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Full Length Research Paper

Lentinan treatment of *Plasmodium yoelii*-infected mice induces apoptosis of regulatory T cells

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To determine the immunomodulatory effects of lentinan, particularly on immune-suppressing regulatory T cells (Treg), in a mouse model of malaria, BALB/c mice were infected with *Plasmodium yoelii* by intraperitoneal (i.p.) injection of 1 × 10^6 red blood cells containing Py17XL, and the infected mice were randomized into either a control group for i.p. injection of PBS or an experiment group for i.p. injection of lentinan. The results show that mean survival was significantly longer for infected mice treated with lentinan (8.10 ± 2.53 days) than for infected controls treated with PBS (5.20 ± 1.20 days; *P*<0.01). Further, IL-12 and IFN-γ expression in spleen cells were significantly higher in the experiment group than the control group (*P*<0.01). When Treg cells were isolated by CD antibody detection from peripheral blood, a higher proportions were undergoing apoptosis in the experiment group than those in the control group (*P*<0.001). To determine the mechanism of cell death in Tregs, we analyzed Bax and Bcl-2 expression. Bax was detected at significantly higher levels, while Bcl-2 was significantly lower in Treg cells from lentinan-treated animals (P<0.001). In conclusion, lentinan significantly delayed progression of *P. yoelii* infection in mice by up-regulating anti-inflammatory cytokines and triggering apoptosis of Treg cells through up-regulation of Bax and down-regulation of Bcl-2, and which should be suggested in the clinical experience in the future.

Key words: Lentinan, *Plasmodium yoelii*, regulatory T cells, apoptosis.

INTRODUCTION

Malaria results from a parasitic infection caused by transmission of *Plasmodium* protozoa by mosquitoes. The disease affects more than 200 million people each year in tropical and subtropical regions, particularly sub-Saharan Africa, and results in more than 700,000 deaths annually (Wozniak et al., 2003). However, much of the pathogenesis of malaria is understood. One key type of immune cell, the $CD4^+CD25^+$ regulatory T cell (Treg) (Zheng et al., 2009), plays a major role in immunosuppression during malaria infection, such that other cells are unable to fight disease progression. Treg cells are a subgroup of T cells with immunomodulatory functions through inter-cell contact and secretion of pro-inflammatory cytokines TGF-β and IL-10 (Wu et al., 2010). Indeed, activation and proliferation of Treg cells in infected mice results in down-regulation of the Th1-type immune response, leading to death (Wu et al., 2007; Amante et al., 2007). Thus, these cells help to promote the pathogenesis of malaria.

Several treatments are commonly used to combat malaria; most often is quinine administration (Sahu et al., 2008). However, a compound derived from shitake mushrooms, lentinan (lent), has shown efficacy in preventing pathogenesis in *Plasmodium*-infected mice and is commonly used in Asian countries as a chemotherapeutic (Zhou et al., 2009). Indeed, lent is an immunomodulator that can effectively enhance the resistance of a host to viruses, bacteria, fungi and parasites, etc (Bisen et al., 2010). Currently, lent is believed to play the role of T-cell immune enhancer, able to activate macrophages, promote lymphocyte proliferation and induce production of interferon, interleukin or other cytokines (Xu et al., 2010). To better understand its actions in reducing the

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pathogenesis of malaria, we treated mice infected with lethal *Plasmodium yoelii* (Py17XL) with lent. We particularly assessed the effects of lent administration on Treg cells and immunosuppression.

MATERIALS AND METHODS

One hundred and twenty female BALB/c mice, 6 to 8 weeks old were provided by the Institute of Laboratory Animals, Chinese Academy of Medical Sciences. Each mouse was intraperitoneally (i.p.) injected with 1×10^6 red blood cells bearing *P. yoelii* (Py17XL). Red blood cells (RBCs) from tail-vein blood samples were stained with Giemsa for analysis and counting using standard methods; the number of infected RBCs was determined. Mice were then randomized to two groups: Controls were treated with PBS and experiments were treated with lent (1 mg/kg body weight, Sigma, USA). PBS or lent was administered 3 times/48 h by i.p. injection. From each group of mice, 20 were used for determining parasitemia and survival time, 20 were used for spleen cell culture, and the remaining 20 were used to study apoptosis of Tregs.

Spleen cell cultures

Spleens were removed by conventional aseptic methods 5 days after mice were infected. Cell suspensions were prepared with 10 ml RPMI-1640 medium containing 5% fetal calf serum. Suspensions were centrifuged at room temperature at 350 ×g for 10 min, then washed with 17 M NH_4Cl to lyse RBCs, followed by two rinses with RPMI-1640 medium. RPMI-1640 medium containing 10% fetal calf serum was used to adjust the concentration of spleen cells to 1×10^7/ml. 500 µl cell suspension were added to each well of a 24-well plate and cultured for 48 h under standard conditions.

Isolation of regulatory T cells and apoptosis detection

Two days after injection of RBCs containing Py17XL, mononuclear cells in peripheral blood from tail-vein blood samples were separated by conventional centrifugal methods. $CD4^+$T cells were separated by negative-antibody cocktail (CD19, CD123 and CD127), and Tregs were separated in combination using CD25-positive magnetic beads. Isolated Tregs were cultured in a 96-well plate, 1×10^4 cells per well.

To assess apoptosis, cells were cultured for 3 days, collected, centrifuged at 1500 rpm for 5 min, washed twice with PBS, and double-stained with Annexin V and Propidium Iodide for 10 min. Apoptosis was detected by flow cytometry within one hour of staining.

Western blotting

Western blotting was used to detect protein expression in spleen cells cultured from infected mice. Briefly, 500 µl protein lysissolution were added to each well of the 24-well plate; plate was incubated for 5 min.

Protein samples were run on SDS-PAGE at 80 V for 2 h and transferred to nitrocellulose at 0.3 A for 60 min. Nitrocellulose was rinsed with PBST, then closed with 5% PBSTM liquid (PBS with 0.05% Tween 20 and 3% milk) for 2 h. Primary antibody [goat anti-mouse Il-12 polyclonal antibody (Upstate Biotech, USA), goat anti-mouse IFN-γ polyclonal antibody (Upstate Biotech, USA), rabbit anti-mouse β-actin polyclonal antibody (Sigma, USA), murine monoclonal Bcl-2 antibody (Upstate Biotech, USA), murine Bax monoclonal antibody (Upstate Biotech, USA)] was diluted 1:400 and incubated with membrane overnight at 4°C.

Membrane was rinsed with 5% PBST, then incubated with goat anti-mouse secondary (Sigma Corporation, 1:4000) or goat anti-rabbit secondary (Sigma Corporation, 1:4000) antibody labeled with horseradish peroxidase, at room temperature for 2 h. After rinsing membrane with PBST for 15 min, DAB substrate kit (Pierce, USA) was applied. Nitrocellulose was exposed, developed and photographed. Bands were scanned and IOD values were calculated relative to β-actin.

Statistical methods

Measurement data are expressed as mean ± standard deviation ($\bar{x} \pm s$). SPSS 13.0 software was applied for analysis of variance (Dunnett's test). Alpha level equaled 0.05, with $P<0.05$ was considered statistically significant.

RESULTS

Delayed parasitemia of *Plasmodium yoelii* following lentinan treatment

Two days after injection of RBCs containing Py17XL, *P. yoelii*-infected red blood cells were detected in peripheral blood samples from both control and experiment mice (Figure 1). In control mice treated with PBS, RBC infection (parasitemia) steadily increased beginning from day 2 following infection, so that by day 6 ~60% of RBCs were infected. In contrast, parasitemia peaked for the first time in lent-treated experiment mice on day 7 following infection; a decrease in parasitemia occurred between days 7 and 9, but on day 10 more than 50% were infected. Mice in both experiment and control group died on different days, all control mice died by day 7 post-infection, however, mice in the experiment group lived as long as day 11. Mean survival time was (5.20 ± 1.20) days in the control group, but was significantly higher at 8.10 ± 2.53 days in the experiment group (t = 4.63, P<0.01).

Lentinan treatment affects IL-12 and IFN-γ protein expression in infected mouse spleens

Spleen cells were harvested 5 days after infection with *P. yoelii,* cultured for 3 days, and proteins were extracted for Western blot analysis of expression of anti-inflammatory cytokines IL-12 and IFN-γ (Figure 2).

Both IL-12 (Figure 2B) and IFN-γ (Figure 2C) were detected at significantly higher levels in spleen cells of infected mice treated with lentinan (experiment) than those treated with PBS (control group; *P*<0.01).

Lentinan treatment increases apoptosis of regulatory T cells in peripheral blood

Treg cells were isolated from peripheral blood and stained with Annexin and PI to detect the proportion of

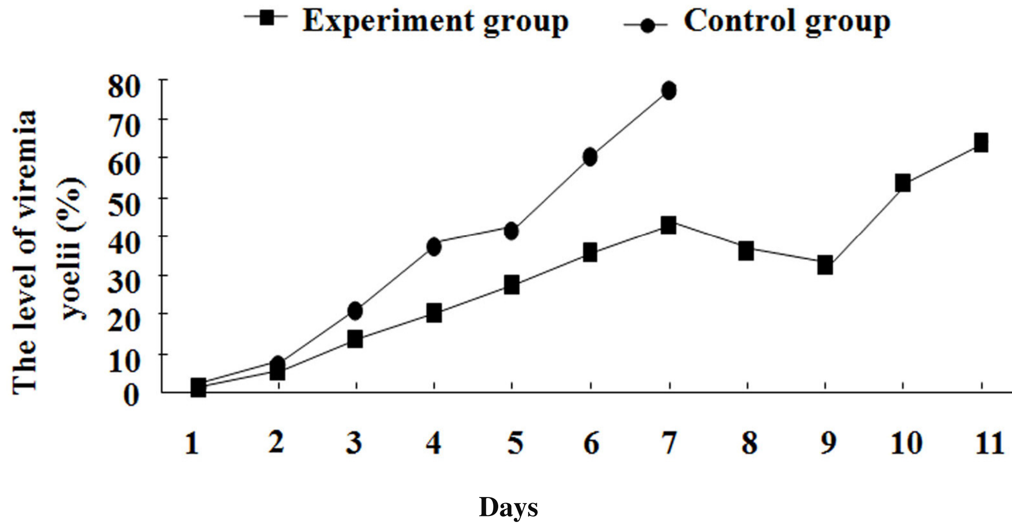

Figure 1. Percent of red blood cells infected with *P. yoelii* from the day of infection to death.

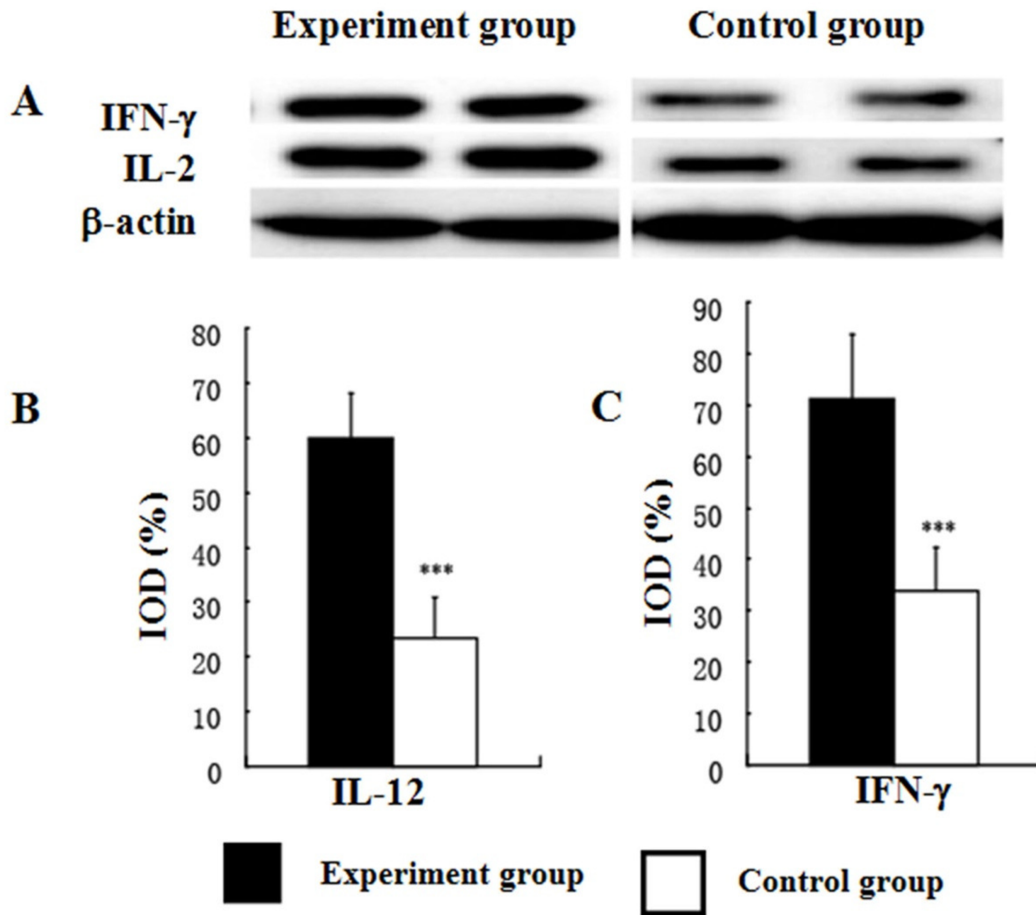

Figure 2. Effects of lent on expression levels of IL-12 and IFN-γ in spleen cells from mice infected by Py17XL. A: Western-blots of IL-12 and IFN-γ, with β-actin as a loading control; B: Comparison of relative integral optical density (IOD) values of IL-12 expression levels in spleen cells from infected mice treated with lent [experiment (black)] or not [control (white)]; C: Comparison of relative IOD values of IFN-γ expression levels in spleen cells from infected mice treated with lent or not (*** P<0.01).

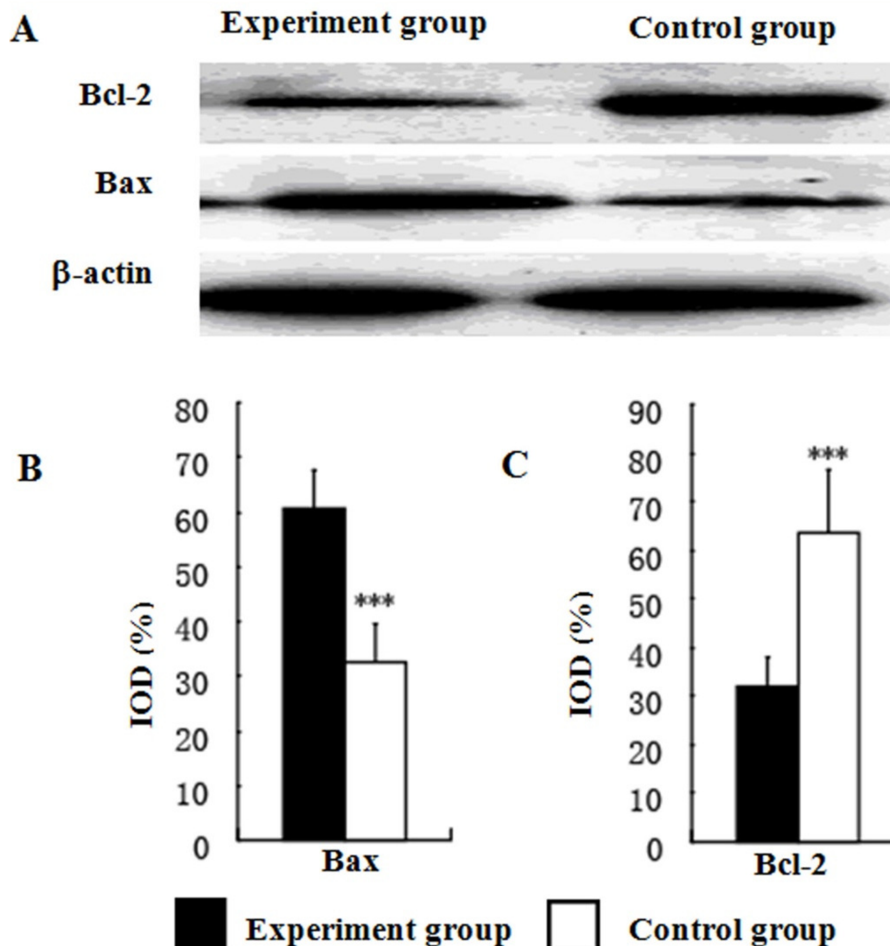

Figure 3. Lent treatment alters expression of Bcl-2 and Bax in Treg cells from infected mice. A: Western-blot analysis of Bcl-2 and Bax expression in Treg cells of infected mice. B: Comparison of relative integral optical density (IOD) values of Bax protein expression levels; C: Comparison of relative IOD values of Bcl-2 protein expression levels ($^{***}P$<0.001).

apoptotic Treg cells by flow cytometry. The proportion of apoptotic Treg cells was significantly higher in samples from animals treated with lent (35.4%) as compared to the controls treated with PBS (0.23%, *P*<0.01).

Lentinan treatment alters expression of Bcl-2 and Bax in Treg cells

To further investigate the increased death of Treg cells in lentinan-treated infected mice, we assessed expression of Bcl-2 and Bax, proteins involved in apoptosis, and in Treg cells by Western blotting (Figure 3A). Significantly higher levels of Bax were detected in experimental (lentinan-treated) Treg cells than in Treg cells from controls (*P*<0.01, Figure 3B). In contrast, Bcl-2 levels were significantly lower in Tregs from experimental mice than those from controls (*P*<0.01, Figure 3C).

DISCUSSION

Our study shows that lent treatment of *P. yoelii*-infected mice can significantly reduce the level of parasitemia and prolong survival. The mechanism behind this delayed pathogenesis appears to be through changes in immune function. Anti-inflammatory cytokines IL-12 and IFN-γ displayed significantly higher protein expression in spleen cells from infected mice treated with lent. Therefore, lent can promote Th1 immunity of infected mice by up-regulating IL-12 and IFN-γ. Indeed, previous studies reported that lent treatment increases TNF-α, IFN-γ and IL-12 protein levels in mice infected with *Listeria monocytogenes*, thereby improving the immunity of mice to this pathogen (Kupfahl et al., 2006).

To further explore the mechanism of enhanced immunity conferred by lent, specifically its effects on Treg cells, we used flow cytometry to assess apoptosis of this cell

population in peripheral blood. The proportion of Treg cells undergoing apoptosis in mice treated with lent was significantly higher than in mice not treated with lent. Additionally, Western blot analysis of apoptosis-related proteins Bcl-2 and Bax in Tregs revealed that Bcl-2 expression was significantly reduced and Bax expression was significantly increased in lent-treated mice. Bcl-2 or B-cell lymphoma leukemia gene-2, is a proto-oncogene that inhibits apoptosis through antioxidation and inhibition of transmembrane calcium flow (Skommer et al., 2010). Bax is related to Bcl-2 but is known to promote apoptosis (Cvejic et al., 2008). Some reports have indicated that Bax expression is closely related to development of malignancies; indeed, in some malignant tumors such as breast (Bachmeier et al., 2011), lung (Xu et al., 2011), bladder (Yoon et al., 2011) and colon cancers (Nehls et al., 2007), Bax expression is significantly reduced. Our results suggest that lent can promote apoptosis of Treg cells by down-regulating Bcl-2 expression levels and up-regulating Bax expression levels.

In summary, the survival time of *P. yoelii*-infected mice improves significantly upon treatment with lentinan, indicating that this compound can confer resistance to the pathogen in a mouse model of malaria. This resistance occurs through: (1) Up-regulation of IL-12 and IFN-γ, thereby triggering Th1 immunity and (2) up-regulation of BAX and down-regulation of Bcl-2, thereby triggering increased apoptosis of immunosuppressing Treg cells.

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