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REGULATORY T CELLS BUT NOT NKT I CELLS ARE MODULATED BY A SINGLE LOW-DOSE CYCLOPHOSPHAMIDE IN A B CELL LYMPHOMA TUMOR-MODEL

M.J. Rico[1], V.R. Rozados[1], L.E. Mainetti[1], M.F. Zacarías Fluck[1], P. Matar[1,2], O.G. Scharovsky[1,3,]*
[1]*Institute of Experimental Genetics, School of Medical Sciences, National University of Rosario, Santa Fe 3100, Rosario 2000, Argentina*
[2]*National Scientific and Technical Research Council (CONICET), Rosario 2000, Argentina*
[3]*Research Council, National University of Rosario, Rosario, Argentina*

***Aim*: Experimental and clinical studies showed that the administration of cyclophosphamide (Cy) in low doses leads to an enhancement of the antitumor immune response. Our objective was to study the modulation, if any, by low dose Cy, of T regulatory (Treg) and natural killer T (NKT) I cells, two cell populations of the innate immune response with opposing effects on the tumors, in a rat B cell lymphoma model. *Methods*: Inbred e rats were challenged s.c. with L-TACB lymphoma and on day 14 animals were distributed in two groups. *Treated*: injected i.p. with cyclophosphamide (10mg/kg of body weight) and *Control*: injected i.p. with saline. Blood samples were taken from days 0 to 21 and the percentage of T regulatory and natural killer T I cells were determined by flow cytometry. Results: We found that the increase of natural and inducible T regulatory cells of peripheral blood achieved during tumor growth was significantly down-regulated by cyclophosphamide. On the contrary, natural killer T I cells were not modulated by the treatment. *Conclusion*: The antimetastatic effect of a single low dose of Cy would be due, at least in part, to downregulation of natural and inducible T regulatory cells.**
***Key Words*: T regulatory cells, NKT I cells, cyclophosphamide, lymphoma.**

The mechanisms of peripheral tolerance that control the quality and intensity of immune responses are exerted by different types of T cells with regulatory function which include specialized subsets of $CD4^+$, $CD8^+$, double negative ($CD4^-CD8^-$) $CD3^+$, γδ T cells, and natural killer T (NKT) cells [1, 2] . Among these, natural T regulatory cells (Tregs) were first described in the 1970s by Gershon [3]. They were originally identified in mice [4] but then, they were also described in rats [5] and humans [6]. Tregs exert their suppressive activity in an antigen-nonspecific way called "bystander suppression" [2]. They suppress a wide variety of immune cells including naive and memory $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, and dendritic cells [7–12]. Tregs are characterized by the constitutive expression of several activation markers as CTLA-4 (CD152) and Foxp3, among others [13–15]. However, Foxp3 is the most specific marker for natural Tregs playing a precise role in the development and function of natural $CD4^+CD25^+$ Tregs [13, 14]. Another subset of regulatory cells are the Type 1 regulatory cells (Tr1) that are defined by their ability to produce high levels of IL-10 and transforming growth factor-β (TGF-β) [16–17], utilizing those cytokines to suppress Th1 and Th2-mediated immune responses. Tr1 cells are inducible, antigen-specific and express normal levels of activation markers such as CD25, following TCR-mediated stimulation. Other important regulators of the immune response are NKT cells [19]. This cell population expresses both functional TCR αβ chains, and NK receptors, such as NK1.1. The cells that recognize antigens presented by the nonpolymorfic MHC class I-like molecule CD1d are true NKT cells [20]. Although type I NKT cells have NK-like cytolytic activity, they are considered regulators of immune responses because they rapidly produce large amounts of Th1 and Th2 cytokines in autoimmune disease, infectious disease, and cancer. Activated NKT cells induce cell death in tumor cells through the expression of cell-death-inducing effector molecules [21].

Both, experimental and clinical studies, revealed that cyclophosphamide (Cy), an alkylating agent commonly used in cancer chemotherapy [22], exerts an apparently paradoxical effect on host immune responses [23]. High doses of Cy usually bring about an impairment of the host defense mechanisms, along with the reduction of primary tumor mass, therefore leading to severe immunosuppression. However, the administration of low doses of Cy leads to an enhancement of the immune response, frequently causing tumor rejection [24, 25].

Our previous results in a rat B-cell lymphoma model (L-TACB) showed that a single low-dose Cy, administered to rats bearing already grown lymphomas, inhibited metastasis development [26]. The antimetastatic action of Cy was mediated by immunomodulation [27]. We demonstrated that IL-10 was the main factor responsible for the induction and development of immunosuppression in L-TACB-bearing hosts [28, 29]. In fact, Cy induced a Th2/Th1 switch in the cytokine profile and increased the proliferative rate of spleen cells [30].

Considering the already demonstrated immunomodulatory effect of low-dose Cy in a B-cell lymphoma tumor model and taking into account that Treg and NKT I cells may be involved in the regulation of the antitumor immune response, we decided to evaluate if low-dose

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*Correspondence: Fax: + 54–341–4804569
E-mail: graciela.scharovsky@gmail.com
Abbreviations used: Cy – cyclophosphamide; Cy-d – cyclophosphamide duplicated dose (20 mg/kg); e rats – IIM e/Fm rats; NKT – natural killer T cells; TGF-β – transforming growth factor-β; Tregs – T regulatory cells; Tr1 – type 1 regulatory cells.

Cy treatment was able to modulate those lymphocyte subpopulations in the same tumor model. Moreover, it has been suggested that Tregs and NKT I might regulate each other. Treg cells suppress the anti-tumor effect and reduce the number of NKT I cells. At the same time, NKT I cells regulate Treg cell function [20].

MATERIALS AND METHODS

Mice. Ten to twelve weeks old female inbred IIM e/Fm rats (*e* rats) [31] were housed and cared at the animal facilities of the School of Medical Sciences, National University of Rosario. Animals were fed commercial chow and water *ad libitum* and were maintained in a 12 h light/dark cycle. All the experiments were done during the first half of the light cycle and in accordance with animal care standards of the institution, which complies with the guidelines issued by the Canadian Council on Animal Care [32].

Tumor. L-TACB is a poorly differentiated B-cell lymphoma that arose spontaneously in an inbred *e* rat [33] and metastasizes exclusively to regional lymph nodes when injected subcutaneously [26]. This tumor is maintained by serial subcutaneous trocar implantation of 1 mm^3 tumor fragments (approximately 10^6 cells) in syngeneic rats.

Drugs. Cyclophosphamide (Cy) (Filaxis Lab., Argentina) was dissolved in sterile distilled water to a concentration of 20 mg/ml and injected at a dose of 10 or 20 mg/kg of body weight.

Experimental model. Circulating T regulatory and NKT I cells were quantified during tumor evolution in individual experiments. Inbred *e* rats were inoculated with L-TACB s.c. (day 0). On day 14 animals were distributed in two groups: control animals injected i.p. with saline and animals treated with a single low dose Cy of 10 mg/kg i.p. Blood samples were obtained on days 0; 7; 14 and 21, and were processed for flow cytometry analysis with the antibodies to the appropriated markers. Different populations of $CD4^+CD25^+$ Tregs were identified: $CTLA\text{-}4^+$, $Foxp3^+$ and $IL\text{-}10^+$ in both experimental groups. Also, we quantified NKT I cells using anti-TCR and anti-CD161 antibodies.

Tregs and NKT I cells quantification. Blood samples from the tail vein were obtained using EDTA as anticoagulant. Peripheral blood mononuclear cells were isolated from peripheral blood samples by centrifugation, using Ficoll-Paque density gradient (Ficoll-Paque PLUS, GE Healthcare, Uppsala, Sweden). The following antibodies were used for immunophenotyping: CD4-PE-Cy5 (BD Pharmingen, USA), CD25-FITC (Serotec, UK), CD152-RPE (Serotec, UK), Foxp3-PE (eBioscience, USA), IL-10-PE (BD Pharmingen, USA), TCR-RPE (Serotec, UK), CD161-FITC (Serotec, UK). Samples were fixed in 1% (wt/vol) paraformaldehyde and analyzed on a Coulter Epics XL (Coulter Corp. Miami, FL, USA). Cells were permeabilized using saponin (0.1% w/v in PBS). Acquired data were analyzed with WinMD1 2.8 data analysis software (Scripps Research Institute, La Jolla, Ca, USA).

Statistics. Non-Parametric ANOVA, Mann — Whitney's U tests were utilized using GraphPad Prism version 3.03 (GraphPad Software, San Diego, CA). Differences between groups were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

First, we studied the kinetics of different subsets of Tregs during tumor growth evolution and after the treatment with low dose Cy. We observed an increase during tumor growth in the percentage of the three cell subsets analyzed (Fig. 1).

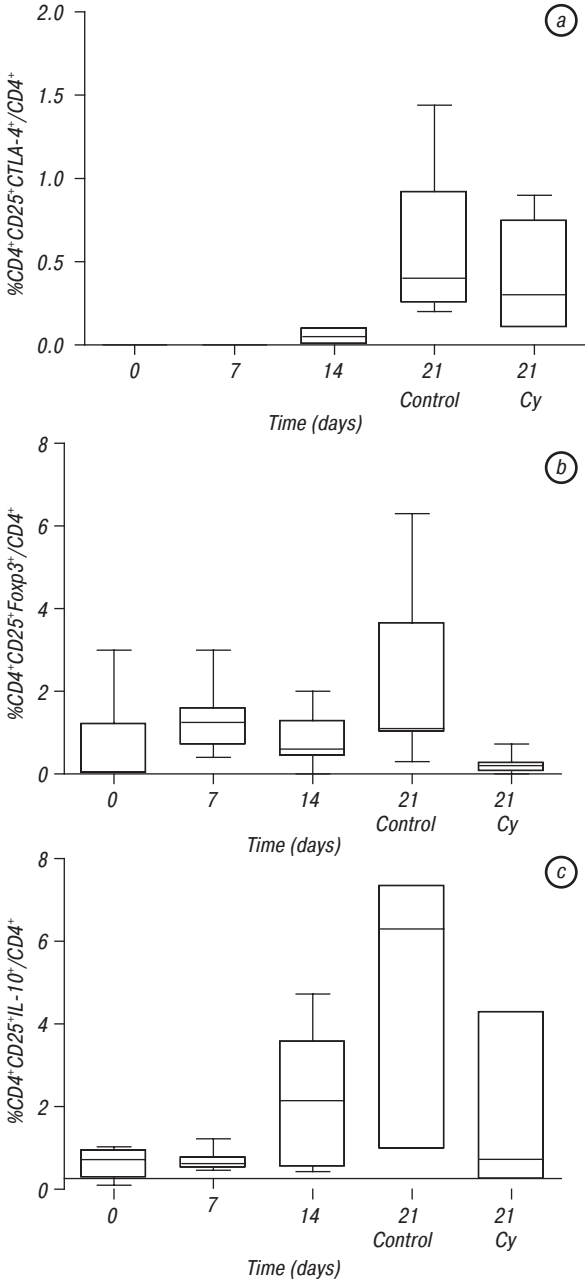

Fig. 1. Effect of cyclophosphamide on circulating Tregs cells during tumor evolution [median (range)]. *a*) $CD4^+CD25^+CTLA\text{-}4^+$ / CD4+ (%): Kruskal — Wallis Test, $P < 0.001$; Mann — Whitney Test, day 21 *vs* day 0: $P < 0.001$; day 21: Control *vs* Cy: ns. *b*) $CD4^+CD25^+Foxp3^+$ / CD4+ (%): Kruskal — Wallis Test, $P < 0.001$; Mann — Whitney Test, day 21 *vs* day 0: $P < 0.05$; day 14 *vs* day 7 and *vs* day 21: ns; day 21: Control *vs* Cy: $P < 0.01$. c) $CD4^+CD25^+IL\text{-}10^+$ / CD4+ (%): Kruskal — Wallis Test, $P < 0.001$; Mann — Whitney Test, day 21 *vs* day 0: $P < 0.01$; day 21: Control *vs* Cy: $P < 0.05$

The percentage of $CD4^+CD25^+CTLA\text{-}4^+$ cells increased during tumor growth ($P < 0.01$); indeed, the levels of these cells on day 21 were higher than those on day 0 [% median (range); day 0: 0.0 (0–0) *vs*. day 21: 0.4 (0.2–1.4);

$P < 0.001$]. At the end of the experiment, the percentage of $CD4^+CD25^+CTLA\text{-}4^+$cells in animals treated with Cy was lower than that of controls, but they did not differ between each other [Day 21: Control 0.4 (0.2–1.4) *vs* Cy: 0.3 (0.1–0.9); ns] (Fig. 1, *a*). The percentage of $CD4^+CD25^+Foxp3^+$ cell subset increased throughout the experiment ($P < 0.001$), being also significant the difference. On day 21, the percentage of $CD4^+CD25^+Foxp3^+$ cells in the treated group was significantly lower compared to that of the control group [Day 21: Cy, 0.2 (0.0–0.7) *vs* Control, 1.1 (0.3–6.3), $P < 0.01$] (Fig. 1, *b*). Moreover, $CD4^+CD25^+IL\text{-}10^+$ cells increased their percentage during the studied period ($P < 0.001$), and the difference between the values on days 0 and 21 was significant [Day 0: 0.7 (0.1–1.0) *vs.* Day 21: 6.3 (1.0–7.4); $P < 0.01$]. Also, the percentage of circulating $CD4^+CD25^+IL\text{-}10^+$ cells in the treated group was significantly lower with respect to the control group on the same day [0.7 (0.2–4.3) *vs.* 6.3 (1.0–7.4), $P < 0.05$] (Fig. 1, *c*).

NKT I is a T cell subset with regulatory properties which can display an antitumor function. We aimed to study the variation of this cell population during tumor evolution. We observed that while from days 0 to 7 the % of NKT I cells increased significantly [12.4 (4.0–30.8) *vs.* 17.8 (6.2–38.2)], ($P < 0.001$)], by day 14 those levels had already returned to the basal ones [8.3 (1.9–22.1)]. The analysis of the effect of Cy treatment on this cell population showed that the treatment decreases its %, although not significantly, on day 21 [Day 21: Control 8.4 (3.8–19.3) *vs* Cy 3.7 (2.4–6.3)] (Fig. 2, *a*).

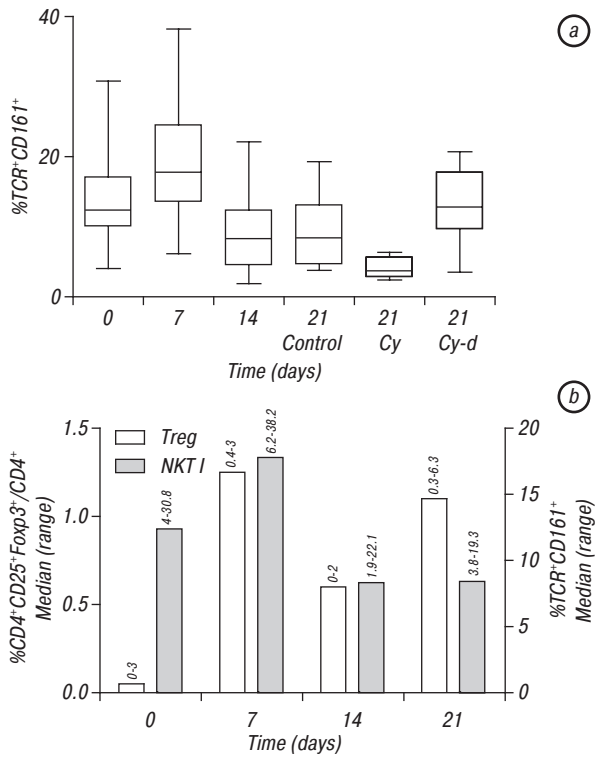

Fig. 2. Effect of cyclophosphamide on circulating NKT I cells during tumor evolution [median (range)]. *a*) % of TCR^+CD161^+ cells, Kruskal — Wallis Test, P<0.001; Mann — Whitney Test, day 7 *vs* day 14: $P < 0.001$; day 21 *vs* day 0: ns; day 21: Cy *vs* Cy-d: $P < 0.01$. *b*) co-evolution of $Foxp3^+$ Tregs and NKT I cells during tumor evolution.

As NKT I and natural Tregs cells exert opposite actions on tumor development we depicted graphically their simultaneous variations along tumor evolution (Fig. 2, *b*). Both cell populations increased their respective levels until day 7. From that day on, while Tregs continue increasing their percentage, NKT I cells decreased, reaching levels even lowers than the basal ones.

Taking into account the lack of modulation of NKT I cells by the antimetastatic dose of Cy, we wanted to know if a different schedule or dose of Cy would be able to increase this cell population. We decided to duplicate the dose previously used in a single injection (Cy-d): when the single dose of Cy was duplicated (20 mg/kg instead of 10), a marginally significant increase in the percentage of NKT I cells with respect to the control group was observed [day 21: Control 8.4 (3.8–19.3) *vs* Cy-d 12.8 (3.5–20.7), $P = 0.055$] (Fig. 2, *a*). Interestingly, when comparing the effect caused on this cell population by a single Cy dose of 10 mg/kg with that of 20 mg/kg (Cy-d), the difference was significant ($P < 0.01$).

The idea that the immune system can recognize and destroy nascent transformed cells was originally integrated in the cancer immunosurveillance hypothesis posed by Burnet and Thomas in 1957 [34]. Almost half a century later, Schreiber and colleagues proposed that cancer immunosurveillance may function as the elimination phase of a process called cancer immunoediting. This process is responsible for eliminating tumors and sculpting the immunogenic phenotypes of tumors that eventually appear in immunocompetent hosts [35]. The importance of the antitumor immune response in determining the fate of an immunogenic tumor makes it interesting and necessary to study the modulation of different subsets of immune cells during tumor development.

$CD4^+CD25^+$ regulatory T cells NKT are two populations of T lymphocytes that can independently regulate adaptive and innate responses. Activated NKT cells seem to modulate quantitatively Treg function through IL-2-dependent mechanisms, whereas Tregs can suppress the proliferation, cytokine release and cytotoxic activity of NKT cells by cell-contact-dependent mechanisms [36].

During L-TACB tumor evolution we observed an increase in the levels of different subsets of $CD4^+CD25^+$ Tregs, namely CTLA-4^+, $Foxp3^+$ and IL-10^+ cells. On the other hand, the significant increase in the percentage of NKT I cells demonstrated between days 0 and 7, was completely reversed by day 14, the level of this cells being even under the basal ones. Taking into consideration that natural $Foxp3^+$ Tregs and NKT I cells exert opposite actions on tumor development, we were interested in learning about their simultaneous variation during tumor growth. We observed that in the first steps of tumor development both cell types, Tregs and NKT I, increased their levels, indicating the existence of a sort of a balance, at least partial, between these cells types with opposite effects. After that moment, the balance is broken because of a significant decrease of the NKT I population, while Tregs showed increased values

with respect to basal ones throughout the experiment, in spite of a transitory non significant decrease on day 14, thus contributing to an immune response modulated towards immunosuppression. We are aware that several other regulatory cell types may have influence in the modulation of the immune response to the tumor. Nevertheless, Tregs and NKT I represent two important cell types involved in innate immunity related to pro- and anti- tumor responses, respectively.

Both, experimental and clinical studies revealed that cyclophosphamide, an alkylating agent commonly used in cancer chemotherapy [22], exerts an apparently paradoxical effect in host immune response. High doses of Cy (i.e. MTD, maximum tolerated dose) bring about, along with a reduction of primary tumor mass, an impairment of the host defense mechanisms, therefore leading to immunosuppression. However, the administration of low doses of Cy leads to an enhancement of the immune response, both in experimental animals and in humans, frequently causing tumor rejection[23, 25, 28, 29].

Previously, we demonstrated that a single low-dose Cy, a treatment completely devoid of toxicity, inhibited metastasis development without affecting primary tumor growth [26]. The antimetastatic action of Cy was mediated by immunomodulation since this effect could be adoptively transferred by immune cells of Cy-treated tumor-bearing rats and the same treatment did not have any effect in immunodeficient nude mice [27]. We demonstrated that IL-10 was the main factor responsible for the induction and development of immunosuppression in L-TACB-bearing hosts and that the antimetastatic immunomodulatory effect of cyclophosphamide was mediated by a reduction in IL-10 levels produced by T-lymphocytes [28]. In fact, Cy induced a Th2/Th1 switch and increased the proliferative rate of spleen cells [30].

Interestingly, the present results showed that the antimetastatic dose of Cy (10mg/kg) induced a significant decrease of the $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+IL\text{-}10^+$ cells. On the other hand, this treatment was not able to change the levels of NKT I cells. Considering the lack of modulation of NKT I cells by the antimetastatic dose of Cy, we studied the level of that cell population after a single dose of 20 mg/kg Cy, and we observed an increase compared to the percentage obtained after the treatment with half a dose. Nevertheless, when compared to the control group, the difference was not significant. Therefore, the decrease of peripheral blood NKT I observed during L-TACB tumor growth could not been reverted by the different treatments utilized.

Hence, the antimetastatic effect of a single low dose of Cy would be due, at least in part, to downregulation of natural and inducible Treg cells. The importance of the innate antitumor immune response in general, and its modulation by treatments with low dose chemotherapeutics in particular, warrants further studies in this area.

Our results may have importance on development of new therapies for metastatic lymphomas considering that a single low-dose cyclophosphamide, a treatment devoid of toxicity, would act inhibiting one of the mechanisms contributing to escape from immune rejection, thus inhibiting malignant growth and progression.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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