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IMMUNOSTIMULATORY ACTIVITY OF MILIFE, A NOVEL IMMUNOMODULATOR OF FUNGUS ORIGIN

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ABSTRACT

Milife is a novel immunomodulator derived from the fungus Fusarium Sambucium. In this study we examined immunomodulatory properties of Milife in 10 months-old BLRB mice. Milife was given to mice orally in a daily dose of 1 mg per mouse, for 2 to 6 days. Groups of mice were sacrificed on days 2, 4, and 6 of treatment, and 3 weeks after completion of a 6 days treatment with Milife, and lymphoid organs were obtained for analysis. Milife administration led to rapid and significant increase in total leukocyte and lymphocyte numbers in peripheral blood that persisted for at least 3 weeks after a 6 days treatment. Cellularity of lymph nodes, bone marrow and thymus increased significantly at days 4 and 6 of treatment, but returned to pretreatment levels after Milife discontinuation. Though total splenocyte numbers did not change dramatically, there occurred delayed increase in $CD4^+$ cells in the spleen 3 weeks following treatment. Preferential accumulation of $CD4^+$ cells was also consistently found in peripheral blood, with the peak being observed at day 6 of treatment. As a result, CD4/CD8 ratio in blood and spleen was significantly higher in treated than in untreated mice. Splenocytes from treated mice proliferated more vigorously in response to Con A. When added in vitro, Milife also mildly costimulated Con A-induced proliferation of splenocytes from intact animals. In conclusion, we have found that Milife can stimulate leuko- and lymphopoesis in BLRB mice, in particular, accumulation of $CD4^+$ T cells in peripheral lymphoid organs. We conclude that Milife may represent an immunomodulator with the potential to correct T cell dysfunction in patients with immunodeficiency.

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INTRODUCTION

It is known that physical and psychological stress, ageing, chronic disease, exposure to environmental toxicants can be associated with immune dysfunction [1-6]. This may lead to increased incidence of allergic, autoimmune and infectious diseases, cancers and immunodeficiency. It has been found that many biologically active substances can modulate lymphocyte functions [7-10,12-22]. Immunomodulators were originally used for the treatment of cancer patients. However, more recently their potential use to correct immune dysfunction in other disease states has been studied too. Some immunomodulators have been shown to affect specifically particular lymphocyte subsets or accessory cells. Thus, Diethyldithiocarbamate influences mainly T lymphocytes [10], Tuftsin, MDP and GMDP are known to activate macrophages [11-14], and Myelopeptides, a group of regulatory peptides originated from the bone marrow, stimulate T lymphocytes, in particular $CD4^+$ T cells [15-18]. All immunomodulators can be subdivided into preparations of natural origin and synthetic agents. The former are typically derived from bacteria, e.g. MDP [12-14], or from fungi, e.g. Bestatin [7] and Z8 [21]. Although immunomodulators specifically targeted to a particular component of the immune system may be needed in certain clinical situations, it is often necessary to stimulate immune system in a nonspecific manner. In this respect, preparations of natural origin may prove more beneficial as they usually represent a mixture of a number of biologically active substances with different properties. A novel immunostimulatory drug, Milife, is a crude extract from the mycelium of fungus Fusarium Sambucium. Milife is a complex preparation containing 18 aminoacids, carbohydrates, phospholypids, nonsaturated fat acids, vitamin B [B1,B6,B12], folic and pantotenic acids, minerals (P, Ca, K, Fe, Zn, Cu, Co, Md, Mn), ubiquinons (Q6, Q9, Q10), as well as some unidentified components. Our preliminary studies of Milife [22] showed that Milife added in vitro stimulated Con A-induced proliferation of and Interleukin-2 production by human peripheral lymphocytes.

The aim of the current study was to examine immunomodulatory activity of Milife in aged BLRB mice that develop spontaneous mammary tumors starting from 10 month age.

MATERIALS AND METHODS

Experimental Animals

BLRB mice [23-24] were bred and maintained in the animal facilities of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry and used in the experiments at the age of 10 months.

Milife Preparation

Milife was obtained from "Dizha" (Russia). For the experiments described below we used crude extract from the mycelium of fungus Fusarium Sambucium, stock VCB-917, which was prepared by suspending 10 g of powdered mycelium in 100 ml of distilled water and shaking the suspension for 48 h at room temperature. Soluble fraction was then obtained by centrifugation and sterilised by filtration (Millipore, 0.22 μm). This stock solution with the concentration equivalent to 0.1 g of powdered mycelium per 1 ml of water was kept frozen at -20° C.

Experimental Design

BLRB mice were treated with Milife at a daily dose of 1 mg per mouse. It was given orally in a volume of 50 μl, dissolved in distilled water, using pipette. Groups of 5 mice received Milife for 2, 4, or 6 days at which times mice were sacrificed and their lymphoid organs were taken for analysis.

To assess delayed effects of Milife, a group of mice was examined 3 weeks after a 6-day treatment. First, mice were anaesthetised under ether and their

blood was collected in tubes containing heparin. Total leukocyte counts were done by diluting blood in 3% solution of acetic acid in water. Blood slides were prepared individually for each mouse to count percentage of lymphocytes. Blood samples from 5 mice in a group were pooled and peripheral blood lymphocytes were separated by gradient centrifugation using Ficoll-Paque (Pharmacia, Piscataway, NJ). Second, lymphoid organs (spleen, thymus, lymph nodes, and bone marrow) were taken from mice, and cell counts in these organs were performed for each mouse in a group individually. Two femurs and axillary plus inguinal lymph nodes from each mouse were taken to provide an estimate of cellularity of bone marrow and lymph node compartments, respectively. The cells were washed three times in HBSS and resuspended in medium RPMI 1640 containing 25 mM HEPES, 2 mM glutamin, 2% fetal bovine serum (FBS; all reagents from Sigma) and 50 μg/ml of gentamicin. Total count of cells in each organ was estimated, and then flow cytometry analysis of cell populations and assessment of mitogen-induced proliferation were carried out.

Control groups of mice received distilled water according to the same schedule as mice treated with Milife.

Assessment of Concanavalin A (Con A)-induced Proliferation of T Lymphocytes

To assess the effect of in vivo Milife treatment on the proliferative capacity of T lymphocytes, splenocytes from treated and untreated mice were stimulated in vitro with ConA (Sigma) in the range of concentrations from 0.6 to 10 ug/ml. Briefly, splenocytes were depleted of erythrocytes by hypotonic shock and resuspended in culture medium RPMI 1640 with 2 mM glutamin and 10% FBS. Cells were cultivated in the presence or absence of ConA in 96-well flat-bottomed microtiter plates (Costar), at 1x106 cells/ml, for 3 days at 37° C in the humidified atmosphere of 5% CO_2 in the air. All cultures were performed in triplicate. To assess the direct effect of Milife on T lymphocyte proliferation, it was added in vitro to splenocytes from intact

mice cultivated with or without ConA. Control cultures received only medium or Con A. Proliferation was measured by colorimetric assay [25]. Briefly, 20 µl of 5 mg/ml MTT solution (4,5-Dimethylthiazolyl-2,5Bdiphenyltetrazolium bromide; Sigma) was added per well for the last 4 h of culture. At the end of incubation, the plates were centrifuged, supernatants were removed, and 100 µl of DMSO was added per well to dissolve formazan crystals. The level of proliferation was estimated by the intensity of MTT reduction as measured by optical density at the wavelength 540 nm using Multiskan MCC/340 reader (Titertek). To compare experiments index of stimulation was calculated for each experiment by dividing optical density in Con A-stimulated culture by that in unstimulated culture.

Flow Cytometry Analysis of Cell Populations

For flow cytometry analysis, peripheral blood lymphocytes, splenocytes and thymocytes from 5 mice in a group were pooled for each organ. Cells were washed 3 times with PBS containing 1% BSA and 0.05% NaN_3, and then stained with the respective antibodies for 30 min on ice. To assess the numbers of $CD4^+$, $CD8^+$ T cells and B-cells, direct staining with the FITC Conjugate of a mAb to mouse CD4, Phycoerythrin (PE) Conjugate of a mAb to mouse CD8a (both from Sigma) and FITC Conjugate of F(ab')2 fragments of rabbit anti-mouse Ig (DAKOPATTS, Denmark) was performed. To assess a total number of T-cells indirect staining was performed. To this end, cells were incubated in the presence of unlabeled murine mAb against Thy-1.2 followed by FITC Conjugate of $F(ab')_2$ fragments of anti-mouse Ig antibody, the same as were used to assess the number of B-cells. The difference between the percentage of positive cells in probes with indirect staining with anti-Thy1.2 plus anti-mouse Ig and in probes directly stained with anti-mouse Ig was considered as the percentage of T-cells. Flow cytometry analysis was performed on cytofluorimeter EPICS-ELITE (Coulter).

Statistical Analysis

Statistical analyses were done by Student's t-test. P values lower than 0.05 were considered significant.

RESULTS

To examine immunostimulatory activity of Milife we used mouse strain BLRB-Rb(8.17)1em [23-24] that was created from unknown C57BL stock in our Laboratory in 1989 year by introducing a Robertsonian translocation into the mouse genom. Female mice, both virgin and bred, of this strain are characterised by high incidence of spontaneous mammary tumors (more than 95 % in bred mice). BLRB male mice also readily develop tumors (100 %) upon implantation of tumor cells from female mice bearing tumors. C57BL/6 mice are much more resistant to the tumor implanted because only about 10 % of C57BL/6 mice develop tumors at much later time then BLRB. This suggests a decreased immune function in these BLRB mice. This work was designed as a previous one to find effective doses and time-dependent influence of Milife to study antitumor activity of Milife in future.

Total Leukocyte and Lymphocyte Numbers in Blood and Lymphoid Organs

Treatment of BLRB mice with Milife led to increased leukocytosis and lymphocytosis in peripheral blood (Fig.1A) and elevated cell counts in lymphoid organs (Fig.1B). A sharp 6-fold increase in total leukocyte number in blood occurred as early as at day 2 of treatment, the levels then decreased by day 6 but still remained elevated throughout the period of observation. Lymphocyte numbers in blood rose in a more gradual manner, reaching highest levels at day 6 of treatment (Fig.1A). Statistically significant, though transient, increase in cellularity also occurred in thymus, lymph nodes, and bone marrow, with maximum cell counts reaching about 300 %, 200 %, and

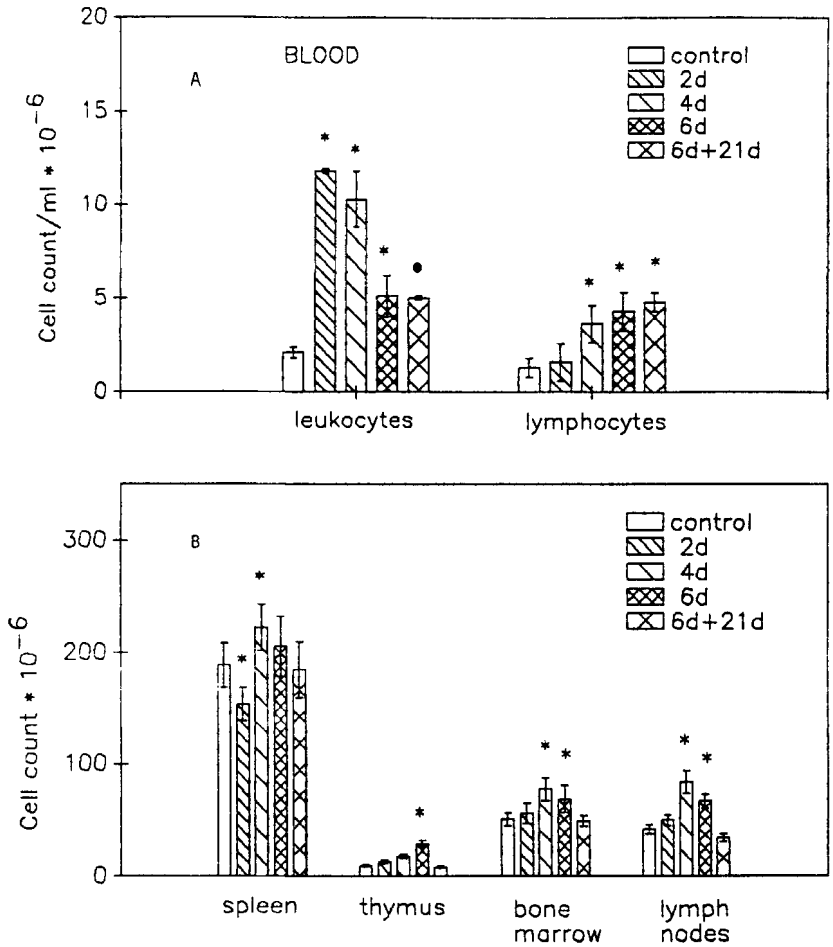

Fig. 1. Kinetic of cellularity of blood (A) and immune organs (B) of BLRB mice fed Milife in vivo in a dose of 1 mg/mouse per/os during from 2 up to 6 days. Error bars represent SEM of three separate experiments. Control mice were fed distilled water during 6 days. A prolonged effect of Milife was estimated after a 21 day rest.

150 % of the respective control levels at days 4 or 6 of treatment (Fig.1B). Changes in total cellularity of spleen were marginal. Upon Milife discontinuation, 3 weeks after a 6-day treatment, cell counts returned to pretreatment levels in all lymphoid organs tested but were still elevated in blood.

Quantification of Lymphocyte Subpopulations

To investigate what lymphocyte subpopulations were affected by Milife treatment we quantified total numbers of B and T lymphocytes, and subsets of $CD4^+$ and $CD8^+$ T cells in blood, spleen and thymus by flow cytometry analysis. In blood, the numbers of both B and T lymphocytes increased steadily from day 2 to reach maximum at day 6 of treatment (Fig.2, panel A). The numbers of $CD4^+$ and $CD8^+$ T cells followed similar kinetics, but the former increased more dramatically than the latter. The effect of Milife was long lasting as all the parameters remained elevated in blood even 3 weeks after Milife discontinuation. The changes in B and T cell subpopulations in spleen were marginal and did not show any consistent pattern, with the exception of the $CD4^+$ T cells that significantly increased after completion of Milife treatment (Fig. 2, panel B). As a result of preferential increase in $CD4^+$ T cell numbers, CD4/CD8 ratio also increased in blood and spleen (Fig. 2, panel D).

In thymus, the numbers of single positive $CD4^+$ or $CD8^+$ cells showed little or no change, and increase in total thymocyte numbers, during Milife treatment, was accounted for by $CD4^+CD8^+$ double positive subset (Fig.3, panel C).

Mitogen-induced Proliferation of T Cells

Finally, we investigated functional competence of T-cells from Milife treated mice by measuring Con A-induced proliferation. Splenocytes from the treated mice consistently showed higher proliferative responses to wide range of Con A concentrations, than splenocytes from untreated animals. The enhancing effect of treatment was most pronounced at day 4 of Milife administration when proliferative response was up to 3 fold higher than that by splenocytes from intact mice (Fig.3A). To assess whether Milife can directly affect T cell proliferation, it was added in vitro to splenocytes from intact mice, unstimulated or stimulated with Con A. In this system, Milife

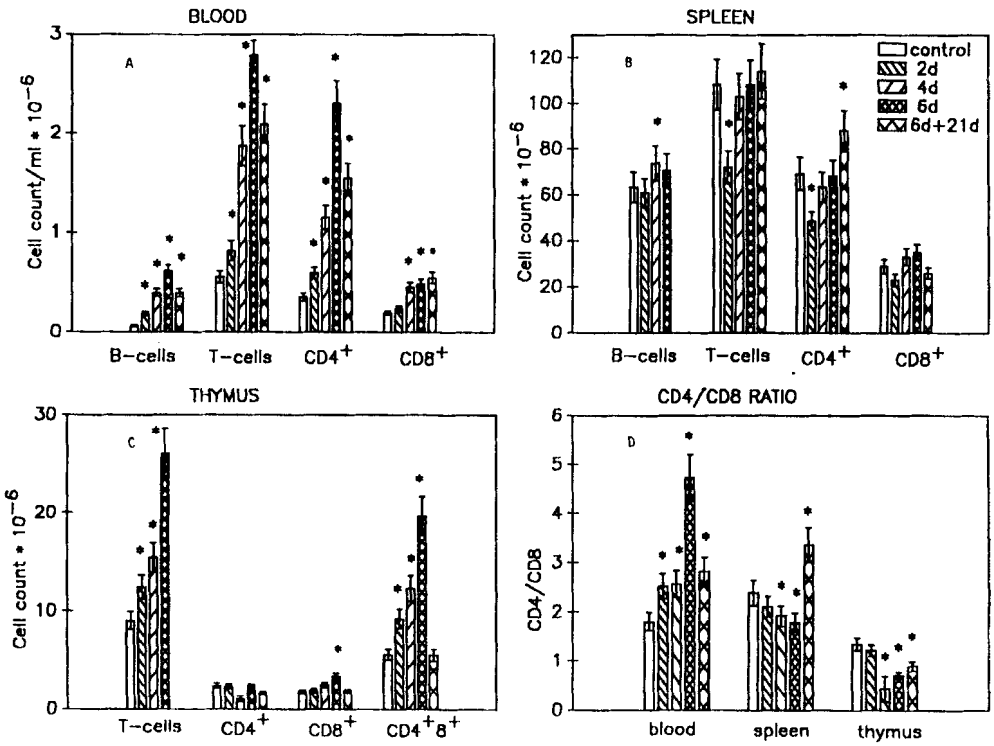

Fig. 2. Effect of Milife treatment on lymphocyte subpopulation kinetic in blood (A), spleen (B) and thymus (C) and CD4/CD8 ratio (D) of BLRB mice fed Milife in vivo in a dose of 1 mg/mouse per/os during from 2 up to 6 days. Error bars represent SEM of three separate experiments. Control mice were fed distilled water during 6 days. A prolonged effect of Milife was estimated after a 21 day rest.

did not have its own mitogenic activity but slightly costimulated ConA-induced proliferation of splenocytes (Fig. 3B).

DISCUSSION

It has been known that a decrease in immune function often follows prolonged and severe disease, chronic physical and psychological stress, and may be associated with pregnancy, nutrition disorder and other conditions [1-6]. This immune dysfunction is typically reversible and can be recovered

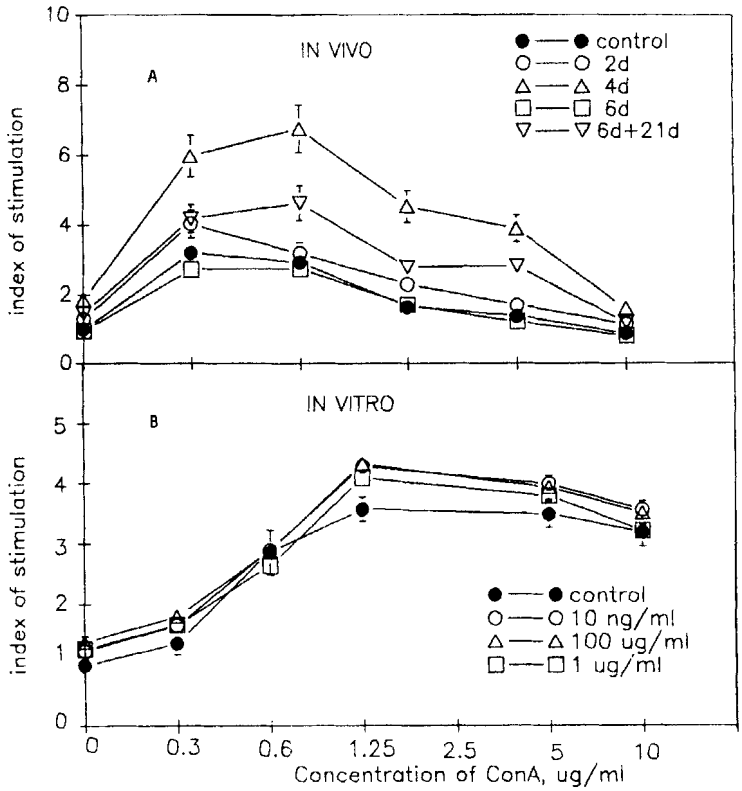

Fig. 3. A. Dose-response curves for Con A-induced proliferation of splenocytes from BLRB mice fed in vivo in a dose of 1 mg/mouse per/os during from 2 up to 6 days. B. Dose-response curves for Con A-induced proliferation of untreated BLRB splenocytes in the presence of Milife in vitro in cultures. Error bars represent SEM of three separate experiments. Control mice were fed distilled water during 6 days. A prolonged effect of Milife was estimated after a 21 day rest.

after a prolonged rest. Nevertheless, such transient immunodeficiency (TID) represents a serious predisposing factor for the development of allergic and autoimmune diseases, cancers, and may eventually progress to a stable immunodeficiency. A person may encounter episodes of TID several times in his life, which may contribute to a decrease in immune function associated with aging.

TID in human is usually characterised by a triad of abnormalities: (a) decreased number of lymphocytes in peripheral blood (less than 1.2 x 10^9/L),

(b) decreased CD4/CD8 ratio (less than 1.2), and (c) depressed lymphocyte function, as assessed by mitogen-induced proliferation and production of interleukin-2. The mechanisms leading to the development of TID are poorly understood. In general, TID apparently results from immune system exhaustion and failure to recover. Administration of immunostimulatory drugs may help the recovery of normal immune activity and thus prevents potential complications of immune dysfunction. Many of the immunomodulators characterised to date have been found to affect certain cell subpopulations in rather selective manner. Thus, MDP, GMDP and Tuftsin stimulate macrophages, while thymic hormones increase the numbers of T cells. However, because TID most likely results from a deficiency in several components of the immune system, immunomodulators with a broad spectrum of activities may be more beneficial for its correction. We believe that to this class of drugs belongs Milife, a novel immunomodulator derived from the mycelium of fungus Fusarium Sambucium.

This study was aimed to investigate the effects of in vivo treatment with Milife in BLRB mice. This mouse strain is characterised by increased susceptibility to the development of tumors, both spontaneous (in mammary gland) in female mice and upon inoculation of autologous tumor cells in male ones. This suggests a decreased immune function in BLRB mice. We have found that Milife can stimulate accumulation of leukocytes and lymphocytes in peripheral blood and lymphoid organs of BLRB mice. The effect of Milife treatment developed quickly, such as significant increase in total leukocyte (6- fold) and lymphocyte (2.4-fold) numbers in blood was first detected at days 2 and 4 of treatment, respectively (Fig.1A). Lymphocyte accumulation in blood was paralleled by an increase in cell counts in the lymph nodes, bone marrow, thymus, and to a lesser extent in spleen (Fig. 1B). Milife treatment led to an increase in both B and T cell numbers in peripheral blood and spleen; among T lymphocytes, $CD4^+$ cells increased to a greater extent, than $CD8^+$ cells, in both compartments (Fig. 2A, B).

These data would suggest that Milife can stimulate leukocyte and lymphocyte production, possibly through bone marrow stimulation. Thus,

because the great majority of peripheral B cells are short-lived cells, and their numbers are maintained through continuous production and export from the precursor pool in the bone marrow [26], a rise in peripheral B cell numbers should have occurred, at least in part, due to their increased production in the bone marrow. In addition, we observed a statistically significant increase in the cellularity of bone marrow (150 % of the control levels) during Milife treatment. At the same time, Milife may also affect cell migration and distribution in different lymphoid organs. Thus, transitory but significant decrease in total splenocyte numbers occurred at day 2, followed by elevation at day 4 of treatment (Fig. 1B), which might indicate temporal redistribution of cells from the spleen to other lymphoid compartments.

Significant changes were found in the thymus in treated mice, where steady accumulation of double positive $CD4^+CD8^+$ thymocytes occurred at days 2 through 6 of Milife administration (Fig. 2C). This could be due, at least in part, to increased migration of T-cell progenitors from bone marrow to the thymus. It can be speculated that, as a result of this process, thymic output of differentiated $CD4^+$ or $CD8^+$ single positive T cells would also increase, thus contributing to the accumulation of mature T cells in the peripheral lymphoid organs during Milife treatment (Fig. 2A, B). An additional mechanism could be direct costimulation of proliferation of mature peripheral T cells by Milife. Indeed, splenocytes from in vivo treated mice showed more vigorous proliferation (Fig. 3A) in response to Con A, than splenocytes from untreated animals. When added in vitro, Milife also slightly costimulated Con A-induced proliferation of splenocytes from intact mice (Fig. 3B). On the other hand, increased responsiveness to Con A might reflect increased functional activity of splenic T cells in Milife treated mice, compared with untreated controls.

It should be noted that some of the effects of Milife treatment were long-lasting and persisted for at least 3 weeks after Milife discontinuation. Of particular importance may be increased blood T and B lymphocyte numbers (Fig. 2A), persistent elevation of $CD4^+$ T cells, increased CD4/CD8 ratio in peripheral blood and spleen (Fig. 2A, B, D), and enhanced functional

activity of splenic T cells (Fig. 3A). All the above allows us to suggest that Milife has a broad lymphocyte stimulatory activity, which makes it a potentially useful drug for the correction of transitory immunodeficiency. Additional studies should establish the exact mechanisms behind immunostimulatory activity of Milife and its applicability for the treatment of human disease.

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