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The effect of high dose cyclophopshamide pretreatment on circulating regulatory T cells and tumour growth in murine DBA/2-SL2 lymphoma model

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Introduction. The aim of this study was to evaluate the role of host immune system, particularly circulating $CD4^+CD25^+FoxP3^{high}$ cells, in the growth regulation of tumours in murine DBA/2-SL2 lymphoma model.

Materials and methods. Murine immune system was suppressed by the intraperitonial injection of high dose cyclophosphamide (CTX). The volume of two SL2 tumours implanted in the same mouse was analysed in CTX pre-treated and control groups. Lymphocytes count was obtained by an automatic haematological analyzer. The frequency of circulating regulatory T cells $CD4^+CD25^+FoxP3^{high}$ was determined in the peripheral blood of mice in each group using regulatory T cells antibody staining kit by flow cytometry.

Results. CTX treatment significantly reduced lymphocyte count (3.84 fold decrease) and $CD4^+CD25^+FoxP3^{high}$ cells percentage (1.83 fold decrease). Primary and secondary tumours in CTX treated group reached significantly smaller volumes than in the control group. Our results indicate that growth of tumours in CTX pre-treated mice is inhibited despite general immunosuppression, contrary to the general observation that occurrence of tumours is increased in immunodeficient / immunosuppressed hosts.

Conclusions. Pre-treatment with CTX decreased tumour volume in murine DBA2-SL2 lymphoma model and this effect can be attributed to systemic CTX effect on murine immune system. In our study we exclude the direct effect of CTX and demonstrate that changes in tumour growth dynamic could be related to the general immunosuppression status and reduced T regs frequency in mice.

Key words: regulatory T cells, tumor immunology, cyclophosphamide

INTRODUCTION

Regulatory T cells are a heterogeneous group comprising naturally occurring $CD4^+CD25^{high}$ Treg cells, Tr1 and TH3 cells, $CD8^+$ regulatory T cells as well as $CD4^+CD25^{high}$ Treg cells developing in the periphery by conversion of $CD4^+CD25^-$ T cells (1). Mouse and human T reg cells are selected in the thymus and in normal conditions represent about 5–10% of total $CD4^+$ T cells in the periphery (2). It is known, that naturally occurring $CD4^+CD25^+$ regulatory T cells (Tregs) have an important role in immunotolerance towards tumours (3), are increased in peripheral blood of cancer patients (4, 5) and are the major regulators of concomitant tumour immunity (6). It is well documented, that T regs are sensitive to cyclophosphamide (CTX) (3).

The intensive research of regulatory T cells began in 1995 when Sakaguchi et al. identified a population of $CD4^+$ T cells highly expressing CD25 and preventing autoimmunity in mice (7). However, CD25 (IL-2R alpha chain) molecule is also expressed on activated effector T cells, and in 2003 more specific intracytoplasmic marker of T reg cells FoxP3 was identified (8). FoxP3 is a transcriptional factor that, in mice, is exclusively expressed in $CD4^+25^+$ Tregs (9). To date FoxP3 is the most specific marker of naturally occurring $CD4^+CD25^+$ regulatory T cells, currently the best defined and characterised subpopulation among the various subgroups of regulatory T cells (3). However, data about the role of $CD4^+CD25^+$ $FoxP3^{high}$ T cells in the regulation of tumour growth in a host organism are scarce.

There is strong evidence about the importance of host immune system in the process of tumourigenesis. The notion that immune system regulates cancer development through a general process of cancer immunoediting is now well established (10–12). Immune system exhibits complex interactions between tumours and the host – it can act as an extrinsic tumour suppressor by effectively eliminating transformed malignant cells (immunosurveillance) and both facilitate tumour progression by sculpting the immunogenic phenotypes of tumours (12). Complementary, the tumour cells also interfere with immune system: the presence

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of primary tumour in patients suppresses T cell and antibody responses (the so-called tumour-induced immunosuppression) and surgical removal of primary tumour restores immunocompetence even when disseminated metastatic disease is present (13).

Recently, we have developed a new method to estimate growth fraction (GF) in tumours (14). This method allows measurement of cell kinetics in an individual tumour at a particular time point and is particularly useful in murine models. We have also shown that GF, defined as the proportion of cells engaged in the cell cycle, decreases during growth of SL2 tumours in DBA/2 mice (14). Thus, implantation of two SL2 tumours several days apart in the same DBA/2 mouse might create a situation in which two tumours with different growth rates are present in the same host. This model could resemble the situation of a primary tumour with distant metastases and be useful in studies of tumour growth dynamic.

Therefore the aim of this study was to evaluate the feasibility of our murine DBA/2-SL2 lymphoma model and determine whether the host immune system, particularly circulating $CD4^+CD25^+FoxP3^{high}$ cells, is involved in the growth regulation of tumours in mice.

MATERIALS AND METHODS

Mice and tumours

Female DBA/2 mice at the age of 8–12 weeks were obtained from the local breeding facility at the Institute of Immunology of Vilnius University, Lithuania.

Spontaneously arisen SL2 lymphoma cells were maintained by weekly intraperitoneal (i.p.) passage in syngeneic DBA/2 mice.

SL2 cells growing i.p. in ascitic fluid were collected from the peritoneal cavity, washed and diluted in RPMI-1640 medium. The primary solid tumours were induced by subcutaneous injection of 10^7 SL2 cells in 0.1 ml of RPMI-1640 medium on the left flank into naïve mice at day 0, and the secondary tumours were induced by subcutaneous injection of 10^7 SL2 cells on the right flank at day 2.

Tumour volume was regarded as the volume of an ellipsoid body and calculated using formula $\frac{1}{6}\pi lb^2$ (the volume of an ellipsoid body) obtained by measuring with calliper perpendicular diameters (length l and breadth b) of tumour.

Experimental research on animals has been conducted according to recommendations of the Lithuanian Ethics Committee for the Laboratory Animal Use.

Induction of immunosuppression

The immunosuppression was induced by high doses of cyclophosphamide (CTX). CTX (Ledoxina, Lemery S. A., Mexico) was dissolved in 0.9% saline and injected i.p. at a dose of 60 mg/kg of body weight daily from day –13 to day –3 (total CTX dose – 600 mg/kg) prior to the implantation of tumours. Mice were weighed daily during the treatment time and doses of CTX were adjusted according to their weight. Control mice received no CTX.

Cell counting and flow cytometry

Peripheral blood was collected from the tail vein of individual mice before the implantation of primary tumours (day 0) and on day 9; EDTA was used as an anticoagulant. Lymphocytes were counted by an automatic haematological counter (HEMAVET/850S, CDC Technologies Inc., Oxford, CT).

Regulatory T (T reg) lymphocytes were analysed using Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego, CA) according to the recommendations of manufacturer.

Briefly, for surface markers staining 50 µl of whole blood were incubated with 5 µl of fluorochrome-labeled anti-CD4-FITC and 5 µl anti-CD25-APC anti-mouse monoclonal antibodies in the dark at room temperature for 30 min. After the incubation, red blood cells were lysed by FACS Lysing Solution (Becton Dickinson, San Jose, CA) for 10 minutes and then washed by Cell Wash (Becton Dickinson, San Jose, CA). To permeabilize lymphocytes, 1 ml of freshly prepared Fixation / Permeabilization (eBioscience, San Diego, CA) working solution was added and samples were incubated for 1 hour at 4 °C in the dark, then washed once with Flow Cytometry Staining Buffer (eBioscience, San Diego, CA) and twice

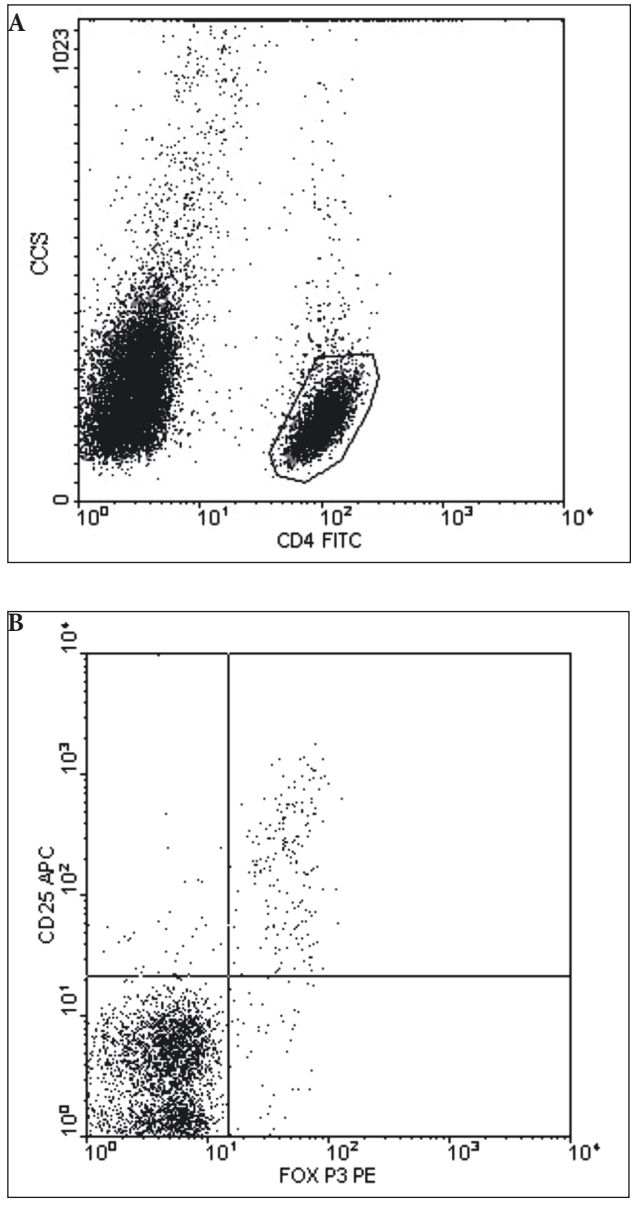

Fig. 1. Analysis of $CD4^+CD25^+FoxP3^{high}$ cells by flow cytometry. Separation of antigen $CD4^+$ expressing T lymphocytes by gating (A), followed by separation of both $CD25^+$ and $FoxP3^{high}$ expressing $CD4^+$ T cell subpopulation (upper right quadrant)

with 1 ml of Permeabilization Buffer (eBioscience, San Diego, CA). To block non-specific Fc receptor binding, 1 μg per test of Fc block (anti-mouse CD16/CD32) was added for 15 min. at 4°C. For intracellular staining 0.5 μg per test of anti-FoxP3- PE antibody (clone FJK-16s) was added and incubated at 4 °C for 30 min. in the dark. Rat IgG2a antibody was used as isotype control.

After washing twice with 1 ml of Permeabilization Buffer (eBioscience, San Diego, CA) samples were resuspended in 500 μl of Flow Cytometry Staining Buffer (eBioscience, San Diego, CA) and multiparameter flow cytometric analysis was performed on FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The data were acquired using CellQuest Software (Becton Dickinson Immunocytometry Systems, San Jose, CA), and 30000 cells were analyzed per sample using WinMDI version 2.8 software.

To identify $CD4^+CD25^+FoxP3^{high}$ cells, the following approach was used. After plotting intensity of side scatter against FITC-labelled CD4 fluorescence, CD4+ cells were visualized as separate subpopulation cloud, which was further gated to separate both CD25 (APC-labelled) and FoxP3 (PE-labelled) expressing cells compartment (Fig. 1). The frequency of FoxP3 positive cells was expressed as a percentage of the total numbers of $CD4^+$ T cells.

Statistics

To compare the data from two independent samples the two-sample Wilcoxon test was used. P-value of 0.05 or less was considered as statistically significant. The data were processed with SAS 9.1 software.

RESULTS

Effect of high dose CTX treatment

To gain evidence about the involvement of the lymphoid system in our model, we used total high dose CTX treatment. Two days after the completion of treatment, primary tumours were implanted. In our model, the time interval between implantation of the primary and secondary SL2 tumour was 2 days. All mice supported the growth of the second tumour.

CTX treatment significantly reduced lymphocyte count (3.84 fold decrease) (Fig. 2 A) and $CD4^+CD25^+FoxP3^{high}$ cells percentage (1.83 fold decrease) (Fig. 2 B) in peripheral blood of mice. This shows that high dose CTX treatment induces immunosuppression by reducing lymphocyte count and decreases $CD4^+CD25^+FoxP3^{high}$ cells frequency. On the 9th day circulating regulatory T cells percentage was restored in CTX treated group (Fig. 2 B).

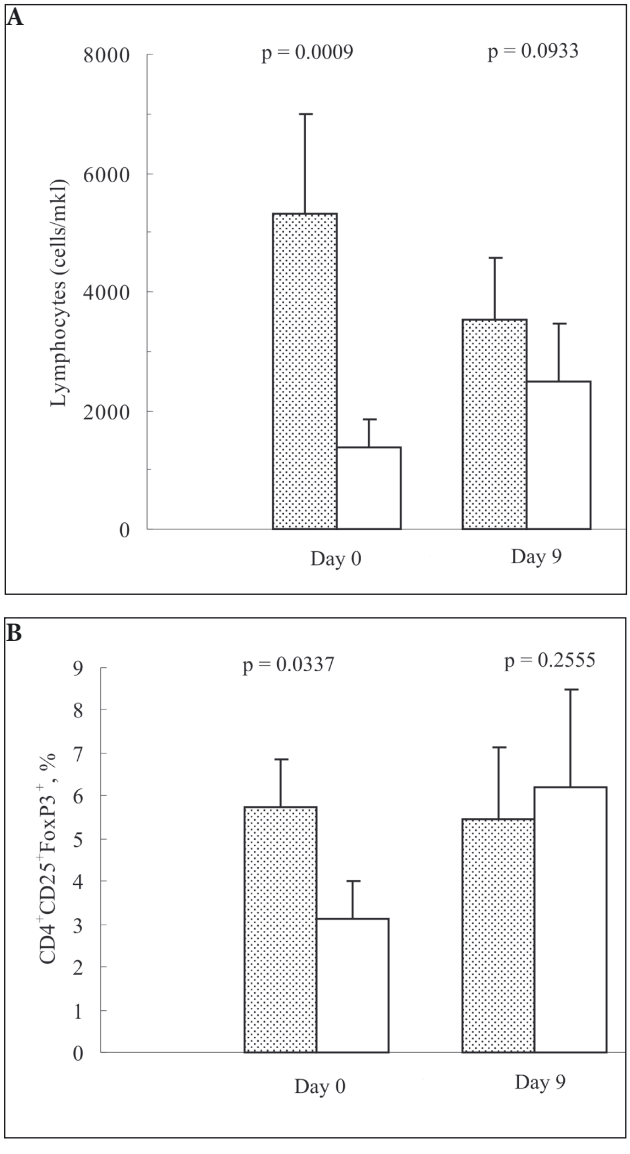

Fig. 2. Lymphocyte counts (A) and percentage of $CD4^+CD25^+FoxP3^{high}$ cells of the total numbers of $CD4^+$ T cells (B) in the peripheral blood of control (dotted bar, n = 7) and cyclophosphamide (CTX) treated (empty bar; n = 7) mice before the primary tumour implantation (day 0) and on the 9th day

Note. CTX was administered at 60 mg/kg/d, total dose 600 mg/kg. CTX treatment significantly reduced lymphocyte count (p = 0.0009) and $CD4^+CD25^+FoxP3^{high}$ cells percentage (p = 0.0337). Results of one representative experiment out of two are shown. Values are given as means ±SD.

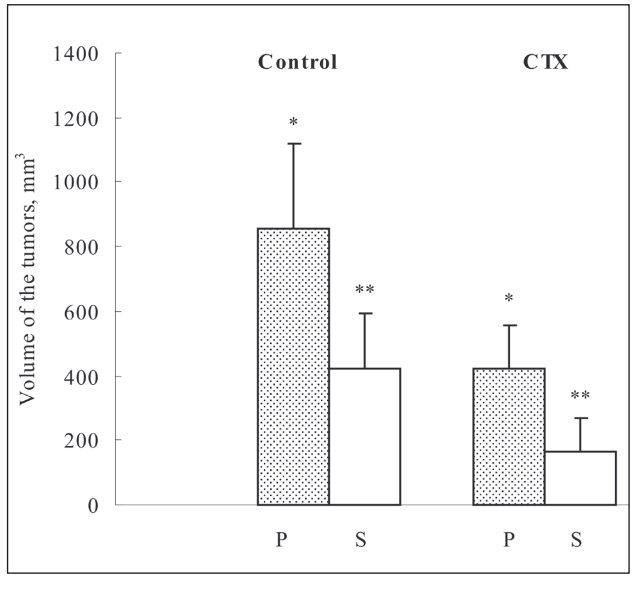

Fig. 3. The volumes of primary and secondary tumours on day 9 in CTX untreated (control; n = 7) and CTX treated (n = 7) mice

Note. Primary (P) and secondary (S) tumours in CTX treated group reached significantly smaller volumes than tumours in control group (* p = 0.0305 and ** p = 0.0100, respectively). Results of one representative experiment out of two are shown. Values are given as means ±SD.

Growth of tumour in immunosuppressed mice

To evaluate the involvement of immune system in the growth of two concomitant tumours we compared the volumes of primary and secondary tumours on day 9 in CTX treated and control mice.

Interestingly, primary and secondary tumours in CTX treated group reached significantly smaller volumes than tumours in control group (Fig. 3).

DISCUSSION

Growth of the secondary SL2 lymphoma tumour in DBA/2 mouse is completely inhibited when the second tumour graft is implanted 7 days or later after the first graft (15, 16). The results of our study show that the time interval of 2 days between the implantation of tumours in our model allows the growth of secondary tumour.

During the growth of SL2 tumour in DBA/2 mice T cell-mediated immunity is generated (15). We interfered with murine immune system by administration of alkylating drug cyclophosphamide (CTX). Prolonged administration of low dose (60 mg/kg) CTX allowed to avoid severe adverse reactions and achieve high total 600 mg/kg dose. The high doses (≥200 mg/kg) of cyclophosphamide (CTX) selectively deplete lymphoid tissue (17) and lead to reduction of $CD4^+$ and $CD8^+$ T cells (18), which is followed by a slow and partial recovery (19). The lymphoid cells are more sensitive to cyclophosphamide than granulocytic cells, and recovery of lymphocyte numbers in spleen occurs only after 2 weeks (20).

CTX is metabolized to 4-hydroxy-cyclophosphamide, which breaks down into the reactive metabolites phosphoramide mustard and acrolein (21). In the mouse, active metabolites of cyclophosphamide can only be detected in plasma for less than 1 h after its administration into the mouse (20). Therefore, after two days, direct immunosuppressive cyclophosphamide effect on the implanted tumour cells can be excluded.

There is equal distribution of Treg cells among the different lymphoid compartments (thymus, blood, lymph nodes, and spleen) in both human and mouse (22), therefore for practical reasons we choose to analyse peripheral blood.

Interestingly, in our experiment primary and secondary implanted tumours reached smaller volumes comparing with control mice in CTX treated mice, what shows that the growth of tumours in CTX pre-treated mice is inhibited despite general immunosuppression, contrary to the general observation that occurrence of tumours is increased in immunodeficient/immunosuppressed hosts (12). Our results could suggest that tumour growth is further suppressed by specific cell population which is sensitive or resistant to CTX treatment. In this work we interfered with mouse immune system by administering high dose of CTX.

It is well documented, that besides the ability to cause general immunosuppression, low doses (20 mg/kg) of CTX can lead to enhanced immune responses against a variety of antigens, including tumour, and selective toxicity of CTX on regulatory T cells has been recently demonstrated (23). However, to our knowledge the effect of high dose CTX treatment on the frequency of FoxP3 expressing T cells in the peripheral blood of mice was not investigated.

We found that high dose CTX decreases the frequency of circulating $CD4^+CD25^+FoxP3^{high}$ cells in mice and this decrease logically could be attributed to the enhanced host antitumour response, that results in decreased primary and secondary tumour volume. This supposition is supported by the observation that naturally occurring T reg cells suppress tumour-specific cytotoxic T lymphocytes (6), and depletion of T reg cells further augments the immune response in mice bearing tumours (3). It was already reported in the literature, that administration of CTX during the latency of tumour induction delays 3-methylcholantrene sarcoma induction in mice (24, 25), and that could be attributed to decreased suppressor cells activity. However, the influence of direct drug toxicity on tumour cells was not ruled out in these studies. In our study we exclude the direct effect of CTX and show that changes in tumour growth dynamic are related to the general immunosuppression status and reduced T regs frequency in mice.

CONCLUSIONS

The results of our study show that DBA2-SL2 model is suitable for the growth studies of several SL2-lymphoma cell populations in the same mouse. Pre-treatment with CTX decreased tumours volume in murine DBA2-SL2 lymphoma model, and this effect is attributed to systemic CTX effect on immune system. Our data suggest that the observed changes in a tumour growth dynamics are related to the general immunosuppression status and reduced T regs frequency in mice. Decreased primary and secondary tumour volume in CTX mice group could be attributed to the enhanced host antitumour response due to reduced frequency of circulating $CD4^+CD25^+FoxP3^{high}$ cells in mice.

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DIDELĖS DOZĖS CIKLOFOSFAMIDO POVEIKIS CIRKULIUOJANČIOMS REGULIACINĖMS T LĄSTELĖMS IR NAVIKŲ AUGIMUI PELIŲ DBA/2-SL2 LIMFOMOS MODELYJE

S a n t r a u k a

Įvadas. Šio tyrimo tikslas buvo įvertinti didelės dozės ciklofosfamido poveikį cirkuliuojančių $CD4^+CD25^+FoxP3^{high}$ T ląstelių kiekiui ir šeimininko imuninės sistemos svarbą navikų reguliacijai pelių DBA/2-SL2 limfomos modelyje.

Priemonės ir metodai. Pelių imuninė sistema buvo nuslopinta intraperitoniškai suleidžiant didelę ciklofosfamido (CTX) dozę prieš navikų implantaciją. Kiekvienai pelei buvo implantuoti du SL2 navikai; jų tūris buvo analizuotas kontrolinėse ir CTX paveiktose grupėse. Limfocitų skaičius buvo matuotas automatiniu hemocitometru, o periferiniame kraujyje cirkuliuojančių reguliacinių $CD4^+CD25^+FoxP3^{high}$ T ląstelių koncentracija buvo nustatyta tėkmės citometrija naudojant monokloninių antikūnų rinkinius.

Rezultatai. CTX reikšmingai sumažino limfocitų skaičių (3,84 kartus) ir $CD4^+CD25^+FoxP3^{high}$ ląstelių procentą (1,83 kartus). CTX paveiktoje pelių grupėje pirminiai ir antriniai navikai užaugo mažesni negu kontrolinėje grupėje. Mūsų rezultatai rodo, kad navikų augimas CTX paveiktoje pelių grupėje yra slopinamas nepaisant bendros imunosupresijos būklės.

Išvados. Paveikus peles CTX iki navikų implantacijos, navikų tūris pelių DBA2-SL2 limfomos modelyje sumažėjo, ir tai gali būti siejama su sisteminiu CTX poveikiu pelių imuninei sistemai. Šiame eksperimente mes atmetėme tiesioginį CTX poveikį navikams ir manome, kad navikų augimo kaitos pokyčius gali nulemti bendra imunosupresijos būklė bei sumažėjęs reguliacinių T ląstelių kiekis.

Raktažodžiai: reguliacinės T ląstelės, navikų imunologija, ciklofosfamidas