



mice, a model characterized by defective cellular immune response [17-21].

### **Materials and Methods:**

#### **Animals**

Eight- to twelve-week-old male BALB/c mice, purchased from the Centro de Investigaciones Biomédicas de Occidente del Instituto Mexicano del Seguro Social (Guadalajara, Jalisco, México), were maintained and bred under conventional laboratory conditions at the University of Guadalajara (Guadalajara, Jalisco, México) according to the guidelines for the use and care of laboratory animals and World Medical Association Declaration of Helsinki (amended by the 52<sup>nd</sup> WMA General Assembly, Edinburgh, Scotland, October 2000). They were used both as experimental animals and to maintain tumoral cell line. Four groups of mice of 7 animals each were studied: control mice (C), control mice with T-activin treatment (CT), lymphoma-bearing mice (L) and lymphoma-bearing mice with T-activin treatment (LT).

#### **Tumor model**

The L-5178-Y murine cell line was used in this study. This is a transplantable murine leukemia cell line which was derived from a thymic tumor induced in a DBA/2 (H-2<sup>d/d</sup>) mouse by methylcholanthrene. In this model, the presence of tumor cells in mice produces cellular immune response suppression and it has been used in previous works to study the antitumor effect of different compounds [17-19, 21-24]. This cell line has been maintained by serially inoculating lymphoblasts into BALB/c mice as follows: one control male BALB/c mouse was inoculated intraperitoneally with 100  $\mu$ l of ascitic fluid containing  $1 \times 10^7$  lymphoblasts. Ten days later, the ascitic fluid was harvested and used to inoculate a new mouse, and so on. For the experiments, tumor-bearing mice intraperitoneally received  $1 \times 10^7$  lymphoma cells resuspended in physiological saline

solution (100  $\mu$ l) and tumor was allowed to growth for 10 days.

#### **T-activin treatment**

Lyophilized T-activin, kindly provided by Dr. Galina Zaitseva Petrovna, was reconstituted with physiological saline solution and filtered with a 0.22  $\mu$ m filter (Nalgene, Hereford, UK). Mice were treated for 10 days with T-activin (0.45  $\mu$ g/g of body weight) administered subcutaneously into the loose skin over the neck. T-activin treatment started the day of lymphoma cell inoculation. Control and tumor-bearing mice without T-activin treatment received physiological saline solution.

#### **Life span and body weight**

Mice from 4 groups were observed daily for 90 days to determine life span and general condition. Additionally ten days after lymphoma cell inoculation, mice from 4 groups were weighed and body weight increment was calculated as follows: weight at the end of treatment minus weight at beginning of treatment. After final weighing, mice were killed by cervical dislocation and ascitic fluid from the peritoneal cavity was aspirated and measured (tumor volume). Fluid was taken with a white blood cell pipette and diluted 100 times with physiological saline solution. A drop of diluted cell suspension was placed in the Neubauer counting chamber and the number of tumoral cells was counted [25]. To determine the status of the cellular immune response, delayed hypersensitivity test to dinitrofluorobenzene (*in vivo* test) and lymphoproliferation assay (*in vitro* test) were performed with mice from 4 groups, the day after the end of treatment.

#### **Delayed hypersensitivity test to dinitrofluorobenzene**

Dinitrofluorobenzene (DNFB; Sigma-Aldrich Corporation, St Louis, MO, USA) was diluted in acetone/olive oil (4:1) immediately before use. On day 4 and 5, starting the day of tumor cell inoculation, mice from each group were

sensitized to DNFB by placing 20  $\mu$ l of 0.7 % DNFB solution on an area of shaved ventral skin. After 5 days, mice received 10  $\mu$ l of 0.4 % DNFB solution (challenge) applied on the left ear. Ear thickness was monitored using a micrometer (The L.S. Starrett Company, Athol, Mass, USA) before challenge and 48 hours after challenge. Increased ear swelling, evidence of *in vivo* cellular immune response [20, 21], was calculated as ear swelling index (percent) as follows:  $[(T_{48}-T_0)/T_0] \times 100$ , in which  $T_0$  and  $T_{48}$  represent ear thickness values before and after challenge, respectively.

#### **Preparation of spleen mononuclear cells**

At the end of treatment, spleen from control and L-5178-Y lymphoma-bearing mice, with or without treatment, was removed under aseptic conditions. It was dissociated by mechanical disaggregation in Hank's Balanced Saline Solution (HBSS). Afterwards, cell suspension was purified by gradient separation on Hystopaque-1077 (Sigma Chemical Co., St Louis, MO, USA), followed by centrifugation for 30 min at 400 x g. Mononuclear cells were recovered and washed two times with HBSS. Cells were then suspended in RPMI 1640 culture medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum [26]. Finally, cells were adjusted to  $1 \times 10^6$  cells/ml.

#### **Splenic T cell proliferation assay**

As evidence of *in vitro* cellular immune response, T cell proliferation assay was performed as follows [27]: mononuclear cells were plated at a concentration of  $2 \times 10^5$  cells/well in a 96-well plastic tissue plate in RPMI-1640 media supplemented with 10% FBS (Sigma Chemical, St Louis, MO, USA), with or without concanavalin-A (ConA, 5  $\mu$ g/ml, Sigma Chemical Co., St Louis, MO, USA). Cultures were incubated for 48 hours at 37 °C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>. They were then pulsed with 1  $\mu$ Ci of <sup>3</sup>H-thymidine (specific activity

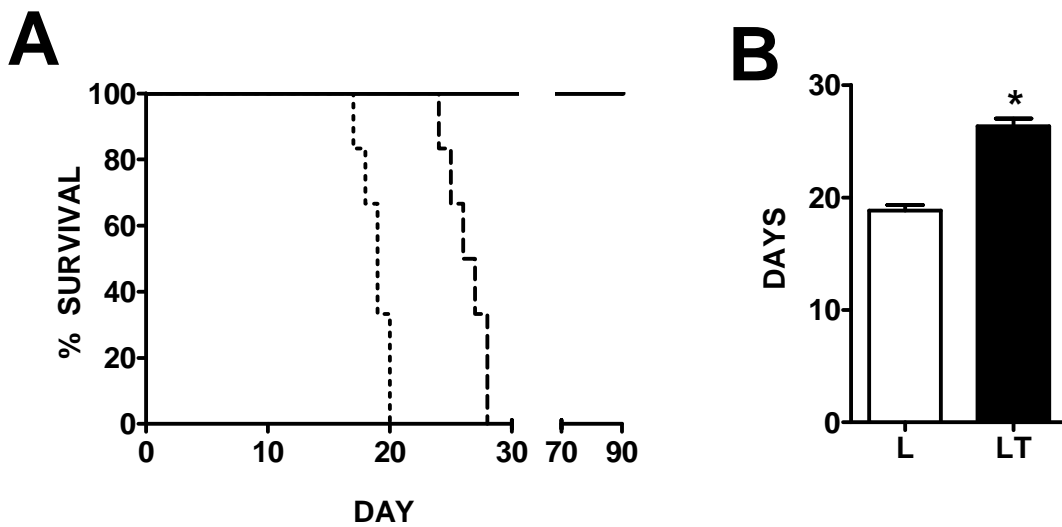
6.7 Ci /  $\mu$ mole; New England Nuclear, Boston, MA, USA). After 24-hr incubation, cells were harvested using an automatic cell harvester (Nunc, Denmark) and the incorporation of <sup>3</sup>H-thymidine was measured in a liquid scintillation  $\beta$ -counter (Beckman Instruments, Irvine, CA). The results were expressed as a stimulation index (SI) according to the following formula [27]: SI = counts per minute (cpm) of stimulated cultures / cpm of unstimulated cultures.

#### **IL-4 and IFN- $\gamma$ quantitative analysis**

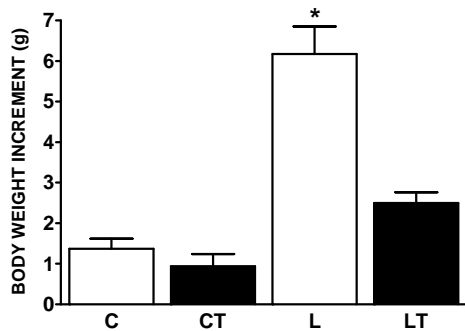
Mononuclear cells ( $2 \times 10^5$ ) were cultured in triplicate and stimulated or not with ConA (5  $\mu$ g/ml, Sigma Chemical Co., St Louis, MO, USA). Cultures were incubated for 48 hr at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>. Cells were then used to identify T cell populations by flow cytometry (see below) and supernatant was tested by ELISA for type 1 cytokine, IFN- $\gamma$ , and type 2 cytokine, IL-4, according to the manufacturer's instructions using commercially available kits from Biotrak (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The sensitivities of these assays were as follows: IFN- $\gamma$  < 10 pg/ml and IL-4: 5 pg/ml.

#### **Flow cytometry**

$1.5 \times 10^6$  mononuclear cells were cultured with 5  $\mu$ g/ml of ConA (Sigma Chemical Co., St Louis, MO, USA) as described. After 48 hr incubation, mononuclear cells were washed with PBS containing 0.1% BSA and 0.01% sodium azide, mixed with monoclonal antibodies anti-CD4-FITC or anti-CD8-PE (Beckman Coulter) and incubated in the dark at room temperature for 30 minutes to detect T-cell subsets expressing CD4<sup>+</sup> (helper) and/or CD8<sup>+</sup> (cytotoxic) surface antigens. At the end of incubation period, the cells were washed two times with PBS, fixed with 0.05% formaldehyde and stored at 4°C in the dark until their analysis. Cells were analyzed with the EPICS XL-MCL flow cytometer



**Figure 1:** Effect of treatment with T-activin on the survival percentage and life span of L-5178-Y lymphoma-bearing mice. A: survival percentage of control mice with and without treatment (—), lymphoma-bearing mice (-----) and lymphoma-bearing mice treated with T-activin (.....). The survival percentage was different among groups ( $p < 0.0008$ ). B: life span of mice with lymphoma, with and without treatment with T-activin. Each bar represents the mean  $\pm$  SEM of n. \*Statistically significant difference,  $p < 0.0001$



**Figure 2:** Effect of treatment with T-activin on body weight increment. Mice were weighed the first day of treatment and 10 days later. Each bar represents the mean  $\pm$  SEM of n. \*Significant differences compared with the other groups ( $p < 0.001$ ). (Beckman Coulter, Krefeld, Germany) and results were expressed as CD4<sup>+</sup> and CD8<sup>+</sup> percentages and as CD4<sup>+</sup>/CD8<sup>+</sup> ratio.

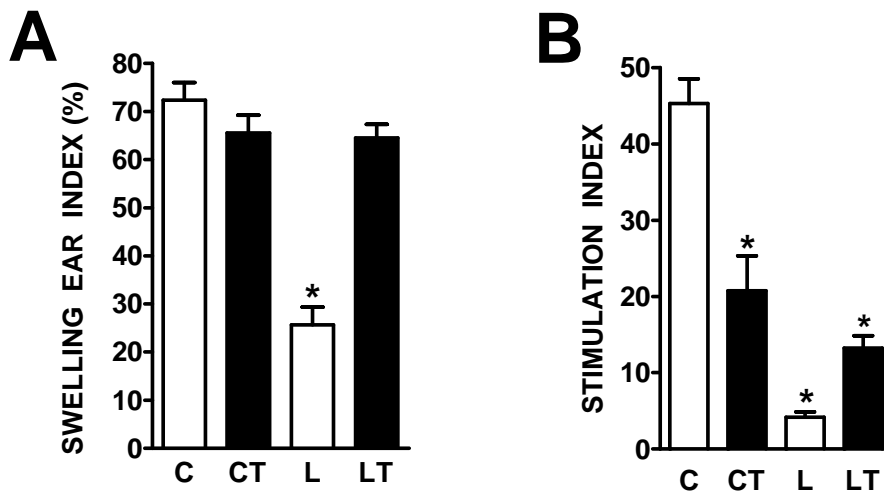
#### Statistical analysis

Data were analyzed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). Data are expressed as mean  $\pm$  SEM. Significant differences were determined by one-way ANOVA and Tukey's post hoc test in all immunological assays. Days of life, tumor volume, tumor cell count and body weight were analyzed by t test. Survival was represented by Kaplan-Meier curves and analyzed by Log rank test.

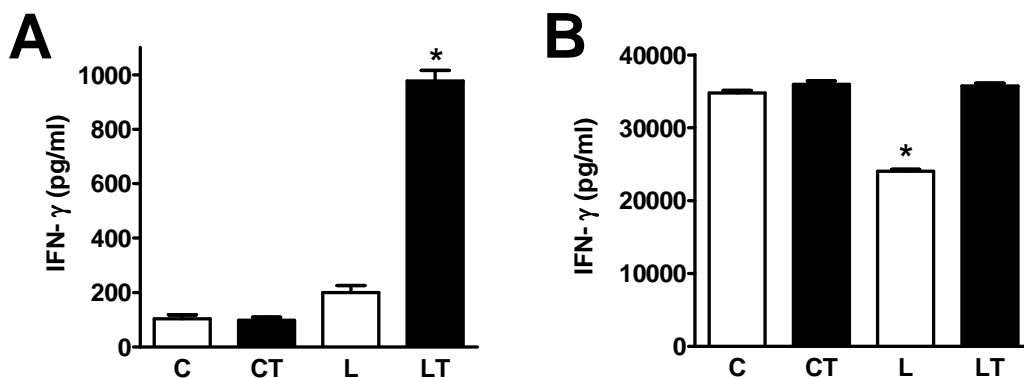
#### Results:

#### T-activin increases life span and reduces tumor volume, tumor cell count and body weight in lymphoma-bearing mice

T-activin treatment significantly increased the life span of lymphoma-bearing mice ( $18.83 \pm 0.47$  vs  $26.33 \pm 0.66$  days) (Figure 1). Lymphoma-bearing mice without T-activin died between 17 and 20 days after tumor cell inoculation whereas all lymphoma-bearing



**Figure 3:** Effect of T-activin treatment on cellular immune response. A: Delayed hypersensitivity test to DNFB, values are means of swelling ear index  $\pm$  SEM. \*Significant differences compared with the other groups ( $p < 0.001$ ). B: Lymphoproliferation, values are means of SI  $\pm$  SEM. \*Significant differences compared with control group ( $p < 0.001$ ) and between CT and L ( $p < 0.01$ ).



**Figure 4:** Effect of treatment with T-activin on IFN- $\gamma$  levels in splenocyte culture supernatants. A: IFN- $\gamma$  production by non-stimulated cells. \*Significant differences compared with the other groups ( $p < 0.001$ ). B: IFN- $\gamma$  production by stimulated cells. \*Significant differences compared with the other groups ( $p < 0.001$ ). Each bar represents the mean  $\pm$  SEM of n.

mice with T-activin were alive. Lymphoma-bearing mice with T-activin began to die on day 24. On the other hand, L-5178-Y lymphoma produced a significant increase in body weight associated with ascitic fluid accumulation in the peritoneal cavity compared with control mice ( $6.17 \pm 0.67$  and

$1.37 \pm 0.24$  g, respectively). T-activin reduced significantly the increase in body weight observed in lymphoma-bearing mice ( $2.5 \pm 0.26$  g) (Figure 2). Although no statistically significant differences were found, there is evidence that this effect is related, at least in part, to a decrease in both, tumor volume

(percentage reduction: 41.91 %) and tumor cell count (percentage reduction: 10.78 %) (Table 1).

#### **T-activin restored immunosuppression state in lymphoma-bearing mice**

The effect of T-activin on cellular immune response *in vivo* and *in vitro* was assayed in delayed hypersensitivity test to DNFB and lymphoproliferative response to ConA, respectively (Figure 3). Presence of lymphoma caused a depressed response to DNFB compared with control mice (swelling ear index:  $25.66 \pm 3.6$  vs  $72.3 \pm 3.6$  %, respectively). T-activin restored this suppressed response to near control mice values (swelling ear index:  $64.4 \pm 2.8$  %). Delayed type reaction to DNFB of control mice without T-activin was similar to control mice with T-activin (swelling ear index:  $72.3 \pm 3.6$  vs  $65.5 \pm 3.7$  %, respectively). Similarly, presence of lymphoma caused a depressed lymphoproliferative response compared with control mice (SI =  $4.17 \pm 0.68$  vs  $45.3 \pm 3.22$ ). The suppressed lymphoproliferation observed in lymphoma bearing mice was only partially

recovered with T-activin treatment compared with control mice without T-activin (SI =  $13.22 \pm 1.62$  vs  $45.3 \pm 3.22$ ). However, there were no statistically significant differences between lymphoma-bearing mice and control mice both treated with T-activin ( $13.22 \pm 1.62$  vs  $20.73 \pm 4.58$ ).

#### **Effect of T-activin on IFN- $\gamma$ and IL-4 levels**

To determine relation between T-activin treatment effect on cellular immune response and type 1 and type 2 cytokine levels, IFN- $\gamma$  and IL-4 cell-free culture supernatant levels were measured (Figures 4 and 5). Figure 4 (panel A) shows that unstimulated cells obtained from control mice, control mice with treatment, and lymphoma-bearing mice without treatment showed no change in IFN- $\gamma$  production ( $103.5 \pm 14.74$ ,  $98.26 \pm 11.52$  and  $200.1 \pm 25.71$  pg/ml, respectively).

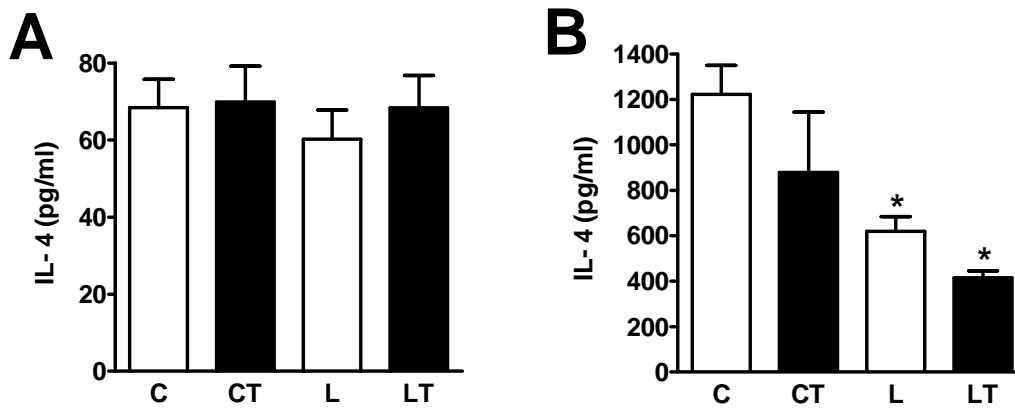
**Table 1:** Effect of treatment with T-activin on tumor volume and tumor cell count

Experimental group	Tumor volume	Tumor cell count
Lymphoma	$2.72 \pm 0.68$	$329.2 \pm 87.92$
Lymphoma + T-activin	$1.58 \pm 0.25$	$293.7 \pm 55.91$

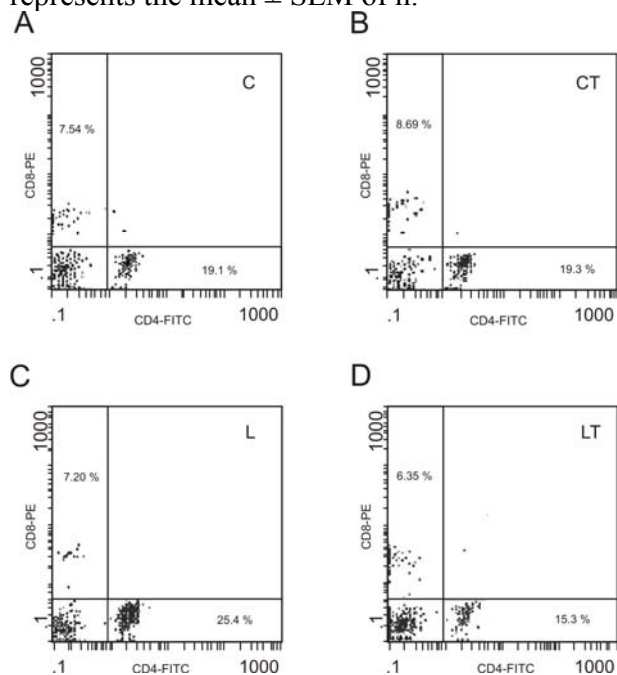
Values represent SEM of the mean

**Table 2.** Effect of T-activin treatment on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets

Experimental group	CD4 <sup>+</sup> (%)	CD8 <sup>+</sup> (%)	CD4 <sup>+</sup> /CD8 <sup>+</sup>
Control	19.1	7.5	2.53
	17.5	7.1	2.46
Control + T-activin	19.3	8.6	2.22
	18.5	8.2	2.24
Lymphoma	25.4	7.2	3.52
	24.5	8.1	3.02
Lymphoma + T-activin	15.3	6.3	2.40
	17.1	6.9	2.47



**Figure 5:** Effect of treatment with T-activin on IL-4 levels in splenocyte culture supernatants. A: IL-4 production by non-stimulated cells. B: IL-4 production by stimulated cells. \*Significant differences compared with control group: C vs L:  $p < 0.01$  and C vs LT:  $p < 0.05$ . Each bar represents the mean  $\pm$  SEM of n.



**Figure 6:** Identification and quantification of CD4<sup>+</sup> and CD8<sup>+</sup> subpopulations. Spleen cells obtained from: A: control mice, B: control mice treated with T-activin, C: mice with lymphoma and D: mice with lymphoma treated with T-activin. Each dot represents a cell labeled with anti-CD4 and/or anti-CD8 monoclonal antibody. The x-axis shows CD4<sup>+</sup> cells and the y-axis shows CD8<sup>+</sup> cells. Double

negative cells (CD4<sup>-</sup>CD8<sup>-</sup>) are shown in the lower left quadrant and double positive cells (CD4<sup>+</sup> CD8<sup>+</sup>) are shown in the upper right quadrant.

However, unstimulated cells from lymphoma-bearing mice with treatment significantly increased IFN- $\gamma$  levels ( $977 \pm 39.31$  pg/ml). On the other hand, stimulated cells (panel B) obtained from lymphoma-bearing mice showed a significant reduction in IFN- $\gamma$  levels ( $24,043 \pm 255.2$  pg/ml), an effect completely reversed by treatment with T-activin ( $35,744 \pm 354.1$  pg/ml). IFN- $\gamma$  levels from control mice, control mice treated with T-activin and lymphoma-bearing mice treated with T-activin were similar ( $34,763 \pm 356.9$ ,  $35,950 \pm 459.7$  and  $35,744 \pm 354.1$  pg/ml, respectively). In contrast, neither the presence of tumor cells nor treatment with T-activin had an effect on IL-4 levels produced by unstimulated cells (Figure 5, panel A), while stimulated cells obtained from mice with lymphoma produced low levels of IL-4 that did not recover with T-activin treatment (Figure 5, panel B).

**T-activin restored CD4<sup>+</sup>/CD8<sup>+</sup> ratio**

In order to inquire if the effect of T-activin observed on cellular immune response and

cytokine levels could be due to a modulation of participating T cell subpopulations, CD4<sup>+</sup> and CD8<sup>+</sup> cells were quantified. Figure 6 shows an example of flow cytometric analysis of CD4 and CD8 positive cells and Table 2 shows CD4<sup>+</sup> percentage, CD8<sup>+</sup> percentage and CD4<sup>+</sup>/CD8<sup>+</sup> ratio data from the four mice groups. Lymphoma produces an unbalanced CD4<sup>+</sup>/CD8<sup>+</sup> ratio average due to increment in percentage of CD4<sup>+</sup> T cells that T-activin treatment modulated and completely reversed (ratio average: 2.495, 3.27 and 2.435 in control mice, lymphoma-bearing mice and lymphoma-bearing mice treated with T-activin, respectively).

#### **Discussion:**

Thymic factors with immunomodulating properties have been introduced in clinical practice to help overcome tumor cell deleterious effects on cellular immune response [3, 12, 28, 29]. However, T-activin antitumoral mechanism is not yet clear. The present study evaluated whether or not T-activin treatment could lead to an improvement in antitumor cell-mediated immunity by modulating cytokine production. It was observed that L-5178-Y lymphoma-bearing mice, when compared with control mice, were defective in mounting both lymphoproliferation and delayed hypersensitivity reaction indicating a reduction in normal T cell function [17-19, 21, 22]. Likewise, L-5178-Y lymphoma-bearing mice showed a body weight increment associated with ascitic fluid accumulation and a life span reduction. T-activin treatment had positive effects on all these parameters. Both delayed hypersensitivity reaction and lymphoproliferation responses showed improvement with T-activin treatment, evidence of a recovery of T cell functions *in vivo* and *in vitro*. One of the first signs observed in lymphoma-bearing mice was the increase in body weight accompanied by abdominal distension due to accumulation of

ascitic fluid containing tumor cells. This malignant ascitic fluid is a manifestation of advanced cancer with poor prognosis, associated with deterioration in quality of life [30, 31], which was observed in the tumor model used. Lymphoma-bearing mice gradually increased in weight and at day 10 of tumor development, when there was significant difference between them and the control mice, cellular immune response suppression was well established and the mice died soon thereafter. Although differences in tumor volume and tumor cell count were not statistically significant, the decrease in both parameters could at least partially explain the effect on body weight. Likewise, the increase in life span was a reliable criterion for determining antitumor efficacy [25] and T-activin was able to significantly increase survival time in the tumor mouse model by about 28.48% in comparison with lymphoma-bearing mice without treatment.

In cancer, type 1 and type 2 cytokines production and CD4<sup>+</sup>/CD8<sup>+</sup> ratio are relevant parameters that determine an effective immune response. Cytokines produced by type 1 immune cells induce the cellular immune response that has a protective antitumor effect and cytokines produced by type 2 immune cells promote the humoral immune response that correlate with progressive tumor growth [32]. On the other hand, antitumor immune responses involve the participation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD4<sup>+</sup> T lymphocytes conferred regulatory signals required for the priming of major histocompatibility complex class I restricted CD8<sup>+</sup> T lymphocytes, main effectors for destroying tumor cells [40, 41]. Changes in CD4<sup>+</sup>/CD8<sup>+</sup> ratio as the disease progresses has been observed in different types of cancer and can be used as a prognosis indicator [42-44]. Thus, effects on cellular immune response observed in L-5178-Y lymphoma-bearing mice treated with T-activin might reflect a shift in type1/type2 immune



response and/or in CD4<sup>+</sup>/CD8<sup>+</sup> ratio. According with the results, T cells from L-5178-Y lymphoma-bearing mice may have a predisposition for entering into the IFN- $\gamma$ -producing effector pathway as a result of T-activin treatment. Results were similar to those obtained with RT-PCR technique [33]. Furthermore, imbalance in CD4<sup>+</sup>/CD8<sup>+</sup> ratio in L-5178-Y lymphoma-bearing mice due to increased CD4<sup>+</sup> has been observed in some types of cancer with poor prognosis [42, 45-47]. This imbalance could at least partially explain the immunosuppression reverted by T-activin. However, it is necessary to identify CD4<sup>+</sup> T cells subpopulation that is being modulated by T-activin to explain a more specific action mechanism.

#### Conclusions:

The results of the present study suggest that T-activin restores imbalance in CD4<sup>+</sup>/CD8<sup>+</sup> ratio and induces a type 1 immune response in tumor-bearing mice through IFN- $\gamma$  production, cytokine with immunomodulatory properties and a well-documented role in protection and eradication of spontaneous and chemically induced tumors [34-39], favoring the activation of cell-mediated immunity, which could explain the antitumoral properties of T-activin.

#### Acknowledgments:

The authors wish to thank Dr. Galina Zaitseva Petrovna for the generous donation of T-activin and Drs. Ana Margarita Rodríguez, Reyna Aydé Herrera Hernández, Adriana Ramírez and Lily Del Toro Arreola for their expert technical assistance.

#### References:

- [1] Koval'skaya NI, Arion VIa, Breusov IuN, Lindner DP. Effect of prolonged administration of T-activin on structure of the thymus. *Bull Exp Biol Med.* 1984, 97(1), 127- 129.
- [2] Arion VY. Thymic peptides as immunoregulators with special reference to T-activin. 1st ed. Routledge; 1989.
- [3] Abdulkadyrov KM, Guseva SA. Clinico-immunologic effect of tactivin in chronic myeloid leukemia. *Gematol Transfuziol.* 1994, 39(5), 19-22.
- [4] Adambekov DA, Litvinov VI, Mambetov KB, Koshmuratov AG, Sabyrbekova TS. Immunity of middle age and aged patients with tuberculosis and its changes during multimodality treatment by using T-activin. *Probl Tuberk.* 1998, 5, 46-48.
- [5] Mosmann TR, Cherwiniski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone: definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 1986, 136, 2348-2357.
- [6] Sad S, Marcotte R, Mosmann T. Cytokine-induced differentiation of precursor mouse CD8<sup>+</sup> T cells into cytotoxic CD8<sup>+</sup> T cells secreting Th1 or Th2 cytokines. *Immunity.* 1995, 2, 271-279.
- [7] Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today.* 1996, 17(3), 138-146.
- [8] Clerici M, Merola M, Ferrario E, Trabattoni D, Villa ML, Stefanon B, et al. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. *J Natl Cancer Inst.* 1997, 89(3), 245-250.
- [9] Kovacs E. Serum levels of IL-12 and the production of IFN-gamma, IL-2 and IL-4 by peripheral blood mononuclear cells (PBMC) in cancer patients treated with *Viscum album* extract. *Biomed Pharmacother.* 2000, 54(6), 305-310.
- [10] Kemp RA, Ronchese F. Tumor-Specific Tc1, but not Tc2, cells deliver protective antitumor immunity. *J Immunol.* 2001, 167, 6497-6502.
- [11] Botella-Estrada R, Escudero M, O'Connor JE, Nagore E, Fenollosa B, Sanmartin O, et al. Cytokine production by peripheral lymphocytes in melanoma. *Eur Cytokine Netw.* 2005, 16, 47-55.
- [12] Geveling NV, Zhogoleva IB. Experience in the use of T-activin in patients with malignant testicular tumors and patients with kidney cancer. *Voprosy Onkologii.* 1986, 32(8), 98-103.
- [13] Guseva SA, Fed'ko AA. Interactions of periferal blood lymphocytes and neutrophils in the stimulation of lymphokine production in chronic leukemia. *Gematol Transfuziol.* 1991, 36(6), 6-9.
- [14] Chuvirov GN, Markova TP. Ultrastructure of the blood lymphoid cells and the dynamics of cellular immunity indices in chronic lympholeukemia patients during immunocorrection with T-activin. *Terapevticheskii Arkhiv.* 1986, 58(9), 67-70.
- [15] Stanojevic BN, Milosevic D, Vuckovic DL, Sasic M, Markovic L. Clinical and immunologic effects of T-activin therapy in early stage melanoma patients. *Neoplasma.* 1996, 43(4), 245-252.

- [16] Mufazalova NA, Treshchalin ID, Treshchalina EM, Sedakova LA, Andronova NV. Effects of T-activin and vitamin E on toxicity and antitumor activity of cyclophosphamide. *Bull Exp Biol Med.* 2004, 137(1), 37-39.
- [17] Daneri-Navarro A, Del Toro-Arreola A, García-Velazco JC, Del Toro-Arreola S, Orbach-Arbouys S, Bravo-Cuellar A. L-5178-Y lymphoma associated immunosuppression in Balb/c mice. *Biomed Pharmacother.* 1995, 49, 39-44.
- [18] Puebla-Pérez AM, Huacuja-Ruiz L, Rodríguez-Orozco G, Villaseñor-García MM, Miranda-Beltrán M, Celis A, et al. Cytotoxic and antitumor activity from *Bursera fagaroides* ethanol extract in mice with L5178Y lymphoma. *Phytother Res.* 1998, 12, 545-548.
- [19] Oronz-Barocio A, Zaitseva G, Chavez-Anaya A, Arceta-Gonzalez VI, Puebla-Perez AM, Alfaro-Bustamante F, et al. Modulation of immune response of BALB/mice bearing lymphoma L5178Y treated with bitter yellow juice of *Aloe vera* (L) in vivo. *Russ J Immunol.* 1999, 4(1), 43-50.
- [20] Schmeda-Hirschmann G, Villaseñor-García MM, Lozoya X, Puebla-Pérez AM. Immunomodulatory activity of Chilean *Cyttaria* species in mice with L5178Y lymphoma. *J Ethnopharmacol.* 2001, 77, 253-257.
- [21] Del Toro-Arreola S, Flores-Torales E, Torres-Lozano C, Del Toro-Arreola A, Tostado-Pelayo K, Ramirez-Dueñas MG, et al. Effect of D-limonene on immune response in BALB/c mice with lymphoma. *Int Immunopharmacol.* 2005, 5(5), 829-838.
- [22] Palacios-Corona R, Ortiz-Navarrete VF, Said-Fernández S, Rodríguez-Padilla C, González-Garza MT. Detection of a factor released by L-5178-Y lymphoblasts that inhibits mouse macrophage-activation induced by lipopolysaccharides. *Arch Med Res.* 1999, 30, 298-302.
- [23] Reynoso-Orozco R, Delgado-Saucedo JI, Puebla-Pérez AM, Calvo-Méndez C, Carr D, Velázquez-Magaña S, et al. Difluorometil ornitina y el extracto acuoso de *Bursera fagaroides* en el modelo de linfoma murino L5178Y en ratones Balb/c: comparación de su efecto en el metabolismo de las poliaminas. *Rev. Salud Anim.* 2008, 30(2), 128-132.
- [24] Preza AM, Jaramillo ME, Puebla AM, Mateos JC, Hernández R, Lugo E. Antitumor activity against murine lymphoma L5178Y model of proteins from cacao (*Theobroma cacao* L.) seeds in relation with in vitro antioxidant activity. *BMC Compl Alternative Med.* 2010, 10, 61-72.
- [25] Dashora N, Sodde V, Bhagat J, Prabhu KS, Lobo R. Antitumor activity of *Dendrophthoe falcata* against Ehrlich ascites carcinoma in swiss albino mice. *Pharmaceutical Crops.* 2011, 2, 1-7.
- [26] Böyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J of Clin Lab Invest.* 1968, 21(197), 77-89.
- [27] Alfaro-Bustamante F, Ramirez-Flores G, Gonzalez-Mendoza A, Islas Rodríguez A, Fafutis-Morris M. Effect of phorbol myristate acetate (PMA) and ionophore A23187 on interleukin-2 levels and proliferation of activated T lymphocytes from patients with lepromatous leprosy. *Int J Lepr Other Mycobact Dis.* 1997, 65(1), 73-79.
- [28] Lempereur L, Cantarella G, Murabito P, Chiarenza A, Fiore L, Zappalà G, et al. Thymic hormones, cancer and behavioural adaptive responses. *Ann Med.* 1999, 31, 40-45.
- [29] Bodey B, Bodey B Jr, Siegel SE, Kaiser, HE. Review of thymic hormones in cancer diagnosis and treatment. *Int J Immunopharm.* 2000, 22, 261-273.
- [30] Parsons SL, Watson SA, Steele RJC. Malignant ascites. *Br J Surg.* 1996, 83(1), 6-14.
- [31] Ayantunde AA, Parsons L. Pattern and prognostic factors in patients with malignant ascites: a retrospective study. *Ann Oncol.* 2007, 18, 945-949.
- [32] Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin Microbiol Rev.* 1996, 9(4), 532-562.
- [33] Barajas MHM, Ramírez FAG, Figuera LE. Effect of T-activin on the gene expression level of cytokines in splenocytes in L-5178-Y bearing Balb/c mice. Human Gemomic Meeting 2003 April 27-30; Cancún, Q.R., México.
- [34] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon- $\gamma$ . *Annu Rev Immunol.* 1997, 15, 749-795.
- [35] Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, et al. Demonstration of an interferon  $\gamma$ -dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci USA.* 1998, 95, 7556-7561.
- [36] Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and Type 2 CD8<sup>+</sup> effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. *J Immunol.* 2000, 164, 916-925.
- [37] Jonasch E, Haluska FG. Interferon in oncological practice: review of interferon biology, clinical applications and toxicities. *The Oncologist.* 2001, 1(6), 34-55.

- [38] Ikeda H, Old LJ, Schreiber RD. The roles of IFN- $\gamma$  in protection against tumor development and cancer immunoediting. *Cytokine Growth F R*. 2002, 13, 95-109.
- [39] Luo Y, Chen X, O'Donnell A. Role of Th1 and Th2 Cytokines in BCG-induced IFN- $\gamma$  production: cytokine promotion and stimulation of BCG effect. *Cytokine*. 2003, 21, 17-26.
- [40] Kenneth H, Hayashi R, Lafond-walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4<sup>+</sup> T cells in the antitumor immune response. *J Exp Med*. 1998, 188(12), 2357-2368.
- [41] Toes REM, Ossendorp F, Offringa R, Melief CJM. CD4 T cells and their role in antitumor immune responses. *J Exp Med*. 1999, 189(5), 753-756.
- [42] Markowska J, Lacki JK, Wiktorowicz K. The usefulness of CD4/CD8 ratio evaluation in monitoring of ovarian cancer patients. *Eur J Gynaecol Oncol*. 1995, 16(1), 54-58.
- [43] Hernberg M. Lymphocyte subsets as prognostic markers for cancer patients receiving immunomodulative therapy. *Med Oncol*. 1999, 16, 145-153.
- [44] Attallah AM, Tabll AA, Ismail MF, Ibrahim TA, El-Dosoky I. Flow cytometric analysis of peripheral blood T-lymphocyte subsets in colon cancer. *Int J Canc Res*. 2006, 2(4), 409-414.
- [45] Chin Y, Janseens J, Vandepitte J, Vandenbrande J, Opdebeek L, Raus J. Phenotypic analysis of tumor-infiltrating lymphocytes from human breast cancer. *Anticancer Res*. 1992, 12(5), 1463-1466.
- [46] Estrela-Lima A, Araújo MS, Costa-Neto JM, Teixeira-Carvalho A, Barrouin-Melo SM, Cardoso SV, et al. Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates. *BMC Cancer*. 2010, 10, 256-269.
- [47] Karlsson M, Lindberg K, Karlén P, Ost A, Thörn M, Winqvist O, et al. Evidence for immunosurveillance in intestinal premalignant lesions. *Scand J Immunol*. 2010, 71(5), 362-368.