin as a control (Figure 1). Injection of Poly (I:C) caused a transient but marked increase in the expression of both mRNAs for IFN-α and IFN-β, compared with untreated control mice (Figure 1A). In CTX-treated mice, the peak of mRNA expression for both IFN-α and IFN-β was almost comparable to that obtained with poly (I:C), but the kinetics was quite different. As shown by densitometric analyses (Figure 1B and 1C), IFN-α and IFN-β mRNA expression in spleen cells from mice injected with poly (I:C) reached its maximum level 6 to 12 hours after treatment. In CTX-treated mice, the maximal expression for both IFN mRNAs peaked between 12 and 24 hours. The apparent delay in the induction of IFN mRNA expression in mice injected with the higher dose of CTX (especially observed for IFN-α) may be somehow dependent on a CTX-induced toxicity. In this regard, there was a consistent decrease in the spleen weight in CTX-treated mice; this appeared more marked in animals treated with the higher dose of CTX. (Figure 1D).

Kinetics of accumulation of Ly-6C expression in the spleen of mice treated with cyclophosphamide

We then evaluated the percentage of Ly-6C hi cells with respect to the number of total spleen lymphocytes. RT-PCR by using 2 sets of primers specific for mouse IFN-α/β with poly (I:C) and processed for total RNA was extracted from spleen cells of mice treated with saline, CTX (83 or 150 mg/kg), or poly (I:C) and processed for FACS analysis. There were 3 mice per group. The data show the mean (± SE) of the percentage of Ly-6C hi cells with respect to the number of total spleen lymphocytes. *P ≤ .05 versus controls; **P ≤ .001 versus controls.

48 hours after injection. No IFN activity was detected in sera from saline-treated control mice. In contrast, sera from poly (I:C)-treated mice exhibited high levels of IFN activity at 12 hours. Considerable levels of IFN (64 U/mL) were also found at 24 hours after poly (I:C) injection, while no IFN activity was detected at the subsequent time point. None of the 6 CTX-treated mice showed any presence of serum IFN at 12 hours after injection. However, at 24 hours, detectable IFN activity was found in 3 out of the 6 CTX-treated mice. At 48 hours, IFN activity (24 to 48 U/mL) was detected in the serum of 4 out of the 6 CTX-treated mice, with no significant difference with respect to the dose of CTX injected. At subsequent times, no IFN activity could be found in the sera of CTX-treated mice (data not shown).

CTX injection results in the up-regulation of Ly-6C expression and bromodeoxyuridine incorporation in spleen lymphocytes

Ly-6C is a lymphocyte activation and differentiation antigen normally present on minor subsets of mature T cells, monocytes, macrophages, and endothelial cells. Previous studies have demonstrated that IFN-α/β is a cytokine capable of specifically enhancing Ly-6C expression.37 By cytofluorimetric analyses, we then evaluated the percentage of Ly-6C positive T cells with respect to the number of total T lymphocytes in the spleens of mice treated with a single injection of CTX (83 mg/kg) 3, 6, 9, and 15 days before sacrifice. In the same experiment, spleen lymphocytes were also analyzed for BrdU uptake. As shown in Figure 2, there was a significant increase of Ly-6C positive cells in CTX-treated mice in comparison with controls at 6 days after CTX treatment. Cell proliferation data, obtained by the BrdU-labeling technique, showed a substantial decrease in the percentage of proliferating cells at day 3 after CTX administration, followed by a significant and long-lasting increase at the subsequent time points. In a subsequent experiment, illustrated in Figure 3, we compared the Ly-6C expression in spleen lymphocytes at various times after treatment of mice with either poly (I:C) or CTX. Ly-6C expression was maximal in splenic lymphocytes 6 days after CTX treatment, whereas poly (I:C) induced a more intense but more transient Ly-6C antigen enhancement that peaked at day 3.

As shown in Figure 4, injection of mice with a potent preparation of anti–IFN-α/β antibodies resulted in a significant inhibition of the CTX-induced up-regulation of Ly-6C expression
on spleen lymphocytes, suggesting that CTX-induced type I IFN was involved in enhancing the expression of this antigen.

Increase of the number of CD44hi T lymphocytes in the spleen and lymph nodes of CTX-treated mice

We then evaluated whether injection of CTX could affect the number of T cells in the spleen and lymph nodes at different times after injection. In general, no statistically significant differences were observed in the total number of CD4 or CD8 T lymphocytes recovered from the spleen or pooled lymph nodes of CTX-treated mice with respect to control injected mice on days 6 and 10, with the exception of a slight increase in the number of CD4 T cells detected in the spleen at 6 days after CTX injection (data not shown). Spleen or lymph node cells from mice treated with a single injection of CTX (83 mg/kg), poly (I:C), or saline were also labeled with biotinylated anti-CD44 and PE-conjugated anti-CD4 or anti-CD8 antibodies and assessed by cytofluorimetric analysis. As shown in Figure 5, there was a marked increase in the percentage of CD44hi T lymphocytes in both the spleen and the lymph nodes of CTX-injected mice, compared with saline-injected control animals. This increase reached its maximum level 10 days after treatment for both CD4+ and CD8+ T-cell subsets. In poly (I:C)-injected mice, there was also a marked increase in CD44hi cell percentage, but the kinetics of this increase appeared to be slightly different in spleen cells as compared with CTX-treated animals.

Figure 5. Increase in the percentage of CD44hi T lymphocytes in the CD4+ and CD8+ T-cell populations from spleens and lymph nodes of mice treated with CTX or poly (I:C). Seven- to 8-week-old DBA/2 mice were injected intraperitoneally with CTX (83 mg/kg) (dotted bars), poly (I:C) (0.15 mg/mouse) (striped bars), or saline (white bars). At different time intervals, spleens and a pool of lymph nodes were taken and disaggregated. Cells were stained for CD44 and for CD4 or CD8 surface antigen before being processed for cytofluorimetric analysis. There were 3 mice per group. The data show the mean (+ SE) value of the percentage of CD44hi cells on CD4 (left panels) or CD8 (right panels) lymphocytes, in the spleen (upper panels) or in the pooled lymph nodes (lower panels).

Discussion

In this study, we have provided the first evidence indicating that CTX is an inducer of type I IFN expression in mice. The induction of type I IFN by CTX was demonstrated not only by the presence of biologically active IFN in the serum of the injected mice at 24 to 48 hours, but also by a progressive accumulation of mRNAs for IFN-α and IFN-β in the spleens, starting at 6 hours and lasting for at least 48 hours after injection. The comparison of the kinetics of induction by CTX with that observed after injection of poly (I:C) (a typical strong inducer of type I IFN in mice) revealed that the peak of IFN-β mRNA induction by poly (I:C) occurred earlier (at 6 hours), but appeared to decrease more rapidly than that observed after CTX injection. As for IFN-α mRNA induction, the kinetics of accumulation of IFN-α mRNA in mice injected with poly (I:C) was similar to that observed in mice treated with the lower dose of CTX (83 mg/kg), whereas a certain delay in the induction kinetics was detected in mice injected with the higher dose of CTX (150 mg/kg). On the whole, these results indicate that CTX is an inducer of type I IFN at a time early after injection, before drug treatment might result in a strong myelosuppression or a detectable decrease in the spleen weight (Figure 1D), substantially before the rebound phenomenon, which starts 5 to 6 days after CTX injection.73,74 Thus, although the mechanisms of type I IFN induction by CTX remain unclear, our results suggest that the type I IFN induction is a direct effect of the chemotherapeutic agent on some mouse cells and does not represent a secondary response to drug-induced myelosuppression or to cell-stimulation events occurring during the rebound phase. This CTX-induced IFN production appears to be capable of directly affecting some lymphocyte functions. In fact, CTX induced an up-regulation in the expression of Ly-6C antigen (a marker known to be specifically induced by type I IFN), and this induction was inhibited by injecting the mice with a potent preparation of anti–IFN-α/β antibodies. Of interest, CTX was also capable of inducing a release of IFN-α/β (ie, BrdU+ T) spleen lymphocytes as well as of modulating the phenotype of T cells, especially by enhancing the number of CD4+ and CD8+ T cells expressing a memory (CD44hi) phenotype in both the spleens and the lymph nodes of the injected mice.

The notion that CTX can induce release of cytokines and growth factors during the rebound events after drug-induced immunosuppression is well documented.38,39 Some studies have recently suggested that CTX can induce a pattern shift of cytokines from Th2 to Th1 in tumor models several days after injection.40 However, our finding that CTX can induce an early and long-lasting expression of type I IFN suggests that this event can be directly important in mediating some of the immunomodulatory effects of CTX observed in a number of experimental systems. CTX is a currently used drug in cancer therapy. Although CTX is generally considered to be a typical chemotherapeutic agent capable of directly inhibiting tumor cell proliferation, many studies have shown that CTX can also induce multiple immunomodulatory effects.7,20 In mouse tumor models, some groups had suggested that CTX can act by inhibiting T suppressor cells.7,21-25 However, the nature of these suppressor cells assumed to be inhibited by CTX has remained elusive.26 In a recent study, in which we have investigated the mechanisms underlying the impressive antitumor response in tumor-bearing mice subjected to a single injection of CTX followed by adoptive immunotherapy,27 we provided...
role in diabetes as well as in the pathogenesis of other autoimmune diseases. Recent studies have revealed that type I IFN is an important cytokine in mice immunized with mucin (I)-mannan fusion protein. In this regard, it is of interest to note that CTL precursors’ frequency in mice immunized with mucin I–mannan fusion protein. This is in contrast to other studies that have reported a decrease in the frequency of CTX-induced cytokines affecting T-cell survival and proliferation of the transferred immune lymphocytes, suggesting that CTX did not act by inhibiting T-suppressor cells but, by rendering the mouse host capable of allowing survival and proliferation of the transferred immune lymphocytes, probably as a result of CTX-induced cytokines affecting T-cell functions.46,47 Notably, type I IFN was important in the response to adoptive immunotherapy in cancer patients. In our previous work, we suggested that in order to attain the optimal effects of adoptive immunotherapy, it should be performed following chemotherapy at a time point when immune function is rebounding.27 Notably, we found that the antitumor response was optimal when immune cells were transferred in tumor-bearing mice approximately 6 hours after a single CTX injection, when an induction of type I IFN mRNA expression is observed in the spleen (Figure 1). We suggest, therefore, that adoptive immunotherapy should be performed shortly after chemotherapy (at a time when type I IFN is initially expressed in some lymphoid tissues), before the rebound overshoot is observed.

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References


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