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Immunosuppressive effect of cyclophosphamide on white blood cells and lymphocyte subpopulations from peripheral blood of Balb/c mice

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ABSTRACT

There has been lack of the uniform standard for establishment of animal immunodepressive models induced by cyclophosphamide (CTX), and the information about the immunosuppressive effect of CTX on peripheral blood lymphocyte subsets in rodents. Here we describe a CTX-induced mouse model and try to establish a feasible immunosuppressive model for studying the fungal pathogenicity. Balb/c mice received two intraperitoneal injections of different CTX doses (50–200 mg/kg) at 2-day intervals. Peripheral whole blood collected at different time-points before and after CTX injection was used to detect white blood cells (WBCs), lymphocytes and their subsets by automated hematology analyzer and flow cytometry, respectively. WBCs and lymphocytes in all groups except CTX50 (50 mg/kg CTX) group commenced to decrease in a dose-dependent manner on day 1, reached the nadir on day 4, rebounded on day 10, and declined again on day 17 after CTX treatment. Low dose (50 mg/kg) CTX produced no obvious change of percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and $CD19^+$ cells, but high doses (100 or 150 mg/kg) yielded a significant decrease of $CD3^+$ and $CD4^+$ cells on day 4 and $CD19^+$ cells on day 10, and increase of $CD8^+$ cells on day 4. The $CD4^+/CD8^+$ ratio decreased on day 4, followed by a rebound thereafter when treated with 3 different doses of CTX. The results indicate that two intraperitoneal injections of CTX at 150 mg/kg at 2-day intervals may establish good immunosuppressive models of Balb/c mice for studying the fungal pathogenicity.

1. Introduction

Animal models are important for studying the pathogenesis, virulence, immunology, diagnosis and treatment of fungal infections. Various species of animals have been used in mycological investigation, with the mouse being the species of choice [1]. The immunosuppressive induction is generally necessary for successful experimental infections of opportunistic fungi [2]. Cyclophosphamide (CTX) is one of the most commonly used anticancer agents and immunodepressant drugs for preventing graft rejection, treating some chronic autoimmune diseases and inducing experimental immunosuppression [3]. CTX is a nonphase-specific cytotoxic agent that can inhibit both humoral and cellular immunity, but its mechanism of action is very sophisticated. CTX has both antineoplastic and immunomodulatory roles [4]. Otherwise, CTX can also reinforce immune responses according to its dose and timing of administration [5]. Therefore, reasonable selection of CTX dose, timing and frequency is pivotal for successful establishment of CTX-induced immunodeppressant animal models, resulting in the effective inhibition of immune function as well as long-term survival of the hosts.

Although there have been many reports on CTX-induced animal models, it is lack of a uniform standard. The dose, route, timing and frequency of CTX administration are different: (1) injection doses are 50–200 mg/kg; (2) injection routes are intraperitoneal, subcutaneous and intravenous; (3) timing of injection is successive or interval; and (4) injection frequency comprises one, two and many times. In addition, the immunological function is generally not performed in CTX-induced mouse models of experimental infections though some authors detect white blood cells (WBCs) and neutrophils [6–11]. Nevertheless, peripheral blood lymphocyte subsets have been seldom detected. Therefore, we determined WBCs, lymphocytes and their subpopulations from peripheral blood of Balb/c mice after two intraperitoneal injections of different CTX doses (50–200 mg/kg) at 2-day intervals. Our goal is to characterize a reliable immunosuppression model in mice for studying the fungal pathogenicity.

2. Materials and methods

2.1. Mice and reagents

Specific pathogen-free male Balb/c mice (6–8 week old, 18–22 g weight) were obtained from Laboratory Animal Center of Guangdong Medical College. The mice were kept at a temperature of 22 °C and 12-h light/dark cycles, and provided with food and water ad libitum. The experiments were approved by the Laboratory Animal Ethical Committee of Guangdong Medical College. CTX injection was purchased from Jiangsu Hengrui Medicine Co., Ltd (China). RBC Lysis Buffer and FITC-,

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PE-Cy5-, and PE-conjugated anti-mouse CD3, CD4, CD8, CD19, and IgG Isotype monoclonal antibodies (mAbs) were products of eBioscience (USA).

2.2. Detection of WBC and lymphocyte counts

Forty-nine mice were randomly divided into 7 groups, each of 7 animals. CTX50, CTX75, CTX100, CTX125, CTX150, CTX175 and CTX200 groups received 50, 75, 100, 125, 150, 175, and 200 mg/kg CTX, respectively. The mice were treated with 2 intraperitoneal injections of equal doses of CTX on days 1 and 4.

Peripheral blood was collected from the retro-orbital plexus of each mouse on day 1 (0 d) before injection as well as on days 1 (1 d), 4 (4 d), 10 (10 d) and 17 (17 d) after first injection. 0.5 ml of blood was placed in a sterile EDTA-anticoagulated tube and counted in a Sysmex KX-21 N Blood Cell Analyser (Sysmex Corporation, Japan).

2.3. Flow cytometry of lymphocyte subpopulations

Twenty-four mice were randomly allocated to CTX50, CTX100 and CTX150 groups, each of 8 ones. CTX injection method was the same as above-mentioned. EDTA-anticoagulated whole blood was collected on day 1 (0 d) before injection as well as on days 4 (4 d), 10 (10 d) and 17 (17 d) after first injection. One hundred microliters of whole blood were gently mixed with 10 μl of anti-mouse mAbs (FITC-CD3, PE-Cy5-CD4, PE-Cy5-CD19, PE-CD8, and negative control IgG) and incubated 20 min at room temperature in the dark. After adding RBC lysis buffer for 10 min, the samples were centrifuged at 1500 ×*g* for 5 min and the supernatant was discarded. The samples were washed with 1 ml phosphate-buffered saline (PBS), centrifuged, and the supernatant was discarded. The cells were resuspended in PBS containing 1% paraformaldehyde, and analyzed on a Coulter Epics-XL flow cytometer (USA). Two-parameter histograms were created by using CELLQUEST software. A total of 5000 lymphocytes were counted in each sample. Cells were illuminated with a 488-nm wavelength argon ion laser using a standard filter combination.

2.4. Statistical analysis

The data were analyzed using AVOVA and least significant difference (LSD) for the equal variances, or Welch and Dunnett T3 test for the unequal variances.

3. Results

3.1. General observation following CTX injection

All mice showed lethargy, lackluster pelage, reluctance to environmental activity, fur piloerection, and reduced food and water intake on day 1 after first injection of CTX, especially on days 3–4, and then restored slowly. Their responsiveness was positively correlated with the dose of CTX. On day 10 after first injection of CTX, 5 mice (1, 2 and 2 cases in CTX125, CTX175 and CTX200 groups, respectively) died within 1 day after blood collection.

3.2. Effect of CTX on peripheral blood WBCs and lymphocytes

WBCs and lymphocytes in all groups except CTX50 group commenced to decrease in a dose-dependent manner on day 1, reached the nadir on day 4, rebounded on day 10, and declined again on day 17 after CTX treatment (Tables 1–2).

3.3. Effect of CTX on peripheral blood lymphocyte subsets

There were a significant descent of percentage of $CD3^+$, $CD3^+CD4^+$ cells and a remarkable increase of $CD3^+CD8^+$ cells on day 4 with a rebound thereafter in CTX100 and CTX150 groups. The $CD4^+/CD8^+$ ratio reduced on day 4, followed by a rebound thereafter in 3 groups. However, the frequency of $CD3^-CD19^+$ cells reached the nadir on day 10 though it was significantly depressed after CTX treatment. The influence of CTX on lymphocyte subpopulations was generally dose-dependent (Fig. 1–5).

4. Discussion

CTX is a prodrug that is bioactivated to produce phosphoramide mustard and acrolein and then alkylates DNA and proteins. The plasma peak levels of CTX metabolites arrive within 2–3 h, and the half-life is 6–9 h after an intravenous dose [12]. The urinary elimination of CTX and its metabolites accomplishes substantially 24 h after the start of treatment [3]. CTX is most toxic to rapidly proliferating tissue such as hematopoietic system, gastrointestinal epithelia, hair follicles and genital glands. Human WBC counts commenced to reduce on day 6, reached nadir between 9 and 12 days, and recovered on day 15 after a single dose of intravenous CTX [3].

In contrast to that in humans, the dynamic change of WBC and lymphocyte counts in rodents is different. After a 7-day treatment with intramuscular injection of CTX (20 mg/kg), peripheral blood lymphocytes decreased by approximately 33% in guinea pigs [13]. A single intraperitoneal injection of 4 mg CTX yielded rapid lymphopenia on days 1–5 in the peripheral blood, spleen, and bone marrow of mice. The lymphopenic recovery in spleen and bone marrow was seen on day 6 post-CTX treatment, but lymphodepletion in peripheral blood was remarkably prolonged and hardly reached normal by day 18 [14]. Daily doses of intraperitoneal CTX (50 mg/kg) reduced the peripheral WBC count by ~85% prior to infection in CD-1 mice [6]. The Balb/c and Swiss Webster mice received 2 intraperitoneal injections of CTX (75–150 mg/kg) 4 days and 1 day prior to infection were neutropenic within 4 days of the first injection and for 4 days after the booster dose [7,8]. Half of CD1 mice showed leukopenia by intraperitoneal injections of CTX (150 mg/kg) 3 successive days

Table 1
Effect of CTX on WBCs at 5 time-points in all groups (mean ± SD).

Groups	*n*	WBCs (10^9/L)				
		0 d	1 d	4 d	10 d	17 d
CTX50	7	7.97 ± 1.90	2.53 ± 0.25$^{\#}$	3.50 ± 0.59$^{\#}$	4.06 ± 1.39$^{\#}$	2.67 ± 0.63$^{\#}$
CTX75	7	9.51 ± 3.78	2.27 ± 0.52$^{\#}$	2.21 ± 0.56$^{\#a}$	4.73 ± 1.10	3.39 ± 1.12$^{\#}$
CTX100	7	6.39 ± 1.82	1.94 ± 0.63$^{\#a}$	1.94 ± 0.78$^{\#a}$	8.67 ± 5.03^{ab}	3.13 ± 0.86$^{\#}$
CTX125	7*	7.26 ± 2.41	1.84 ± 0.70$^{\#a}$	1.39 ± 0.49$^{\#a}$	7.73 ± 3.55^{ab}	5.05 ± 1.82^{abc}
CTX150	7	7.46 ± 2.20	1.19 ± 0.47$^{\#abcd}$	1.00 ± 0.46$^{\#ab}$	6.26 ± 2.67	3.16 ± 0.9$^{\#d}$
CTX175	7*	7.87 ± 2.96	1.01 ± 0.56$^{\#abcd}$	1.01 ± 0.44$^{\#ab}$	8.56 ± 1.37^{ab}	3.82 ± 1.02
CTX200	7*	7.14 ± 1.73	0.61 ± 0.44$^{\#abcde}$	0.40 ± 0.43$^{\#abcd}$	5.00 ± 1.30^{cf}	4.44 ± 1.43^{a}

* There were 6, 5 and 5 mice in CTX125, CTX175 and CTX200 groups on day 17, respectively.
$^{\#}$ $P<0.05$ when compared with day 0.
^{a,b,c,d,e,f} $P<0.05$ when compared with CTX50, CTX75, CTX100, CTX125, CTX150 and CTX175 groups.

Table 2
Effect of CTX on lymphocytes at 5 time-points in all groups (mean ± SD).

Groups	*n*	Lymphocytes (10^9/L)				
		0 d	1 d	4 d	10 d	17 d
CTX50	7	6.29 ± 2.29	1.86 ± 0.41$^{\#}$	2.37 ± 0.34$^{\#}$	2.01 ± 0.75$^{\#}$	1.50 ± 0.37$^{\#}$
CTX75	7	8.14 ± 3.57	1.71 ± 0.47$^{\#}$	1.46 ± 0.16$^{\#a}$	2.40 ± 1.26$^{\#}$	1.99 ± 0.80$^{\#}$
CTX100	7	5.29 ± 1.68	1.56 ± 0.48$^{\#}$	1.34 ± 0.53$^{\#a}$	4.74 ± 3.22^{ab}	1.94 ± 0.55$^{\#}$
CTX125	7*	5.93 ± 2.33	1.21 ± 0.27$^{\#ab}$	0.49 ± 0.61$^{\#a}$	4.51 ± 1.98^{ab}	2.68 ± 1.09^{a}
CTX150	7	6.21 ± 2.06	0.86 ± 0.25$^{\#abc}$	0.56 ± 0.54$^{\#a}$	3.56 ± 1.10	2.03 ± 0.91$^{\#}$
CTX175	7*	6.66 ± 2.75	0.70 ± 0.36$^{\#abcd}$	0.29 ± 0.36$^{\#abc}$	4.63 ± 0.99^{ab}	2.10 ± 0.68$^{\#}$
CTX200	7*	5.97 ± 1.60	0.34 ± 0.29$^{\#abcde}$	0.00 ± 0.00	2.84 ± 0.87$^{\#c}$	2.54 ± 0.88$^{\#a}$

* There were 6, 5 and 5 mice in CTX125, CTX175 and CTX200 groups on day 17, respectively.
$^{\#}$ $P<0.05$ when compared with day 0.
^{a,b,c,d,e} $P<0.05$ when compared with CTX50, CTX75, CTX100, CTX125 and CTX150 groups.

before and 1 day after the bacterial challenge [9]. Subcutaneous injection of CTX 4 days before infection (150 mg/kg), 1 day before infection (100 mg/kg), and 2 days after infection (100 mg/kg) produced neutropenia for 4 days in ICR/Swiss mice [10]. Intravenous injection of CTX (200 mg/kg) 3 days prior to infection resulted in a state of profound neutropenia 3 days after administration in CD1 mice, but WBC count began to recover 4 days after this nadir [11].

In this study, we adopt the intraperitoneal administration of CTX because the intraperitoneal injection is most common in mouse immunocompromised models and the immunosuppressive results can be easily compared, though the intravenous and subcutaneous routes are possibly more reliable. Meanwhile, differential count of neutrophils had to be discarded because it was impossible when WBC count was $\leq 3.0\times10^9$/L. We found that the first CTX injection of 50–200 mg/kg immediately lead to the decrease of WBC and lymphocyte counts in Balb/c mice, and the booster dose enhanced the depression. They arrived at nadir on days 1–4 after CTX injection, recovered partly on day 10, and fell again on day 17. The inhibition of WBCs and lymphocytes was related to CTX doses tested. These results are similar to the previous studies of experimental animal models. The partial recovery of peripheral leucopenia and lymphopenia may be related to the mobilization of hematopoietic progenitor cells (HPCs), because CTX can increase circulating HPCs by affecting the distribution of primitive hematopoietic stem cell and more mature progenitor cell subpopulations in the marrow and blood of mouse [15]. A single intraperitoneal injection of 4 mg/kg CTX caused a significant decline of peripheral blood stem cells (PBSCs) on day 4 in C57BL/6 mice, and a progressive increase of PBSCs was shown on days 4–10 [16]. However, it is unclear why WBC and lymphocyte counts reduce again. Following the administration of a maximum tolerated, split-dose chemotherapy protocol of cyclophosphamide, cisplatin, and carmustine, polymorphonuclear neutrophils were found first to reconstitute the host while lymphocytes returned to normal levels by 30–60 days in Balb/c-ANN mice. It is suggested that there could be a prolonged secondary impairment of the lymphopoietic system in this model [17]. Therefore, we speculate that the after effect of CTX-induced myeloid inhibition might be implicated in the repeated decrease of WBC and lymphocyte counts. In addition, 5 mice could die of hemorrhagic shock other than severe immunosuppressionon because their deaths occurred immediately after blood collection and on day 10 post-CTX injection in different groups.

Host defense mechanism against fungi includes innate and adaptive immunity [18]. Adaptive immunity is very important though innate immunity is a primary host-defense mechanism [1]. It is now accepted that cell-mediated immunity (CMI) is pivotal in determining resistance or susceptibility to fungal infections, but certain types of antibody response are protective [18]. In order to further understand the effect of CTX on the CMI and humoral immunity, the dynamic change of lymphocyte subsets was then examined by flow cytometry. On the selection of CTX doses and blood collection timepoints, the following factors were taken into account: (1) lymphocyte count was null 4 days after administration of 200 mg/kg CTX; (2) there were generally no significant differences in WBC and lymphocyte counts between 2 adjacent groups; (3) WBC and lymphocyte counts generally reached the nadir on day 4.

The suppressive effects of CTX on lymphocyte subpopulations of lymphoid organs in rodents have been widely investigated, but there is little information about changes of peripheral blood lymphocyte

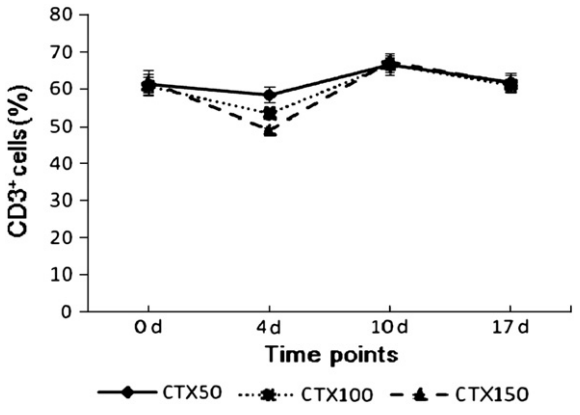

Fig. 1. Dynamics of the percentage of $CD3^+$ cells following treatment with 50, 100 and 150 mg/kg CTX at 4 time-points. The percentage of $CD3^+$ cells decreased on day 4 in CTX100 and CTX150 groups ($P<0.05$), followed by a rebound thereafter in 3 groups. Data are expressed as mean ± SEM, and obtained from groups of 8 mice.

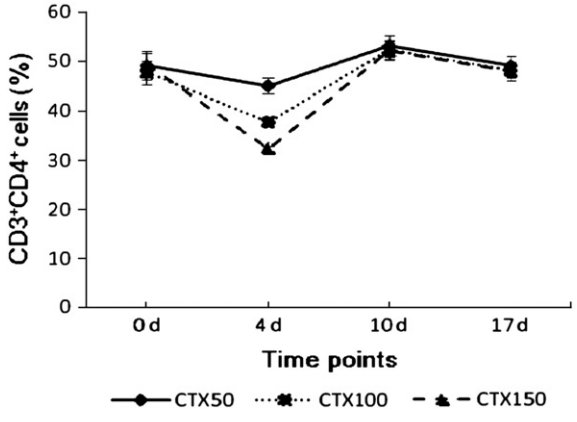

Fig. 2. Dynamics of the percentage of $CD3^+CD4^+$ cells following treatment with 50, 100 and 150 mg/kg CTX at 4 time-points. The percentage of $CD4^+$ cells decreased on day 4 in CTX100 and CTX150 groups ($P<0.05$), especially in CTX150 group, followed by a rebound thereafter in 3 groups. Data are expressed as mean ± SEM, and obtained from groups of 8 mice.

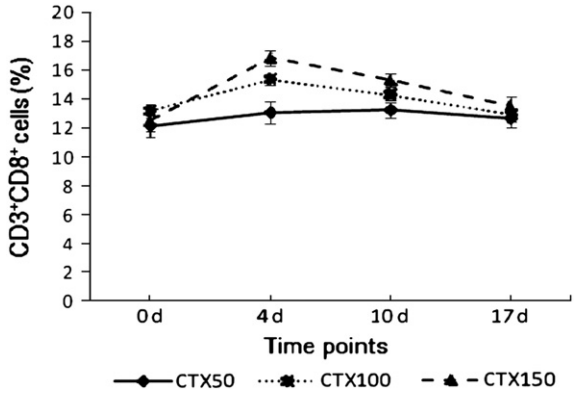

Fig. 3. Dynamics of the percentage of $CD3^+CD8^+$ cells following treatment with 50, 100 and 150 mg/kg CTX at 4 time-points. The frequency of $CD8^+$ cells showed no dynamic change in CTX50 group, but its initial increase was followed by a return to normal values in CTX100 and CTX150 groups. The relative number of $CD8^+$ cells in CTX150 group was superior to that in CTX50 group ($P<0.05$). Data are expressed as mean ± SEM, and obtained from groups of 8 mice.

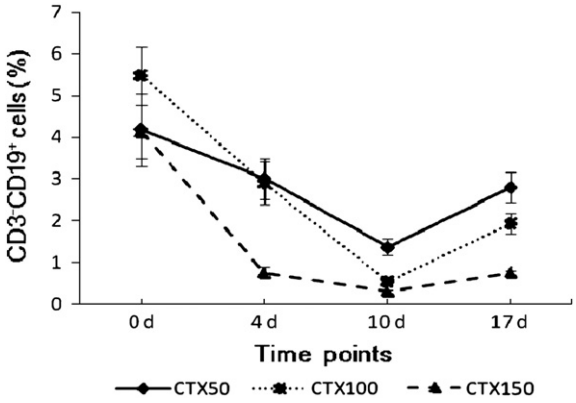

Fig. 5. Dynamics of the percentage of $CD3^-CD19^+$ cells following treatment with 50, 100 and 150 mg/kg CTX at 4 time-points. The frequency of $CD19^+$ cells showed a remarkable decrease in CTX100 group after 10 days and in CTX150 group after 4 days and a mild increase on day 17 in 3 groups. The relative number of $CD19^+$ cells in CTX150 group reduced significantly compared with other groups after 4 days ($P<0.05$). Data are expressed as mean ± SEM, and obtained from groups of 8 mice.

subsets. A single injection of 300 mg/kg CTX decreased the numbers of background Ig-secreting cells in lymphoid organs on day 7 and abolished the functional capacity of B cells on day 1, followed by a gradual recovery with a substantial overshoot peaking about 40 days [19]. Injection of 250 mg/kg CTX to DBA/2 mice decreased the counts of T and B cells in the bone marrow and spleen [20]. A single intraperitoneal injection of 200 mg/kg CTX in normal C57BL/6 mice reduced numbers of splenic $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes but did not alter the $CD4^+/CD8^+$ ratio [21]. Intraperitoneal injection of CTX (200 mg/kg) significantly reduced the number of $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes of Balb/c mice on days 3 and 5, but did not decrease their percentage [22]. The substantial loss of $CD4^+$ and $CD8^+$ subsets was observed in the spleens of C3HeB/FeJ (H-2 k) mice treated with a single dose of CTX at 100 or 200 mg/kg within 5 days, and more modest cell loss was detected in animals dosed with 5 consecutive daily injections at 20 mg/kg [23].

Our study demonstrated that two intraperitoneal injections of 50 mg/kg CTX at 2-day intervals evoked no obvious change of percentage of total ($CD3^+$), helper ($CD3^+CD4^+$), and cytotoxic ($CD3^+CD8^+$) T cells and B ($CD3^-CD19^+$) cells. However, higher doses (100 or 150 mg/kg) significantly reduced the number of $CD3^+$ and $CD4^+$ cells and elevated that of $CD8^+$ cells on day 4 with a rebound thereafter, while they induced the nadir of the frequency of $CD19^+$ cells on day 10. The $CD4^+/CD8^+$ ratio decreased on day 4, followed by a rebound thereafter when treated with 3 different doses of CTX. In line with the above-mentioned reports, these results suggest that CTX can also exert a profound depression on CMI and humoral immunity in a dose-dependent manner. The significant reduction of $CD4^+/CD8^+$ ratio is due to the decrease of $CD4^+$ cells and increase of $CD8^+$ cells. It has been shown that activated T cells are more sensitive to alkylating agents than activated B cells [24], so the nadir of $CD3^+$ and $CD4^+$ cells precedes that of $CD19^+$ cells. Considering CTX can induce apoptotic cell death in variety of tissues (including thymus), and the starting time for apoptotic induction in the thymus is 8 h after CTX exposure regardless of its doses [25], the decrease of $CD3^+$, $CD4^+$ and $CD19^+$ cells could be caused by CTX-induced apoptosis.

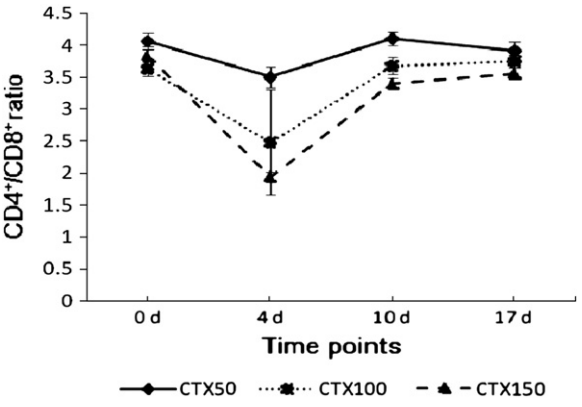

Fig. 4. Dynamics of the ratio of $CD4^+$ to $CD8^+$ cells following treatment with 50, 100 and 150 mg/kg CTX at 4 time-points. The $CD4^+/CD8^+$ ratio decreased on day 4 ($P<0.05$), followed by a rebound thereafter in 3 groups. The $CD4^+/CD8^+$ ratio was lower in CTX100 and CTX150 groups than that in CTX50 group after 4 days ($P<0.05$). Data are expressed as mean ± SEM, and obtained from groups of 8 mice.

It is well known that T cells can provide the protection against fungal infections, while recent studies can also testify the protective role of B cells in some fungal infections [1]. High doses (≥175 mg/kg) of CTX yielded the aggressive immunocomprise and general health deterioration unfavorable for fungal invasion in large amounts and long-term survival of the animals, while low doses (<150 mg/kg) resulted in a mild immunodepression unsuitable for successful inoculation. WBC and lymphocyte counts and the frequency of $CD4^+$ and $CD19^+$ cells were significantly lower in CTX150 group than those in CTX100 group after CTX treatment. The results demonstrate that the peripheral immunologic profile exhibited by 150 mg/kg CTX-treated mice could be more favorable to inoculation. Furthermore, the critical period for the development of disseminated trichosporonosis was shorter than 3 weeks after infection in immunocompromised mice, while the reimmunosuppression can reinforce the latent trichosporonemia to develop into disseminated trichosporonosis [26]. During 17-day observation period, we found that WBC and lymphocyte counts as well as percentage of $CD3^+$ and $CD4^+$ cells and $CD4^+/CD8^+$ ratio generally reached the nadir 4 days after the first CTX injection (i.e., 1 day after the second injection), suggestive of the optimal time for the inoculation of pathogenic fungi. Considering that the opportunistic fungi could enter the body by microbial inoculation when the body's immune competence is inhibited by the initial treatment with immunodepressants [26], and the natural course of fungal infection can be observed, serial boosters of CTX administration seem to be unnecessary.

In conclusion, this study shows that two intraperitoneal injections of different CTX doses (50–200 mg/kg) at 2-day intervals can produce a dose-dependent inhibition of innate and adaptive immunity in Balb/c

mice, and the booster dose can reinforce the depression. The optimal immunodepression in Balb/c mice could be induced by two intraperitoneal injections of CTX at 150 mg/kg, which may serve as a susceptible animal model for opportunistic fungus.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.intimp.2011.04.011.

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