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Inhibitory effect of fluoxetine on lymphoma growth through the modulation of antitumor T-cell response by serotonin-dependent and independent mechanisms

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ABSTRACT

Fluoxetine, a selective serotonin reuptake inhibitor, is widely used for the treatment of depressive symptoms of cancer patients. However, there are contradictory evidences about its effects on immunity and cancer. Thus, we studied the effects of fluoxetine on tumor growth and on antitumoral T-cell-mediated immunity. In vivo chronic fluoxetine treatment inhibited tumor growth, and increased latency of appearance of solid tumors and survival of mice. Fluoxetine administration also increased mitogen-induced T-cell proliferation and Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ) expression, without altering $CD4^+/CD8^+$ ratio. In vitro, fluoxetine did not affect tumor cells proliferation, but it exerted a direct effect on T lymphocytes. Both fluoxetine and serotonin stimulated proliferation induced by a suboptimal mitogen concentration but inhibited proliferation at the optimal one. When both drugs were combined the results indicated that the effects of fluoxetine are in part independent of its ability to elevate serotonin extracellular levels. Finally, continue fluoxetine administration in nude mice – devoid of T lymphocytes – did not modify tumor progression, thus supporting the hypothesis of an immuno-modulatory effect of this drug on T cells that drives tumor growth control. These findings indicate, for the first time, that fluoxetine inhibits tumor growth through modulation of T-cell-mediated immunity by the already known serotonin-dependent pathway and by a novel independent mechanism.

1. Introduction

Between the most important diseases of the present time there are several neuropsychopathologies, including depression, anxiety, and schizophrenia. Such psychiatric illnesses mainly affect the normal function of the nervous system, but they are also related to alterations of the immune system function and to the evolution of certain types of cancer. Additionally, stressful situations such as cancer and infectious diseases are considered to promote the onset of depressive disorders, and for this reason patients are treated with antidepressant as a co-adjuvant to chemotherapy. Selective serotonin reuptake inhibitors (SSRIs) are among the most commonly used antidepressants, due to their

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efficacy, safety and tolerability [1]. SSRIs block the reuptake of serotonin into the presynaptic nerve terminals, resulting in enhanced synaptic serotonin levels [2]. Fluoxetine is an antidepressant drug that belongs to SSRIs and is the first choice for the treatment of depression, bulimia nervosa and obsessive–compulsive disorder. Antidepressant therapy has been shown to be associated with alterations of the immune function and cancer prognosis. Nevertheless, there are conflicting experimental findings about the effect of fluoxetine on the immune system. Pellegrino and Bayer [3,4] found that acute but not chronic fluoxetine administration decreases mitogen-induced T lymphocyte proliferation. By contrast, it was demonstrated that fluoxetine was able to reverse the stress-induced suppression on T lymphocyte population without effect on unstressed mice [5,6]. Instead, we found that fluoxetine exerts a dual effect upon in vitro T-cell proliferation, depending on the degree of lymphocyte activation [7]. In addition, antidepressant treatment has been reported to produce changes in tumor evolution, both towards a down- and an up-regulation, whereas others have found no differences [8–11].

The first aim of this work was to evaluate the effects of chronic fluoxetine treatment on the neoplastic pathology. With that purpose, we evaluated the influence of this antidepressant in the in vivo biological behavior of a T-cell lymphoma growing on syngeneic wild type mice and on athymic (nude) mice. In addition, we analyzed the in vitro proliferation of tumor cells in the presence of this drug. We also studied the in vivo effects of fluoxetine on the T-cell-mediated immunity, namely the T-cell lymphoproliferative response to selective mitogen Concanavalin A (Con A), the $CD4^+/CD8^+$ subset balance by dual-flow cytometry, and the expression of the antitumoral cytokines Tumor Necrosis Factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) by real-time RT-PCR. Finally, we analyzed the in vitro effects of fluoxetine and serotonin upon mitogen-induced T-cell proliferation in order to better understand the mechanisms of action of this antidepressant drug.

2. Materials and methods

2.1. Animals

Inbred female BALB/c (H-2^d) mice between 60 and 100 days old were purchased from the Instituto Nacional de Tecnologia Agropecuaria (INTA, Castelar, Buenos Aires, Argentina). Inbred female nude (*nu/nu*) mice between 60 and 100 days old were purchased from the Comision Nacional de Energia Atomica (CNEA, Ezeiza, Buenos Aires, Argentina). Animals were cared for in accordance with the 'Guide for the Care and Use of Laboratory Animals' of the United States National Institutes of Health. All the animals were housed in groups and maintained on a 12/12 h light/dark cycle under controlled temperatures between 18 and 22 °C. Food and water were available ad libitum. Two weeks before the beginning of the experiments, phases of the estrous cycle were monitored daily in order to verify that all mice have a synchronized estrous cycle.

2.2. Fluoxetine treatments

Mice were orally given 15 mg/kg/day of fluoxetine (generously given by Gador Capital Federal, Buenos Aires, Argentina), in a fresh solution prepared in the drinking water. Animals did normally drink the volume of water required for the daily dose (15 μl), as we previously described [12]. This treatment did not induce behavioral changes in normal animals (data not shown). For lymphoma evolution analysis, wild type animals were randomly assigned to four groups: one group received fluoxetine treatment during 4 weeks before tumor injection, the second group received fluoxetine treatment after tumor injection until they died (i.e., approximately 4 weeks), the third group always received fluoxetine and the fourth group used as control never received fluoxetine (see Fig. 1A). For immune system status analysis, wild type mice were divided in two groups: one group of mice was daily administrated with fluoxetine for 4 weeks, and the other group was left untreated. Animals were sacrificed at the fourth week of treatment. For lymphoma evolution analysis in nude mice, one group of animals was continuously treated with fluoxetine before and after tumor injection, and other group was left untreated.

2.3. Lymphoma model

LBC cell line is an aggressive T-cell lymphoma derived from an early T-cell lymphocyte precursor in BALB/c mice (H-2^d). Intraperitoneal transplantation of LBC cells into mice resulted in an aggressive T-lymphoblastic lymphoma that infiltrated lymph nodes, thymus, spleen, liver, ovary, and uterus [13]. LBC T lymphoma cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Invitrogen), 100 μg/ml of streptomycin (Invitrogen) and 50 μM 2-β-mercaptoethanol (Sigma–Aldrich, St. Louis, Missouri, USA). Before implantation, LBC cells were washed with phosphate buffered saline (PBS), counted according to Trypan blue staining (Sigma–Aldrich) and resuspended in PBS. Syngeneic animals from the indicated experimental groups were inoculated subcutaneously with 1×10^6 LBC cells in 100 μl PBS to generate a solid tumor. In these conditions, about 70–80% of animals did not reject tumor cells. It is worth to note that in all our experiments those animals bearing solid tumors were used. Tumor length and width were measured every day using calipers, and tumor volume was calculated as $V = \pi/6 \times \text{length} \times \text{width}^2$.

2.4. LBC proliferation assay

The effect of fluoxetine or 5-hydroxytryptamine hydrochloride (i.e., serotonin, Sigma–Aldrich) on LBC cells proliferation was evaluated. Proliferation was determined by culturing 2×10^5 cells/ml in 96-well plates in 100 μl triplicate aliquots in supplemented medium. Aliquots of 100 μl of fluoxetine or serotonin were added to the microculture final concentrations ranging from 1×10^{-10} M to 1×10^{-4} M. Cells were pulsed with 0.75 μCi [^{3}H]-thymidine (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and then were cultured at 37 °C in a 5% CO_2 atmosphere for 24 h. Thymidine incorporation was measured by scintillation counting after retention over GF/C

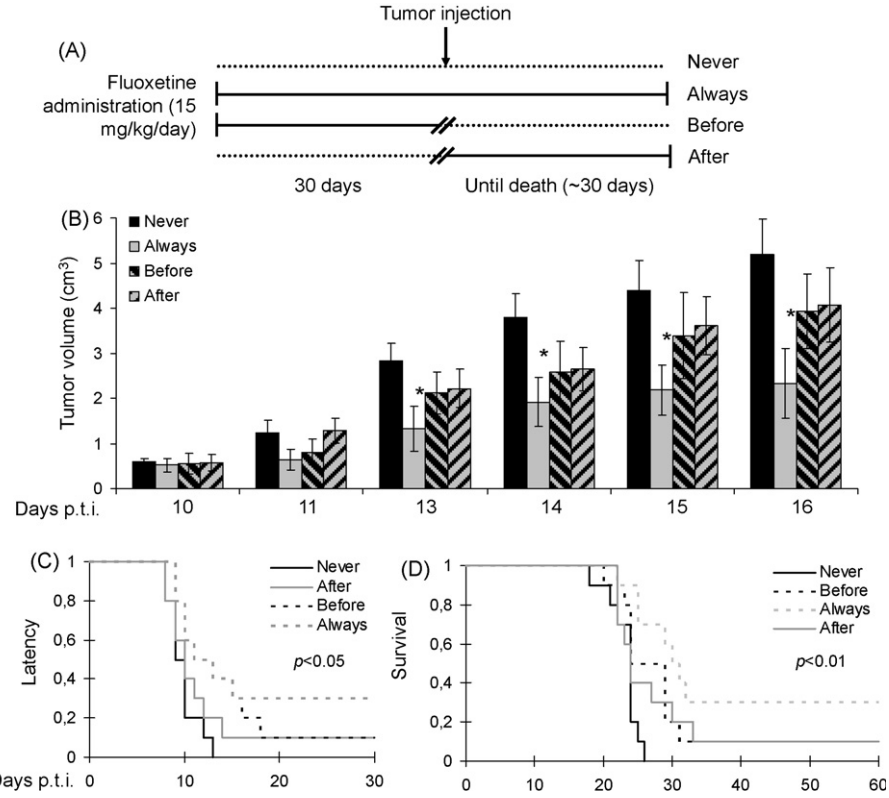


Fig. 1 – Effect of chronic fluoxetine administration on LBC T lymphoma growth. (A) Animals were treated with 15 mg/kg/day of fluoxetine for at least 4 weeks, according to four schedules: Never, Always, Before and After tumor implantation. Mice were inoculated subcutaneously with 1×10^6 LBC cells to generate a solid tumor. (B) Tumor length and width were measured and tumor volume was calculated as $V = \pi/6 \times L \times W^2$. Values are expressed as means ± standard error for each day post-tumor injection (p.t.i.). Statistical significance was determined using one-way ANOVA followed by Dunnett's contrast for post hoc analysis ($n = 10$ mice per group, $^*p < 0.05$). (C) Latency is expressed as the number of animals without palpable tumors against days post-tumor injection. (D) Survival is expressed as the number of live animals against days post-tumor injection.

glass-fiber filters (Whatman, Brentford, Middlesex, UK) of the acid-insoluble macromolecular fraction.

2.5. *T-cell suspensions, culture conditions and proliferation assays*

Lymphocytes from lymph nodes were obtained as previously described [6]. Briefly, mice were sacrificed and lymph nodes were quickly removed and disrupted through a 1 mm metal mesh. Cell suspensions were centrifuged and washed twice with RPMI 1640 supplemented with 10% FBS, 2 mM glutamine and 100 μg/ml of streptomycin. Cell viability was estimated according to Trypan blue exclusion criteria and was higher than 90%. Proliferation was determined by culturing 2×10^5 cells per well in 96-well plates in a final volume of 0.2 ml in triplicate aliquots of supplemented medium. Increasing concentrations of Con A (Sigma–Aldrich) were added to the microcultures. Cells were cultured at 37 °C in a 5% CO_2 atmosphere. Mitogenic activity was measured by adding 0.75 μCi [^{3}H]-thymidine per well for the last 24 h of culture. Thymidine incorporation was measured as described for LBC cells. Mitogen-stimulated cells displayed the expected proliferation kinetic, with a peak of proliferation at the third day of culture. To analyze the in vitro effect of fluoxetine and/or of serotonin on the T-cell lymphoproliferative response, co-incubation of lymphocyte suspensions from normal animals was carried out with Con A at final concentrations of 0.5 or 1 μg/ml and serotonin at final concentrations ranging from 1×10^{-10} to 1×10^{-4} M, fluoxetine at final concentrations ranging from 1×10^{-8} to 1×10^{-5} M; and fluoxetine at final concentrations ranging from 1×10^{-8} to 1×10^{-5} M plus serotonin at a final concentration of 1×10^{-6} M.

2.6. *Lymphocyte subsets determination by flow cytometry*

$CD4^+$ T-helper/inducer and $CD8^+$ T-cytotoxic/suppressor lymphocytes were determined in lymph node cell suspensions by flow cytometry [6]. Briefly, 1×10^6 cells in 100 μl of cytometry buffer (2% bovine fetal serum and 0.01% NaN_3 in PBS) were stained with 0.50 μl of fluorescein-conjugated anti-mouse $CD4^+$ (CD4-FITC) and 0.50 μl of phycoerythrin-conjugated anti-mouse $CD8^+$ (CD8-PE) monoclonal antibodies (eBioscience, San Diego, California, USA), washed twice and fixed with 2% formaldehyde in PBS. Lymphocytes were identified by FACS analysis using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA). Optimal

amounts of antibodies were used and 8000–10,000 events were analyzed per tube. Dot plots of two-color fluorescence analysis as well as percentages of lymphocytes expressing CD4 and CD8 were determined. Isotype controls (IgG1-FITC/IgG2a-PE, Sigma–Aldrich) were used for each assay to determine non-specific staining.

2.7. Quantitative real-time reverse transcription polymerase chain reaction

Lymph nodes were homogenized in Trizol Reagent (Invitrogen) and total RNA was isolated following manufacturer's instructions. PolyA$^+$ mRNA was isolated from total RNA using the PolyATract mRNA isolation System (Promega, Madison, Wisconsin, USA). Complementary DNA (cDNA) was synthesized by retrotranscription using oligodT and SuperScript II Reverse Transcriptase enzyme (Invitrogen). Real-time RT-PCRs were carried out as we previously described [12], in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). cDNA amounts present in each sample were determined using SYBR Green PCR Core Reagents kit (Applied Biosystems). Each RT-PCR quantification experiment was performed in duplicates. Primer sequences were designed using Primer Express software (Applied Biosystems) and purchased from Invitrogen. To verify that the SYBR Green dye detected only one PCR product, all the reactions were subjected to a heat dissociation protocol following the final cycle of the PCR. Oligonucleotide sequences used were: TNF-α forward 5′-GCA CCA CCA TCA AGG ACT CAA-3′, TNF-α reverse 5′-TTG CAG AAC TCA GGA ATG GAC A-3′, IFN-γ forward 5′-TGC TGA TGG GAG GAG ATG TCT AC-3′, IFN-γ reverse 5′-ACC TGA CAC ATT CGA GTG CTG T-3′, β-actin forward 5′-CAA CTT GAT GTA TGA AGG CTT TGG T-3′, β-actin reverse 5′-ACT TTT ATT GGT CTC AAG TCA GTG TAC AG-3′. Polymerase chain reaction products detection was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. As we previously shown [12], values were referred to β-actin as a housekeeping gene for data normalization, because no significant differences between groups using cyclophilin or glucose-6-phosphate-dehydrogenase (G6PDH) mRNA expression levels were found.

2.8. Statistical analysis

Group means were analyzed for statistical significance using unpaired two-tailed Student *t*-test or one-way analysis of variance (ANOVA) with Dunnett's contrast for post hoc effects. For survival analysis, Kaplan–Meier curves were constructed and compared using Log-Rank test. Differences between means were considered significant if $p < 0.05$.

3. Results

3.1. Fluoxetine improves T lymphoma prognosis

In order to study if antidepressant treatment has any effect on the biological behavior of tumors, animals were treated with 15 mg/kg/day of fluoxetine according to four schedules (Fig. 1A): a group of animals was orally administered with fluoxetine during 1 month until tumor injection and then this treatment was interrupted (Before), in another group of animals, fluoxetine treatment begun after tumor injection and lasted until death for approximately 1 month later (After), a third group of mice was continuously treated with fluoxetine before and after tumor injection (Always), and the last group of control animals was left untreated (Never). Given that fluoxetine exerts its antidepressant effect in humans after long-term administration and that steady-state plasma levels are attained after 4–5 weeks of continuous drug administration, we treated mice with fluoxetine for at least 4 weeks. In order to generate solid tumors, syngeneic mice treated as described above were subcutaneously injected with 1×10^6 LBC T lymphoma cells. As shown in Fig. 1(B), animals that were always treated with fluoxetine displayed a markedly reduced tumor growth compared to control animals. On the other hand, when fluoxetine treatment was interrupted, tumor growth was increased, but never to values achieved by untreated animals. Finally, when treatment begun after injection, tumor proliferation was partially inhibited. However, this was not enough to reach values achieved by animals continuously treated with fluoxetine.

Table 1 – Effects of fluoxetine administration on the latency of appearance of solid tumors and on animal survival

Treatment	Latency (days)	Survival (days)
Never	9.80 ± 0.51	23.20 ± 0.73
Always	12.90 ± 0.86*	29.00 ± 1.19**
Before	12.20 ± 1.15*	26.40 ± 1.22*
After	10.50 ± 0.71	26.00 ± 1.42*

Values obtained from statistical analysis of Fig. 1C and D, are expressed as the corresponding means ± standard deviation of both parameters. Statistical analysis was performed comparing control group against treatments using Log-Rank test ($n = 10$ mice per group, $^*p < 0.05$, $^{**}p < 0.01$).

Additionally, animals treated with fluoxetine prior to neoplastic cells injection had a delayed latency of appearance of solid tumors (Fig. 1C and Table 1). Moreover, animals treated continuously with fluoxetine had a greater survival than control animals, whereas early and late treatments resulted in intermediate values (Fig. 1D and Table 1). Although the most remarkable difference was found when animals were continuously (Always) treated with fluoxetine, it is worth noting that antidepressant treatment after tumor development significantly increased survival rate, not limiting the present results.

To further analyze the clinical relevance of these findings, we tested cytokine levels in animals bearing solid tumors treated or not with fluoxetine after LBC cells injection (After, see Fig. 1A). We measured TNF-γ and IFN-α expression based on their importance in T-cell-mediated antitumor immunity. We found a significant increase of IFN-γ and TNF-α mRNA expression levels as measured by real-time RT-PCR in lymph node cells from fluoxetine-treated mice in comparison to untreated mice (Fig. 2). Thus, fluoxetine effects on T lymphoma prognosis would be related to its modulation on T-cell-mediated antitumor immunity.

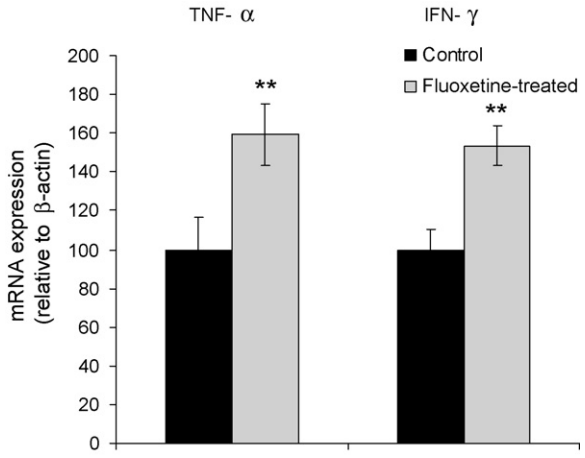


Fig. 2 – Effects of fluoxetine on TNF-α and IFN-γ expression in animals bearing tumors. Animals were subcutaneously injected with LBC cells and treated or not with fluoxetine. After 2 weeks, animals were killed, and total RNA and polyA$^+$ mRNA were isolated from lymph nodes and used for cDNA synthesis. Cytokine expression was evaluated by real-time RT-PCR using SYBR Green dye. Values are expressed as means ± standard error normalized with β-actin gene as housekeeper. Statistical significance was determined using unpaired *t*-test ($n = 6$ mice per group, $^{**}p < 0.01$).

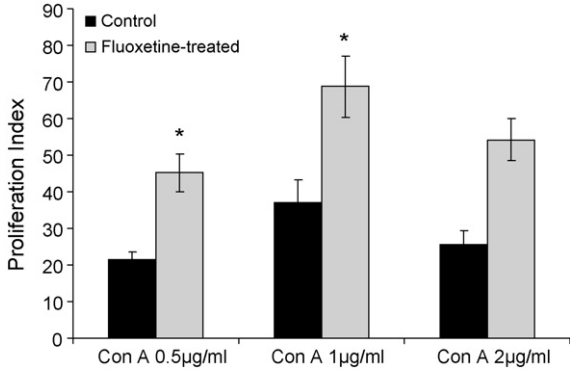


Fig. 3 – Effect of chronic fluoxetine treatment on T-cell proliferation. Cells from lymph nodes of mice treated with 15 mg/kg fluoxetine for 4 weeks and untreated animals were stimulated with Con A at 0.5, 1 and 2 μg/ml. Values are expressed as means ± standard error of the stimulated/basal ratio (proliferation index). Statistical significance was determined using unpaired *t*-test ($n = 6$ mice per group, $^{*}p < 0.05$).

3.2. *Fluoxetine does not act directly on tumor cells to inhibit their growth*

Next, we analyzed if fluoxetine inhibition of tumor growth was due to a direct effect on T lymphoma cells. With this purpose, we assayed LBC proliferation, co-incubating these cells with fluoxetine at a range of concentrations between 10^{-10} and 10^{-4} M in supplemented medium, by the evaluation of [^{3}H]-thymidine incorporation. As it can be seen in Table 2, fluoxetine had no effect on T lymphoma cell proliferation at any concentration tested. Moreover, we also evaluated if serotonin, which is the main target of fluoxetine, affected LBC proliferation at the same range of concentrations. No changes were found on lymphoma cells proliferation in the presence of serotonin (Table 2).

Table 2 – In vitro effects of fluoxetine and serotonin on LBC proliferation

Drug concentration	LBC proliferation (dpms)	
	Fluoxetine	Serotonin
Basal	59634 ± 7149	63955 ± 4031
10^{-10} M	61054 ± 3462	65392 ± 6240
10^{-9} M	57979 ± 8910	64164 ± 9870
10^{-8} M	62881 ± 3313	61240 ± 4428
10^{-7} M	61517 ± 6276	64164 ± 2317
10^{-6} M	60586 ± 9737	68645 ± 10129
10^{-5} M	67281 ± 3666	63028 ± 2726
10^{-4} M	63442 ± 4566	60615 ± 3912

LBC T lymphoma cells were induced to proliferate with FBS at different fluoxetine or serotonin concentrations and [^{3}H]-thymidine incorporation was evaluated. Values are expressed as means ± standard error. Unpaired *t*-test was used for statistical analysis, and no significant differences were found.

3.3. *Fluoxetine enhances T-cell immunity*

3.3.1. *Fluoxetine increases T lymphocyte reactivity*

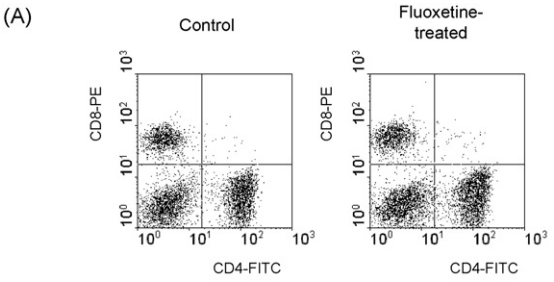
Given that fluoxetine improved lymphoma prognosis, especially when it was administered prior tumor injection, but this was not a direct effect of the drug on LBC cells, we asked whether fluoxetine was able to enhance immune response in normal animals not bearing tumors. Thus, we analyzed the effects of fluoxetine administration for 4 weeks on lymphocyte proliferation to the T-cell selective mitogen Con A. As shown in Fig. 3, a significant increase in T-cell proliferation was observed in fluoxetine-treated animals. These results indicate that fluoxetine treatment enhances the in vitro proliferative response of T cells.

3.3.2. *Fluoxetine does not alter CD4$^+$/CD8$^+$ lymphocyte subsets*

In order to evaluate whether fluoxetine-induced T-cell immunity enhancement was due to a modification on CD4$^+$ T-helper/inducer and CD8$^+$ T-cytotoxic/suppressor lymphocytes, these populations were determined in lymph node cell suspensions by dual fluorescence flow cytometry using specific antibodies (α-CD4-FITC and α-CD8-PE). No changes were observed in CD8$^+$ or CD4$^+$ T lymphocytes subset distribution on fluoxetine-treated animals in comparison to control mice (Fig. 4).

3.3.3. *Fluoxetine increases antitumoral cytokine production*

We also investigated if fluoxetine exerts any effect on cytokine production. We measured TNF-α and IFN-γ expression in lymph node cells from fluoxetine-treated mice in comparison to normal mice. Real-time RT-PCR revealed an increase on



(B)

	$CD4^+$	$CD8^+$	$CD4^+$ $CD8^+$	$CD4^-$ $CD8^-$
Control	37.50 ± 3.81 %	13.00 ± 1.74 %	1.92 ± 0.55 %	47.59 ± 5.58 %
Fluoxetine-treated	39.16 ± 3.00 %	15.59 ± 2.01 %	0.98 ± 1.36 %	44.27 ± 4.79 %

Fig. 4 – $CD4^+/CD8^+$ T lymphocyte subsets distribution on control and fluoxetine-treated animals. Mice were daily treated with 15 mg/kg fluoxetine for 4 weeks. $CD4^+$ T-helper/inducer and $CD8^+$ T-cytotoxic/suppressor lymphocytes populations were determined in lymph nodes cell suspensions by dual fluorescence flow cytometry using specific antibodies (α-CD4-FITC and α-CD8-PE). (A) Representative dot plots of two-color fluorescence analysis of lymphocytes expressing CD4 and CD8 from each treatment. (B) Percentage of $CD4^+/CD8^+$ T lymphocytes subsets, expressed as means ± standard error of percentage of lymphocytes expressing CD4 and/or CD8. Statistical significance was determined using unpaired t-test ($n = 6$ mice per group).

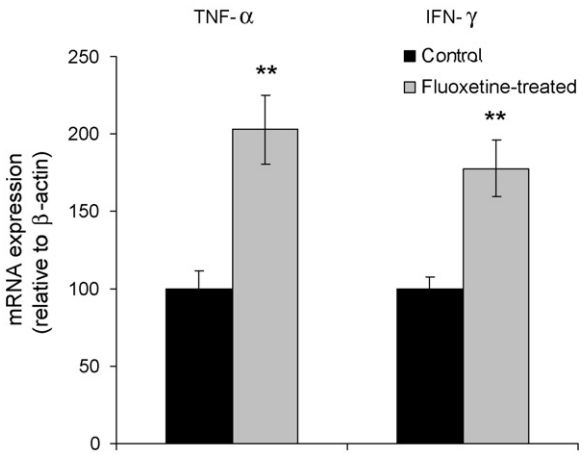


Fig. 5 – Fluoxetine action on TNF-α and IFN-γ mRNA expression in lymph node cells. Total RNA and $polyA^+$ mRNA were isolated from lymph nodes of untreated and fluoxetine-treated animals, and used for cDNA synthesis. Cytokine expression was evaluated by real-time RT-PCR using SYBR Green dye. Values are expressed as means ± standard error normalized with β-actin gene as housekeeper. Statistical significance was determined using unpaired t-test ($n = 5$ mice per group, $^{**}p < 0.01$).

both TNF-α and IFN-γ mRNA expression levels in lymph node cells from fluoxetine-treated mice respect to normal animals (Fig. 5). Thus, fluoxetine improves the production of key cytokines involved in T-cell-mediated antitumoral immunity.

3.4. *Fluoxetine does not alter tumor growth in athymic mice*

We asked whether fluoxetine effects on tumor growth were mainly due to their effects on T cells and not other cell types that are also modulated by this drug. With this purpose, we used athymic mice which are devoid of T lymphocytes. Nude mice were treated continuously with fluoxetine before and after tumor injection (Always, see Fig. 1A). Athymic mice treated with this antidepressant displayed a similar tumor progression than untreated controls (Fig. 6), hence supporting our hypothesis of a modulator effect of fluoxetine on T cells that drives tumor growth inhibition.

3.5. *Fluoxetine acts directly on T cells to modulate mitogen-induced proliferation by serotonin-dependent and independent mechanisms*

In order to test more deeply the mechanisms underlying the effects of fluoxetine on T cells, we assayed the in vitro proliferation induced by suboptimal and optimal Con A concentrations co-incubated with fluoxetine at a range of concentrations from 10^{-8} to 10^{-5} M in comparison to co-incubation with serotonin at a range of concentrations from 10^{-10} to 10^{-4} M. As shown in Fig. 7(A), low doses of fluoxetine stimulated the proliferation induced by the submitogenic concentration of Con A (solid dots), being the optimal stimulatory effect of fluoxetine at $10^{-8}/10^{-7}$ M, and decreased thereafter giving an inhibitory effect at 10^{-5} M. On the contrary, at the optimal Con A concentration fluoxetine exerted an inhibitory effect at all concentrations

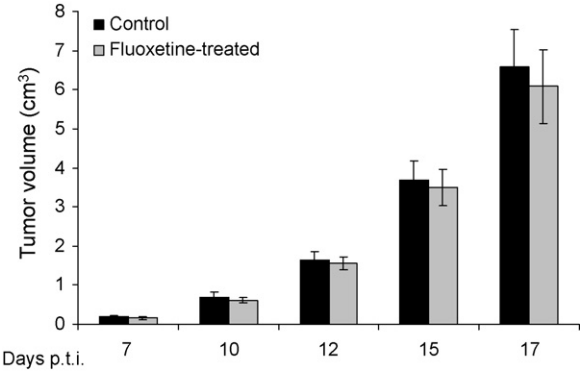


Fig. 6 – Effect of chronic fluoxetine administration on LBC T lymphoma growth in athymic mice. Nude animals were treated with 15 mg/kg/day of fluoxetine before and after tumor implantation. Mice were inoculated subcutaneously with 1×10^6 LBC cells to generate a solid tumor. Tumor length and width were measured and tumor volume was calculated as $V = \pi/6 \times L \times W^2$. Values are expressed as means ± standard error for each day post-tumor injection (p.t.i.). Unpaired t-test was used for statistical analysis ($n = 4$ mice per group), and no significant differences were found at any day tested.

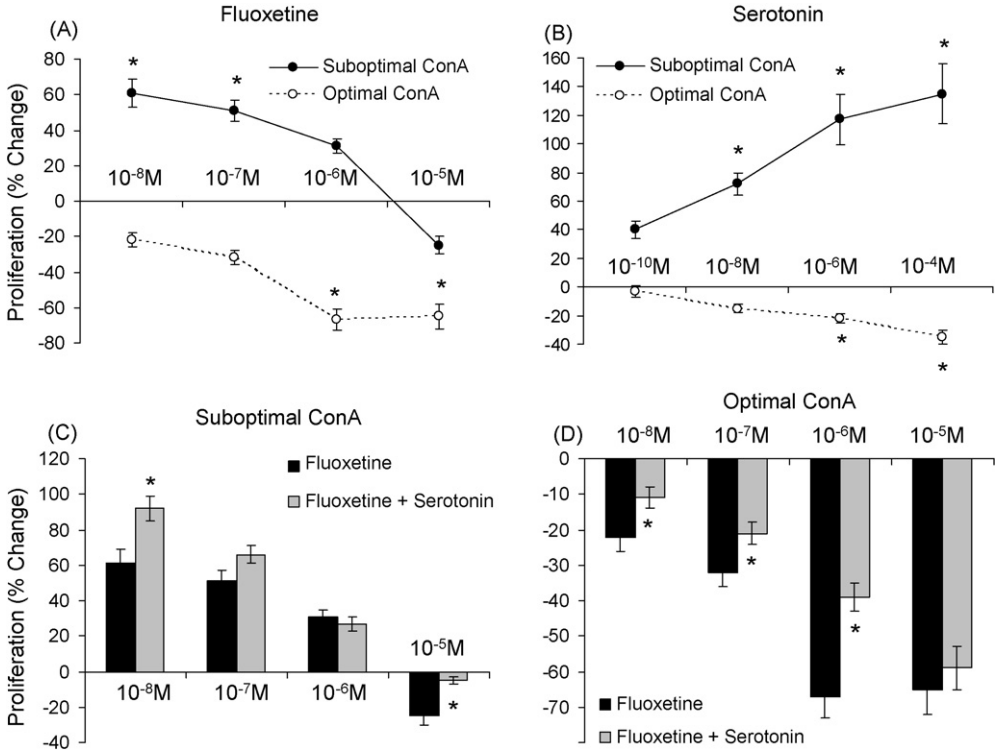


Fig. 7 – In vitro effects of fluoxetine and/or serotonin on T-cell proliferative response. T lymphocytes from normal mice were stimulated with suboptimal (●) or optimal (○) concentrations of Con A (0.5 and 1 μg/ml respectively) alone or in the presence of the indicated concentrations of fluoxetine (A), serotonin (B). Panel (C) and (D) show the effect of fluoxetine alone (black bars) or in the presence of serotonin (grey bars) on T-cell proliferation at suboptimal and optimal Con A concentration respectively. Results show the [^{3}H]-thymidine incorporation expressed as percentage of change respect to proliferation in the absence of drugs. Values represent the means ± standard error at the third day of culture. Statistical significance was determined using unpaired *t*-test ($n = 5$, $^*p < 0.05$).

tested (open dots). No effect was observed for concentrations lower than 10^{-8} M of fluoxetine (data not shown). It is important to note that fluoxetine alone had no effect on non-stimulated T-cell incorporation of [^{3}H]-thymidine (data not shown). A similar effect was produced by serotonin treatment, which was able to stimulate the suboptimal Con A induced proliferation (Fig. 7B, solid dots) and to inhibit the optimal one (Fig. 7B, open dots) showing the maximal stimulatory and inhibitory effect at 10^{-5}/10^{-6} M. Both effects were directly related to the concentrations used (Fig. 7B). On the contrary, the stimulatory effect of fluoxetine was inversely related with the concentration used, whereas the inhibitory effect was directly related to fluoxetine concentrations (Fig. 7A). Additionally, we tested combinations of serotonin and fluoxetine on mitogen-induced T-cell reactivity. As it can be seen in Fig. 6, serotonin (10^{-6} M) decreased the inhibitory effect of 10^{-8}–10^{-6} M fluoxetine observed for optimal Con A proliferation (Fig. 7D) and increased the maximal stimulatory (Fig. 7C) effect of 10^{-6}/10^{-7} M fluoxetine found for suboptimal Con A proliferation. Moreover, serotonin was able to attenuate the inhibitory effect of 10^{-5} M fluoxetine on proliferation induced by the suboptimal concentration of Con A but no the one observed for the optimal concentration of Con A (Fig. 7C and D).

4. Discussion

Here, we investigated the influence of fluoxetine on the biological behavior of an aggressive T-cell lymphoma. The results indicated that fluoxetine inhibits tumor growth, delays its appearance, and extends survival of mice. Despite that these effects were maximal in animals treated continuously with fluoxetine, and that reduction of tumor volume was only observed in animals continuously treated with the antidepressant, all fluoxetine treatments ameliorate tumor progression in terms of survival. An especially relevant observation is the fact that the survival was improved in animals treated with fluoxetine after tumor injection. Moreover, these animals also displayed an increase of the antitumor cytokines TNF-α and IFN-γ, highlighting the clinical relevance of the present results. The lack of significant differences in tumor volume growing in animals treated with fluoxetine after LBC cells injection could be related to the fact that LBC is a very aggressive lymphoma and that untreated animals bearing tumors have a mean survival time of 23.2 ± 0.7 days makes it difficult to analyze the full action of the antidepressant given after tumor injection, as it takes 4–5 weeks to reach steady-state plasma levels.

Several studies have shown that some antidepressants are able to affect tumor cell viability. Brandes et al. [14] showed that fluoxetine increases proliferation of B16f10 melanoma cells and C3 fibrosarcoma cells both in vivo and in vitro. Arimochi and Morita [15] found that fluoxetine and certain tricyclic antidepressants result on cytotoxicity on human HT29 colon carcinoma cells. The SSRIs 6-nitroquipazine, zimelidine and fluoxetine, inhibit the proliferation of prostate cancer cells in a concentration-dependent manner [16]. Paroxetine and fluoxetine are able to induce apoptosis in rat glioblastoma and human neuroblastoma cells [17,18]. The SSRI citalopram and the tricyclic antidepressants (TCAs) imipramine and clomipramine can evoke apoptosis of acute myeloid leukemia HL-60 cells via a caspase-3-dependent pathway [19]. Recently, it was demonstrated that three SSRIs – fluoxetine, paroxetine and citalopram – act directly on Burkitt lymphoma cells to trigger rapid and extensive programmed cell death [20]. Thus, one possibility is that fluoxetine acts directly on tumor cells to inhibit lymphoma progression. However, here we found that fluoxetine has no effect on LBC tumor cell proliferation in vitro in concentrations as higher as 10^{-4} M, in agreement with a previous report in other cell lines [21]. Moreover, serotonin, the target of fluoxetine, neither affected LBC proliferation. It is possible that the effect of SSRIs on tumor growth depends on the cell type studied. In this regard it was demonstrated a complete insensitivity of the Jurkat T-cell leukemia line, despite these cells carry readily detectable immunoreactive serotonin transporter [22].

Given that fluoxetine does not act directly on lymphoma cells, this antidepressant could be acting at the immune system level to inhibit tumor growth. In support to this a fluoxetine-dependent increment in antitumor cytokines IFN-γ and TNF-α was found in bearing tumor animals. To test this hypothesis, we investigate the T-cell reactivity, $CD4^+/CD8^+$ subset balance and the expression of the antitumoral cytokines TNF-α and IFN-γ after fluoxetine administration in animals without tumor challenge treated or not with fluoxetine. Our results showed that oral administration of fluoxetine produces an increase of Con A-induced T-cell proliferation. It is important to note that in a previous report we did not find any effect of fluoxetine chronically administered by intraperitoneal way in control animals [6]. It is possible that this differential effect could be due to the distinct administration routes. In fact, manipulation and injection would be additional stressors for the animal that could mask fluoxetine stimulatory effects. $CD8^+$ cytotoxic T lymphocytes play an immunologic role as the specific tumor terminator. On the other hand, $CD4^+$ helper T lymphocytes serve as controllers on $CD8^+$ T-cell-dependent tumor termination [23]. The $CD4^+/CD8^+$ ratio in tumor infiltrating lymphocytes and/or in peripheral blood has been proposed as an indicator of progressive tumor and/or worse prognosis of patients [24,25]. According to our results, fluoxetine is not implicated in changes of $CD4^+/CD8^+$ ratio that could be related in the improvement of tumor progression by fluoxetine treatment. However, an important increase of IFN-γ and TNF-α mRNA expression levels was found in fluoxetine-treated animals. These results suggest that chronic fluoxetine administration improved Th1-type immune response. Th1 cytokines play a critical role in generating antitumor responses, by activating natural killer cells, T-cytolytic cells and macrophages [26]. IFN-γ is a well-known cytokine playing a crucial role between innate and cognate immunity. Its relevance has been paradigmatically pioneered by Schreiber and co-workers [27] who showed a role for IFN-γ in antigen processing and presentation in tumor immunosurveillance. Besides, IFN-γ is also critical as an anti-angiogenic agent [28] and in natural killer-mediated lysis of tumor cells [29]. TNF-α has been described as a powerful anti-cancer effector cytokine produced by immune cells such as macrophages and lymphocytes. It mediates tumor cell killing primarily by apoptosis [30–32].

Moreover, both cytokines are crucial for the management of T-cell lymphomas in both rodents and humans [25,33,34]. In agreement with these evidences, our results indicate that fluoxetine administration produces an increase of T-cell proliferation and a higher IFN-γ and TNF-α production and suggest that this regulation of T-cell response could at last control tumor evolution. As fluoxetine is known to have effects on many cell types including vascular endothelial and smooth muscle cells [35,36], it is possible that the observed tumor growth inhibition could be due to an anti-angiogenic activity. Even if it is immunity related, the target could be antitumor macrophage or NK cell activity that is regulated by fluoxetine [3,37]. To test if other cell types are be involved in the inhibition of tumor growth by fluoxetine, further experiments on nude mice, which are devoid of T lymphocytes, were performed. Results showed that athymic mice treated with fluoxetine displayed a similar tumor progression than untreated controls. Therefore, although the participation of other cell types cannot be excluded, these results strongly indicate that fluoxetine-induced modulation of T-cell-mediated immunity is a crucial component of the mechanism that leads to an inhibition of tumor growth.

In order to test more deeply the mechanisms underlying the effects of fluoxetine on T cells, we analyzed if fluoxetine is able to modulate directly T lymphocyte reactivity. According to our previous results [7] we found that fluoxetine exerts a dual effect upon T-cell proliferation, depending on the degree of lymphocyte activation. Fluoxetine increased the proliferation induced by suboptimal Con A concentration, but at the optimal one it inhibited the T-cell reactivity. Additionally, serotonin, the main target of fluoxetine, has been shown to affect various functions of immune cells [for review see 38]. The presence of serotonin receptors on T cells was demonstrated by pharmacological studies [39,40] and by mRNA expression [41,42]. In order to study the participation of serotonin in fluoxetine effects on T-cell activity we analyzed the effect of serotonin alone and in combination with fluoxetine on mitogen-induced proliferation. Serotonin displayed different actions than fluoxetine when performing concentration response curves with suboptimal mitogen concentrations. Additionally, the combination of both drugs showed no synergic actions, as serotonin was able to increase the maximal stimulatory effect of fluoxetine but did not modify the lower ones, and always partially reversed the inhibitory effect of fluoxetine. These results indicate that the effects of fluoxetine are in part independent of its ability to elevate serotonin extracellular levels. This mechanism could include the action through a novel receptor or novel intracellular pathway coupled to serotonin transporter. In

accordance, a recent study has shown antidepressant-induced suppression of cell proliferation and cytokine secretion, unrelated to inhibition of monoamine reuptake, and a subsequent increase in the availability of noradrenaline/serotonin at receptors on immune cells [43]. It is important to note that these authors found that fluoxetine suppresses IFN-γ production and mitogen-induced T-cell proliferation. However, human lymphocytes are treated ex vivo with concentrations ranged up to 1 μM of fluoxetine that in our hand also result in an inhibitory effect of T-cell proliferation. Similarly, Taler et al. [44] showed that paroxetine and sertraline at concentrations higher than 1 μM induce an inhibition of cell proliferation and TNF-α secretion on ex vivo treated human T lymphocytes. These effects seem to be related to the suppressive effect of SSRIs on the expression of genes involved in proliferative and inflammatory responses of lymphocytes. Nevertheless, it is worth noting that the role of serotonin-activated central nervous system receptors after fluoxetine administration on fluoxetine antitumoral effects cannot be ruled out by the present experiments.

Finally, our results show that fluoxetine is effective to delay the tumor progression regulating the antitumor immune response by serotonin-dependent and independent mechanisms, pointing to a potential therapeutic action of fluoxetine on cancer treatment. The complete mechanism involved at molecular, cellular and functional levels are now under study.

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