Cyclophosphamide Enhances the Antitumor Efficacy of Adoptively Transferred Immune Cells through the Induction of Cytokine Expression, B-Cell and T-Cell Homeostatic Proliferation, and Specific Tumor Infiltration

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Abstract

Purpose: Immunotherapy is a promising antitumor strategy, which can be successfully combined with current anticancer treatments, as suggested by recent studies showing the paradoxical chemotherapy-induced enhancement of the immune response. The purpose of the present work is to dissect the biological events induced by chemotherapy that cooperate with immunotherapy in the success of the combined treatment against cancer. In particular, we focused on the following: (a) cyclophosphamide-induced modulation of several cytokines, (b) homeostatic proliferation of adoptively transferred lymphocytes, and (c) homing of transferred lymphocytes to secondary lymphoid organs and tumor mass.

Experimental Design: Here, we used the adoptive transfer of tumor-immune cells after cyclophosphamide treatment of tumor-bearing mice as a model to elucidate the mechanisms by which cyclophosphamide can render the immune lymphocytes competent to induce tumor rejection.

Results: The transfer of antitumor immunity was found to be dependent on CD4+ T cells and on the cooperation of adoptively transferred cells with the host immune system. Of note, tumor-immune lymphocytes migrated specifically to the tumor only in mice pretreated with cyclophosphamide. Cyclophosphamide treatment also promoted homeostatic proliferation/activation of transferred B and T lymphocytes. Optimal therapeutic responses to the transfer of immune cells were associated with the cyclophosphamide-mediated induction of a "cytokine storm" [including granulocyte macrophage colony-stimulating factor, interleukin (IL)-1β, IL-7, IL-15, IL-2, IL-21, and IFN-γ], occurring during the "rebound phase" after drug-induced lymphodepletion.

Conclusions: The ensemble of these data provides a new rationale for combining immunotherapy and chemotherapy to induce an effective antitumor response in cancer patients.

Over the last decade, several new strategies of immunotherapy have been evaluated in cancer patients, but consistent clinical responses have not been obtained thus far. The lack of clinical responses points out the need to overcome the escape mechanisms exploited by tumors by designing novel strategies based on the combination of immunotherapy with other anticancer treatments. One emerging strategy is focused on the manipulation of the host before immunotherapy with the aim of reducing regulatory T cells and expanding/activating tumor-specific T lymphocytes (1). Several reports have shown that nonmyeloablative chemotherapeutic treatments (2), as well as irradiation (3), can modulate the immune response and enhance the antitumor activity of adoptively transferred lymphocytes through a process known as homeostatic proliferation. Under lymphopenic conditions, the residual naive and memory T cells proliferate to reconstitute a nearly normal lymphocyte pool. This expansion seems to require self-peptide/MHC recognition and interleukin (IL)-7 for naive CD4+ and CD8+ T cells (4, 5) and for memory CD4+ T cells (6), whereas proliferation of memory CD8+ T cells needs both IL-7 and IL-15 (7). The expanded T cells show an activated phenotype and express memory phenotype markers; moreover, they acquire the ability to rapidly secrete IFN-γ and become cytotoxic effectors when stimulated with cognate antigen (8).

It is widely accepted that chemotherapeutic agents can exert multiple effects on host immune and nonimmune cells and that this action may be relevant for the induction of an antitumor immune response (9). Among them, the immuno-modulatory effects of cyclophosphamide, an alkylating agent widely used to treat solid organ malignancies as well as lymphoproliferative and autoimmune disorders, have long been studied (10) and are still matter of current investigation.
In particular, several preclinical studies have shown that cyclophosphamide can enhance the antitumor activity of adoptively transferred T cells (2, 11–13) as well as of tumor vaccines (14, 15). Of note, the combination of lymphodepleting chemotherapy followed by the adoptive transfer of autologous tumor-reactive lymphocytes has also been evaluated in phase I/II clinical trials and was shown to mediate significant tumor regression in patients with IL-2 refractory metastatic melanoma (16, 17). Several mechanisms have been suggested to explain cyclophosphamide immunomodulatory activity. As an example, Matar et al. (18) described a Th2/Th1 shift in cytokine production of tumor-bearing rats treated with low-dose cyclophosphamide. In addition, some reports showed that chemotherapy can increase the efficiency of immunotherapy by removing tumor-induced suppressor T cells (19–22). In a previous study, we showed that a single injection of cyclophosphamide enhances proliferation, long-term survival, and antitumor activity of adoptively transferred tumor-immune cells and proposed that this effect was mediated by the production of soluble growth factors induced by cyclophosphamide administration (2). We also showed that cyclophosphamide induced the expression of type I IFN in vivo and modulated the phenotype of T cells, with expansion of CD4+ and CD8+ T cells exhibiting a memory (CD44hi) phenotype (12). In spite of all this, the specific mechanisms by which cyclophosphamide could render the transferred immune cells so effective in eradicating established tumors in mice remained elusive. In the present study, we evaluated the fate of transferred tumor-immune cells when injected into cyclophosphamidetreated tumor-bearing mice and investigated the factors involved in their antitumor activity.

Materials and Methods

Mice. Six- to 7-week-old C57BL/6 (Thy 1.2 and Thy 1.1 strains) and DBA/2 mice were obtained from Charles River Breeding Laboratories (Calco, Italy). Seven-week-old severe combined immunodeficient (SCID) mice were obtained from Harlan (Udine, Italy). Mice were housed in the facilities of the Cell Biology and Neurosciences Department at the Istituto Superiore di Sanita` (Rome, Italy). All mice were treated in accordance with the European Community guidelines. Tumor cells. RBL-5 and B16 melanoma cells were cultured in RPMI 1640 supplemented with 10% FCS. RBL-5 (8 × 10^5) or B16 (1 × 10^5) melanoma cells were implanted s.c. into C57BL/6 or SCID mice for tumor growth. Two million 3Cl-8 Friend leukemia cells were implanted s.c. into DBA/2 mice for tumor growth (23).

Chemotherapy and radiotherapy of recipient mice. C57BL/6, DBA/2, or SCID tumor-bearing mice were injected i.p. with 100 mg/kg cyclophosphamide (Sigma, St. Louis, MO). DBA/2 tumor-bearing mice were subjected to sublethal (5 Gy) total body irradiation.

Immunization of donor mice. C57BL/6 mice received three s.c. inocula, at a time interval of 2 weeks, of a tumor lysate obtained from three freeze-thaw cycles of 5 × 10^6 RBL-5 cells. DBA/2 mice were injected s.c. with 2 × 10^6 genetically modified Friend leukemia cells producing IFNα (IFNo1-Cl-I1; ref. 24).

Adoptive cell transfer, depletion, and neutralization experiments. Spleen and lymph node cell suspensions from vaccinated and nonvaccinated donor mice, prepared as described in ref. 2, were pooled and laid over Lympholyte-M Gradient (Cederlane, Hornby, Ontario, Canada) for lymphocyte enrichment. When indicated, cells were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). Where indicated, CD4+, CD8+, and natural killer cells were depleted by using complement-mediated cytotoxicity as described previously (2). Fifty million (a spleen equivalent number) CFSE-labeled or unlabeled cells (as indicated in the figure legends) were injected i.v. in tumor-bearing, cyclophosphamide-treated, or untreated mice. In neutralization experiments, 0.5 mg of anti-mouse IL-7 antibody or control antibody (R&D Systems, Minneapolis, MN) was injected i.p. 2 days after cyclophosphamide treatment and 2 days after immune cell adoptive transfer.

Flow cytometry. Phycoerythrin-conjugated anti-CD4, phycoerythrin-conjugated anti-CD8, biotinylated anti-CD3, and biotinylated anti-CD19 monoclonal antibodies (mAb) were purchased from BD Biosciences (Milan, Italy). Biotinylated antibodies were detected with peridinin chlorophyll protein–conjugated streptavidin. Cells were analyzed on FACSort flow cytometer (Becton Dickinson). A total of 5,000 CFSE+ events per sample was collected, and the percentage of cells that had proliferated were determined by gating on the lineage-positive CFSEdim subset.

Immunofluorescence. For histologic analysis, tumor tissues were embedded in OCT and snap frozen; 5-μm thick sections of embedded tissues were cut and air dried. OCT was removed by washing in PBS, and sections were immunostained with biotinylated anti-CD90 (Thy 1.1) mAb (BD Biosciences) followed by incubation with streptavidin conjugated with Alexa Fluor 488. Confocal laser scanner microscopy observations were done using a Leica TCSSP2AOPS apparatus (Leica Microsystems, Wetzlar, Germany).

[3H]Thymidine incorporation assays. Splenocytes (2 × 10^6) were cocultivated with the same number of irradiated (Co, 20,000 rads) RBL-5 tumor cells. After a 4-day incubation at 37°C, [3H]thymidine (0.5 μCi/well) was added to the culture and plates were incubated for additional 18 h. The cells were harvested onto a glass fiber paper using a semiautomated cell harvester, and [3H]thymidine uptake was measured in a liquid scintillation counter.

Determination of tumor-specific antibodies. To measure specific antibody levels in serum samples, standard direct ELISAs were done as described previously (25). Briefly, 96-well plates were coated with RBL-5 tumor lysate (4 × 10^6 cells). Peroxidase-conjugated anti-mouse IgG (Pierce, Rockford, IL) was used at a dilution of 1:75,000. Orthophenylenediamine (Sigma) was used as enzymatic substrate. Absorbances were read in a microplate autoreader at 490 nm wavelength. Results are expressed as reciprocal end point titers where a threshold of positivity for absorbance values was calculated as the average + 3 SD of all dilutions from three control mouse sera. For a given sample, the end point titer was determined as the first dilution below the threshold of positivity.

IFN-γ enzyme-linked immunospot assay. CD8+ lymphocytes were isolated from splenocytes by magnetic separation using MACS CD8a microbeads according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Macrogamocytes/macrophages were obtained by plastic adherence for 3 h at 37°C. Nitrocellulose-bottomed 96-well plates (MultiScreen, Millipore, Bedford, MA) were coated with anti-IFN-γ mAb (Mabtech, Nacka, Sweden). After washing, CD8+ lymphocytes were mixed with adherent cells and plated with RBL-5 in duplicate at 5:1 responder to stimulator ratio. Plates were incubated overnight at 37°C. After incubation with biotinylated anti-IFN-γ mAb and streptavidin-enzyme conjugate (Mabtech), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) was added to each well. The reaction was terminated by washing with tap water on the appearance of dark spots. The spots were counted using the 4-Plate ELISPOT Reader V2.1 (Aelvis GmbH, Hannover, Germany).

Real-time PCR. Total RNA from bone marrow cells was obtained by RNeasy (Qiagen, Milan, Italy) purification according to the manufacturer’s instructions. Total RNA from spleens and lymph nodes was purified by Trizol reagent (Invitrogen, Carlsbad, CA) extraction. cDNA templates were obtained by reverse transcription (Promega, Madison, WI). Quantitative measurements of specific transcripts were acquired by real-time PCR using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Milan, Italy). PCR amplifications were done with QuantiTect SYBR Green PCR reagents (Qiagen). To verify that a single
**Results**

**Combination of cyclophosphamide or sublethal irradiation and adoptive transfer of tumor-immune splenocytes is curative in mice with established syngeneic tumors.** Figure 1 shows the dramatic effects on established Friend leukemia cells (Fig. 1A) or RBL-5 lymphoma (Fig. 1B) of the combined treatment with cyclophosphamide and splenocytes deriving from mice immunized against tumors (tumor-immune splenocytes). In both tumor models, the single cyclophosphamide administration or the adoptive transfer of tumor-immune splenocytes alone was devoid of any significant effect on tumor growth and survival. On the contrary, the sequential treatment with cyclophosphamide followed 5 h later by the i.v. infusion of immune lymphocytes resulted in complete tumor regression in 100% of mice with no tumor recurrence (up to 100 days after treatment). The adoptive transfer of splenocytes from untreated mice did not affect tumor growth either alone or in combination with cyclophosphamide treatment (data not shown).

Further experiments showed that the injection of immune spleen cells up to 3 days after cyclophosphamide administration did not significantly alter the effect of treatment, whereas lack of efficacy was observed when the adoptive cell transfer was done beyond day 3 (data not shown). Similar therapeutic effects were observed when adoptive transfer of immune splenocytes was combined with sublethal (5 Gy) total body irradiation (Fig. 1A), suggesting that common myelotoxic effects induced by both cyclophosphamide and radiotherapy could condition the host environment, allowing adoptively transferred immune cells to eradicate established tumors.

**The essential roles of CD4+ T cells from donor mice and of the immune system of recipient mice.** To determine which cell population was responsible for the transfer of the antitumor activity, ex vivo depletion experiments were carried out on splenocytes derived from mice immunized against RBL-5 cells before their adoptive transfer into cyclophosphamide-treated RBL-5 tumor-bearing mice. As shown in Fig. 1C, the antitumor effect of cyclophosphamide treatment combined with infusion of CD4+ T-cell–depleted splenocyte suspension was significantly reduced with respect to standard cyclophosphamide-immune spleen cell treatment. In contrast, depletion of CD8+ T cells (Fig. 1C), as well as of natural killer cells or macrophages (data not shown), did not affect the therapeutic effectiveness. These data are consistent with the concept that CD4+ T cells are indeed the main cells responsible for the transfer of the antitumor immunity.

To evaluate whether the transfer of antitumor activity by immune splenocytes required the cooperation of the immune system of tumor-bearing recipient mice, we implanted s.c. RBL-5 tumors in immunodeficient SCID mice and in immunocompetent C57BL/6 mice as control before subjecting them to the sequential chemoimmunotherapy protocol. The results shown in Fig. 1D indicate that no therapeutic effect was observed in SCID mice, whereas immunocompetent mice treated with cyclophosphamide and immune splenocyte infusion underwent complete tumor regression.

**Cyclophosphamide-driven homing of adoptively transferred lymphocytes.** To assess the fate of adoptively transferred cells, RBL-5 tumor-bearing mice were infused with CFSE-labeled splenocytes from vaccinated or nonvaccinated donors. Figure 2A shows that, in the absence of cyclophosphamide treatment, the transferred immune cells were unable to home neither to the spleen nor to the lymph nodes of recipient mice, and similar results were obtained when transferred cells were derived from naive donors (data not shown). Conversely, chemotherapy...
pretreatment clearly induced the homing of transferred CD19⁺, CD8⁺, and CD4⁺ cells to secondary lymphoid organs independently on whether the lymphocytes were derived from immunized or naive donor mice. Of interest, the number of transferred CD19⁺ B cells deriving from immunized mice was increased with respect to that of cells derived from naive mice. To assess whether transferred splenocytes were able to home to secondary lymphoid organs only because of the increased “space” generated by cyclophosphamide-induced lymphopenia, CFSE-labeled tumor-immune splenocytes were injected into tumor-bearing SCID mice. As illustrated in Fig. 2B, the number of CFSE-positive immune cells detected in the spleen was higher in SCID than in C57BL/6 mice. However, this number was still thrice lower than that found in the spleen of C57BL/6 mice pretreated with cyclophosphamide. These findings suggest the involvement of other cyclophosphamide-induced mechanisms, in addition to that of the replenishment of a lymphopenic host. Of special note, only adoptively transferred cells derived from tumor-immunized donor mice, but not from control mice, were able to infiltrate the tumor tissue (Fig. 2C). These results were confirmed by immunofluorescence analysis of tumor sections derived from cyclophosphamide-treated Thy 1.2⁺ C57BL/6 mice injected with naive or tumor-immune cells from congenic Thy 1.1⁺ animals (Fig. 2F and G). It is worth noting that RBL-5-specific splenocytes were unable to migrate into an antigenically unrelated B16 melanoma tumor (Fig. 2C), thus showing that homing of transferred immune cells to the tumor was an antigen-driven phenomenon. Moreover, the adoptive transfer of RBL-5-specific splenocytes in combination with cyclophosphamide was not effective in eradicating B16 melanoma tumor (data not shown).

Effect of cyclophosphamide treatment on homeostatic and antigen-specific proliferation. The increased number of transferred splenocytes recovered in the spleen and lymph nodes of cyclophosphamide-treated mice could be due to cell proliferation. To investigate this issue, transferred splenocytes were labeled with CFSE and proliferation was analyzed by means of flow cytometry in cyclophosphamide-conditioned hosts. Figure 3A shows that transferred splenocytes underwent several rounds of proliferation in secondary lymphoid organs 10 days after their transfer into cyclophosphamide-treated mice regardless of whether cells were derived from tumor-immunized or naive donor mice, thus showing that the proliferative stimulus induced by cyclophosphamide was nonspecific. These data were confirmed by an in vitro [³H]thymidine uptake assay in which proliferation of spleen cells derived from tumor-bearing mice, treated with cyclophosphamide alone or in combination with immune splenocyte adoptive transfer, was evaluated in the presence or absence of irradiated RBL-5 cells (Fig. 3B). Spleen cells from mice treated with cyclophosphamide and adoptive immune cell transfer displayed a spontaneous [³H]thymidine uptake even in the absence of in vitro specific antigenic stimulation (Fig. 3B, top). Of interest, when the same splenocytes were cultured in the presence of irradiated RBL-5 cells, [³H]thymidine uptake was considerably higher (Fig. 3B, bottom). Of note, cells from mice treated with cyclophosphamide alone underwent a weak spontaneous proliferation both in the presence and in the absence of antigen. Taken together, these data suggest that cyclophosphamide pretreatment can induce an environmental conditioning, which affects both

Fig. 2. Effect of cyclophosphamide treatment on the homing of adoptively transferred lymphocytes to the lymphoid organs and to the tumor mass. A to C, C57BL/6 or SCID (where indicated) mice were injected s.c. with RBL-5 or B16 melanoma (where indicated) cells. Mice were divided into three groups, and 11 d later, some mice (n = 3) were treated i.p. with cyclophosphamide (CTX) followed after 3 d by i.v. infusion of CFSE-labeled immune (or naive) splenocytes. One group received immune splenocytes alone. A and B. 5 d after the adoptive cell transfer, CFSE⁺ absolute cell number and phenotype were analyzed by flow cytometry in spleen and lymph nodes. A, white columns, CD19⁺ lymphocytes; striped columns, CD8⁺ T lymphocytes; black columns, CD4⁺ T lymphocytes. C. CFSE⁺ cells infiltrating the tumor mass were evaluated in RBL-5 as well as in B16 melanoma tumor. Results of one representative experiment out of three. Spleen tissue sections from Thy 1.2⁺ (D) or Thy 1.1⁺ (E) C57BL/6 mice were stained with anti-CD90 mAbs and analyzed by confocal laser scanner microscopy. Bar, 40 μm.
Interestingly, the percentage of proliferating CD19+ B cells was correlated with elevated and sustained levels of IFN-γ-producing lymphocytes, but it is particularly effective on the proliferation/expansion of adoptively transferred cells. Moreover, although cyclophosphamide treatment induces both naive and immune T lymphocyte nonspecific proliferation, it strongly enhances antigen-driven proliferation of tumor-immune lymphocytes.

**Correlation between in vivo proliferation of different lymphocyte subpopulations, antibody production, and tumor-specific CD8+ T-cell frequency.** The phenotypic characterization of CFSE-labeled transferred cells showed that CD19+ and CD8+ lymphocytes underwent in vivo proliferation in both spleen (Fig. 4A and C) and lymph nodes (data not shown) 10 days after transfer into cyclophosphamide-treated recipient mice. Interestingly, the percentage of proliferating CD19+ B cells was higher when transferred lymphocytes were derived from vaccinated rather than from naive animals (Fig. 4A). This could be indicative of an increased activation of transferred CD19+ B cells and correlated with elevated and sustained levels of tumor-specific serum antibodies with respect to the barely detectable antibody levels found in mice treated with naive cells (Fig. 4B). No tumor-specific antibody response was detectable in tumor-bearing mice untreated or treated with cyclophosphamide alone (data not shown).

In contrast to B lymphocyte proliferation, the levels of dividing CFSE-labeled CD8+ T lymphocytes were rather similar in mice treated with either immune or naive splenocytes (Fig. 4C). To evaluate and quantify tumor-specific CD8+ T-cell response, the frequency of IFN-γ-producing lymphocytes in response to RBL-5 cells was evaluated by enzyme-linked immunospot analysis. Figure 4D shows that, 3 days after the adoptive cell transfer, the number of IFN-γ-producing cells increased in cyclophosphamide pretreated mice regardless of whether the transferred cells were derived from naive or vaccinated animals. On the contrary, at day 10, the number of IFN-γ-producing cells was thrice higher when cyclophosphamide-treated mice were injected with immune rather than with naive cells. Of note, although the number of IFN-γ-producing lymphocytes was augmented in mice receiving immune cells without cyclophosphamide conditioning with respect to untreated mice, this number was further increased when mice were pretreated with cyclophosphamide, suggesting that a marked expansion of activated tumor-immune cells can occur in vivo only after cyclophosphamide treatment.

**Cytokine gene expression after cyclophosphamide treatment disconnection and role of IL-7 in the homing/proliferation of adoptively transferred cells.** We evaluated the expression level of several cytokines related to hematopoietic and lymphopoietic systems by real-time PCR in bone marrow, lymph nodes, and spleen of tumor-bearing C57BL/6 mice at different time points after a single cyclophosphamide administration (Fig. 5A). In bone marrow cells, the expression of GM-CSF, IL-1β, IL-7, IL-2, IL-21, IFN-γ, IL-10, and IL-13 was significantly increased with respect to untreated mice (only changes higher than 2-fold were considered). This increase occurred early on day 2 after cyclophosphamide administration, whereas on days 4 and 7 the mRNA expression returned to baseline levels. On the contrary, IL-4 and IL-6 mRNA levels were decreased at each time point, whereas IL-15 and tumor necrosis factor-α were increased in the absence (top) or presence (bottom) of irradiated RBL-5 cells as stimulators. A, cyclophosphamide alone; C, cyclophosphamide plus immune cells; C, untreated mice. Spleenocyte proliferation was measured by [3H]thymidine uptake assay. Points, mean of [3H]thymidine uptake of spleen cell suspensions from three individual mice tested in triplicate; bars, SE.
expression was not affected by cyclophosphamide. In the spleen, no significant changes in gene expression were observed for IL-2, IL-21, IFN-γ, IL-4, IL-10, tumor necrosis factor-α (TNF-α), IL-6, and IL-13 gene expression was analyzed by real-time PCR. 2 d after cyclophosphamide injection, bone marrows (BM) and spleens were removed from SCID mice and IL-7 and IL-15 gene expression was analyzed. Relative mRNA levels were calculated by the comparative cycle threshold (Ct) method and normalized by β-actin expression. Columns, mean fold change of mRNA level of five treated mice with respect to the mean level of five untreated controls; bars, SE. *, P \leq 0.05 versus untreated control; **, P < 0.005 versus untreated control. C. After cyclophosphamide treatment, mice were inoculated with CFSE-labeled immune splenocytes and anti-IL-7 antibody (Ab) or control antibody as described in Materials and Methods. Ten days after the adoptive cell transfer, the percentage of CFSE+ cells was analyzed by flow cytometry in the spleen. Results of one representative experiment out of three.

As shown in Fig. 1, treatment with cyclophosphamide and adoptive cell transfer was ineffective in tumor-bearing SCID mice. To evaluate whether the lack of efficacy is due to a defect in the cyclophosphamide-mediated induction of homeostatic cytokines, the expression of IL-7 and IL-15 genes was analyzed in SCID mice (Fig. 5B). The transcript levels of these cytokines expression profile was similar to that found in the spleen (data not shown).
were compared between cyclophosphamide-treated and untreated mice in bone marrow and spleen 2 days after cyclophosphamide administration (i.e., when the transcript levels of IL-7 and IL-15 are maximal in immunocompetent mice). Similarly to what was observed in C57BL/6 mice, in SCID bone marrow cells the expression of IL-7 was up-regulated by cyclophosphamide treatment, whereas IL-15 expression was not changed. In contrast, cyclophosphamide administration did not augment the expression of IL-7 in the spleen of SCID mice. These findings suggest that, at least for these two cytokines, bone marrow compartment responds to cyclophosphamide treatment in a similar way in SCID and immunocompetent mice, whereas in periphery the substantial absence of lymphocytes can account for the lack of IL-7 up-regulation.

Among the cytokines analyzed in C57BL/6 mice, IL-7, known to support T lymphocyte homeostatic proliferation and survival, was the most up-regulated. To assess the role of this cytokine in the homing and proliferation of transferred lymphocytes, IL-7 was in vivo neutralized in mice treated with cyclophosphamide and adoptive cell transfer. The number of transferred cells recovered in the spleen of anti-IL-7 antibody-treated mice was diminished compared with control antibody-treated mice (Fig. 5C). Nevertheless, IL-7 neutralization did not reduce the proliferation of adoptively transferred cells (data not shown), suggesting that IL-7 is essential only for the retention/survival of transferred cells in this model.

Lastly, we analyzed whether the adoptive transfer of splenocytes by itself or in combination with cyclophosphamide administration could modulate the gene expression of selected cytokines in C57BL/6 mice. Figure 6A shows that cytokine transcript levels detected in spleen and bone marrow cells of mice treated with either tumor-immune or naive splenocytes in the absence of cyclophosphamide were comparable with the levels found in untreated mice. Moreover, the injection of either type of cells following cyclophosphamide administration did not substantially influence the cytokine gene expression profile induced by cyclophosphamide alone. Taken together, these results suggest that cyclophosphamide treatment discontinuation itself was responsible for the observed modulations in cytokine expression. Finally, we measured the levels of IFN-γ and IL-10 in the sera of mice subjected to different treatment regimens. The analysis of the plasma levels of these two cytokines revealed a significant influence of immune cell adoptive transfer in cyclophosphamide-treated tumor-bearing mice (Fig. 6B). In fact, whereas cyclophosphamide alone did not significantly increase the amount of plasma IFN-γ with respect to controls, the levels of this cytokine were almost doubled in cyclophosphamide-treated mice infused with tumor-immune splenocytes. Conversely, cyclophosphamide plus immune cell administration significantly reduced IL-10 production compared with controls, thus suggesting a polarization toward a Th1 type of immune response.

Discussion

In the present study, we provided new information on the cellular and molecular mechanisms by which cyclophosphamide conditioning can enhance the activity of adoptively transferred tumor-immune cells, thus allowing the regression of established tumors. Of note, the chemoimmunotherapeutic approach was shown to be highly effective in both immunocompetent and immunodeficient mice, confirming the versatility of this approach in different tumor models. The ability of cyclophosphamide to enhance the efficacy of adoptive immunotherapy may be due to several mechanisms, including the modulation of cytokine expression and the augmentation of the antitumor effect of transferred cells. The results of our study suggest that cyclophosphamide treatment can improve the homing and proliferation of transferred lymphocytes, possibly through the modulation of cytokine expression.

Fig. 6. Cytokine mRNA levels and protein plasma levels in mice subjected to cyclophosphamide treatment and adoptive cell transfer. C57BL/6 mice were injected s.c. with 8 × 10⁶ RBL-5 cells. Eleven days later, mice were treated i.p. with cyclophosphamide or left untreated as control. Five hours (A) or 3 d (B) after cyclophosphamide administration, some mice received an i.v. inoculum of immune or naive splenocytes. A, black columns, no cell infusion; white columns, tumor-immune cell infusion; gray columns, naive cell infusion. Bone marrows and spleens (Spl) were collected 2 d after cyclophosphamide injection and GM-CSF, IL-1β, IL-7, IL-2, IFN-γ, IL-10, IL-4, IL-6 and tumor necrosis factor-α gene expression was analyzed by real-time PCR. Relative transcript levels were calculated by the comparative cycle threshold (C T) method and normalized by β-actin expression. Columns, average of three determinations; bars, SE. B. 10 d after the adoptive cell transfer, mice were bled and IFN-γ and IL-10 plasma levels were measured by ELISA. Columns, mean of three separated experiments evaluated in duplicate; bars, SE.
strategy was capable of inducing the complete eradication of two different established tumors transplanted into diverse mouse strains (DBA/2 and C57BL/6 mice). Although the adoptive transfer of tumor-immune cells was not curative by itself, it became highly effective in inducing tumor eradication when preceded by either cyclophosphamide administration or irradiation. It is well known that both chemotherapy and irradiation can induce lymphodepletion, which is then followed by a spontaneous expansion of the remaining T cells in the periphery to restore the original pool size (29). The potent therapeutic efficacy induced by either treatment may therefore depend on common homeostatic mechanisms acting during the recovery phase that follows lymphopenia.

Depletion experiments, aimed at identifying the cell subpopulations responsible for the transfer of the antitumor activity, showed that CD4+ T cells were essential for tumor rejection. This finding may provide an explanation for the failure of most chemoimmunotherapeutic strategies, which have been generally based on the infusion of CD8+ T-cell clones (30, 31). Of interest, to achieve an optimal antitumor effect, the adoptive cell transfer requires an intact immune system in the recipient host, as shown by experiments carried out in SCID mice, suggesting the need for cooperation between transferred and resident immune cells.

Currently, little is known about the fate of adoptively transferred cells in a lymphopenic host. Dummer et al. (3) showed that lymph node cells, transferred from naive syngeneic animals, can migrate into secondary lymphoid organs and tumor only in sublethally irradiated tumor-bearing mice. We showed here that cyclophosphamide treatment can efficiently promote the survival of transferred cells and their homing to both spleen and lymph nodes independently on whether lymphocytes were derived from immunized or naive donor mice. According to Maine and Mule (32), this phenomenon could be explained by the cytotoxic activity of cyclophosphamide that creates empty “space” within the lymphoid compartment. However, our experiments in SCID mice show that at least two mechanisms operate in augmenting adoptively transferred cell homing/proliferation: the induction of lymphopenia that produces “space” and the up-regulation of homeostatic cytokines. It has been emphasized that the infiltration of adoptively transferred tumor-reactive T cells to the tumor tissue is a prerequisite for antitumor efficacy (33, 34). In this regard, our study clearly shows that pretreatment of recipient animals with cyclophosphamide is indispensable to promote transferred lymphocyte migration to the tumor site; however, this effect was evident only on cells endowed with tumor specificity and correlated with tumor regression.

In addition to augmenting homing/survival, cyclophosphamide treatment also enhances the ability of transferred cells to proliferate into the recipient host, acting through both nonspecific and antigen-specific mechanisms. In fact, cyclophosphamide nonspecifically enhanced homeostatic proliferation of either immune or naïve transferred cells; nevertheless, in vitro experiments showed that, although the expansion of tumor-immune cells was induced by cyclophosphamide even in the absence of tumor antigen, the proliferation rate was further increased when splenocytes were cultured in the presence of antigen-expressing tumor cells.

An important question is whether cells undergoing homeostatic proliferation acquire effector functions, which may subsequently lead to tumor rejection. Although current immunotherapeutic strategies for the treatment of solid tumors tend to focus on the cellular arm of the immune response, it has been shown that both humoral and cellular immunity are required to induce a complete tumor eradication (35). Remarkably, we have shown here that cyclophosphamide treatment, in combination with adoptive transfer of tumor-immune cells, resulted in a raise of both the frequency of tumor-specific CD8+ T cells and antibody levels. Although the effect of cyclophosphamide on the homing/survival and proliferation of lymphocytes in secondary lymphoid organs is nonspecific, altogether these findings indicate that only cells endowed with antitumor specificity can be induced by cyclophosphamide-driven homeostatic mechanisms to mount an effective antitumor immune response.

In a previous study, we had shown that cyclophosphamide acts through the production of yet unidentified soluble factors sustaining the proliferation, survival, and activity of transferred lymphocytes (2). To gain more insights into the cytokine milieu induced by cyclophosphamide treatment, we analyzed the expression levels of several factors regulating hematopoiesis, homeostatic proliferation, and T-cell functions. We observed the overexpression of some hematopoietic growth factors (i.e., GM-CSF, IL-1β, and IL-7). GM-CSF is known to induce myelorestitution following chemotherapy-induced myelosuppression (36). IL-1β was also shown to strongly enhance myelorestitution in combination with IL-2 when given after cyclophosphamide treatment in a mouse model (37). Of special interest, IL-7, which is a critical cytokine for murine T and B lymphopoiesis (38), was the most up-regulated cytokine in bone marrow cells from cyclophosphamide-treated mice.

It has been suggested that chemotherapy-induced lymphopenia can enhance T-cell–mediated tumor immunotherapy by reducing the number of cells that compete for homeostatic cytokines (39). We have shown here, for the first time, that cyclophosphamide administration actively induces the expression of cytokines regulating homeostatic expansion. In fact, 2 days after cyclophosphamide treatment, IL-7, IL-2, and IL-21 were strongly overexpressed in the bone marrows of treated mice. Of note, the expression of IL-7 and IL-15 mRNA was also increased in the spleen. It is generally assumed that homeostatic proliferation is mainly driven by cytokines sharing a common γ chain in their receptors (IL-2, IL-7, IL-15, and IL-21). Among these cytokines, IL-7 has a pivotal role, as it was shown to be indispensable for homeostatic expansion of naive CD8+ and CD4+ T cells in lymphopenic hosts and for CD8+ T-cell survival in normal hosts (4). IL-7 neutralization experiments showed that, in our model, this cytokine has a major role in promoting the retention/survival of transferred lymphocytes; however, IL-7 is not the only factor controlling cyclophosphamide-induced homeostatic proliferation of transferred cells. It is likely that cell proliferation is guided by a synergy of different homeostatic cytokines, including IL-15 and IL-21. IL-15 is involved in the control of memory CD8+ T-cell survival and division (40) and can enhance the in vivo antitumor activity of adoptively transferred, tumor-reactive CD8+ T cells (41). In addition, IL-21 can synergize with either IL-7 or IL-15 in driving the proliferation of CD8+ T cells, in inducing the differentiation of B cells into plasma cells and in enhancing the activity of natural killer cells (42). Of note, some homeostatic cytokines do not induce regulatory T cells; in fact, IL-7 administration in patients...
selectively decreases the percentage of circulating CD4⁺ regulatory T cells (43) and culture of regulatory T cells in the presence of IL-7 and IL-15 abrogates their suppressive activity (44). Moreover, it has recently been shown that the α chain of IL-7 receptor is expressed at low levels in regulatory T cells (45). Altogether, these observations suggest that the cyclophosphamide-induced homeostatic cytokines may redirect the immune system from a state of tolerance to an effective antitumor immunity.

Finally, we analyzed the expression of cytokines involved in the polarization toward a Th1 or a Th2 type of immune response. Real-time PCR experiments showed that the Th1 cytokines IL-2 and IFN-γ were overexpressed 2 days after cyclophosphamide administration, whereas IL-4 (Th2 cytokine) and IL-6 were down-regulated. The finding that IL-10 and IL-13 mRNAs were also increased on cyclophosphamide treatment suggests that cyclophosphamide can initially induce a Th0 phenotype, which is followed by a polarization toward a Th1 type of immune response, as suggested by the increase of IFN-γ and the decrease of IL-10 plasma levels in mice treated with cyclophosphamide and immune cell adoptive transfer. Of note, IL-13 has been shown to affect the activation and maturation of dendritic cells (46) and to prevent the blockade of their differentiation induced by tumor cells (47) and may therefore counteract escape mechanisms exploited by tumors at this level.

The modulation of cytokine gene expression was predominantly observed in the bone marrow, whereas in the spleen and lymph nodes most mRNA levels remained constant; these results suggest that the “cytokine storm” originates in a primary lymphoid organ rather than in the periphery. The preferential induction of homeostatic cytokines in the bone marrow is also in agreement with the recent observation that this organ is the preferred site for homeostatic proliferation of memory CD8⁺ T cells (48). Remarkably, the induction of cytokine mRNA expression was maximal within 2 days from cyclophosphamide injection and decreased by day 4, indicating the need to perform the adoptive cell transfer at early time points after chemotherapy.

Current clinical trials are based on the ex vivo selection and in vitro expansion of tumor-reactive lymphocytes before reinfusion into the patient after nonmyeloablative lymphodepletion. However, the adoptive transfer of CD8⁺ T clones that had been multiply stimulated proved ineffective (31), whereas treatment with less-expanded tumor-infiltrating lymphocytes caused objective responses in ~50% of treated patients (17). Furthermore, tumor regression was shown to correlate with the persistence of cells expressing an early effector (CD27⁺CD28⁺) phenotype (49). Studies in murine models have confirmed that the acquisition of full effector functions in vitro can impair the in vivo antitumor efficacy of adoptively transferred CD8⁺ T cells (50). According to these studies and our findings, we propose that the selection of efficacious tumor-specific T cells and their expansion and acquisition of effector functions may occur spontaneously in vivo through chemotherapy-mediated homeostatic mechanisms acting on vaccine-stimulated transferred cells.

The results obtained in this study clearly show that several requirements are essential for achieving a successful eradication of the tumor: (a) the induction of lymphopenia that produces “space” and allows the homing of transferred immune lymphocytes to lymphoid organs and ultimately to the tumor site; (b) the induction of a “cytokine storm” that drives the homeostatic proliferation, expands and activates antitumor lymphocytes, and redirects the immune response from tolerance toward antitumor efficacy; (c) the transfer of help from a vaccinated animal; and (d) the cooperation between transferred cells and the host immune system. Collectively, these findings reveal novel mechanisms by which chemotherapy can markedly enhance the antitumor response of adoptively transferred immune lymphocytes and open new perspectives for combining chemotherapy and immunotherapy in cancer patients.

**Acknowledgments**

We thank M. Venditti and M. Spada for technical assistance and A. Mariani and A. Fresolone for secretarial assistance.

**References**


immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;34:336 – 44.


Cyclophosphamide Enhances the Antitumor Efficacy of Adoptively Transferred Immune Cells through the Induction of Cytokine Expression, B-Cell and T-Cell Homeostatic Proliferation, and Specific Tumor Infiltration

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