Pachyman treatment improves $CD4^+CD25^+$ Treg counts and serum interleukin 4 and interferon γ levels in a mouse model of Kawasaki disease

MAO-PING CHU[1], DAN WANG[1], YING-YING ZHANG[1], BAO-QING LI[3], AI-HUA ZHOU[1], XI-WEN CHEN[2] and YAN QIAN[1]

[1]Department of Pediatrics, Heart Center, the First Affiliated Hospital of Wenzhou Medical College; [2]Experimental Animal Center, Wenzhou Medical College; [3]Department of Laboratory Medicine, the Second Affiliated Hospital of Wenzhou Medical College, Wenzhou, Zhejiang 325000, P.R. China

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Abstract. The aim of this study was to explore the effect of pachyman, a mushroom extract, on $CD4^+CD25^+$ regulatory T cells (Tregs), serum interleukin 4 (IL-4) and interferon γ (IFN-γ) levels in a mouse model of Kawasaki disease. *Lactobacillus casei* cell wall extract was diluted to 1 mg/ml in PBS and administered to mice by intraperitoneal injection to establish a model of Kawasaki disease. Sixty female mice were used in this study, 40 of which were randomly assigned to a model (normal saline by gavage, n=20) or experimental group (200 mg/kg/day pachyman by gavage, n=20). The remaining 20 mice were disease and treatment-free, and were used as the control group. Compared to the control mice, mice in the model group exhibited a significantly lower percentage of $CD4^+CD25^+$ Tregs and significantly higher serum IL-4 and IFN-γ levels ($P<0.05$). However, $CD4^+CD25^+$ Tregs significantly increased and IL-4 and IFN-γ levels significantly decreased in experimental mice following pachyman treatment ($P<0.05$). Further analysis showed a negative correlation between $CD4^+CD25^+$ Tregs and IL-4/IFN-γ levels ($P<0.05$). In conclusion, pachyman improves immune function in a mouse model of Kawasaki disease by upregulating $CD4^+CD25^+$ Tregs, which may inhibit the cytokine secretion of Th_1 and Th_2 cells.

Introduction

Kawasaki disease, a vasculitis syndrome of unknown etiology, typically occurs in infants and young children and it is characterized by fever accompanied by mucocutaneous and lymph node damage. The most serious characteristic of this disease is inflammation of the arteries, which produces coronary artery lesions, including aneurysm and stenosis, and may result in acute myocardial infarction or sudden death (1). In recent years, the incidence of Kawasaki disease has risen, making it the most common acquired pediatric ischemic heart disease (2). Furthermore, one study has shown that a history of Kawasaki disease is closely associated with the development of coronary heart disease in adulthood (3). However, despite the severity of the complications of Kawasaki disease, its etiology and pathogenesis remain unclear; a better understanding of this disease may, in fact, result in better treatment paradigms.

Although known to be an autoimmune disorder, the underlying mechanism initiating Kawasaki disease pathology is controversial. One proposed mechanism is the superantigen theory, suggesting that superantigen-induced abnormal cell activation is the initiating and key step (4). By contrast, other investigators have proposed that Kawasaki disease is caused by the normal antigen-induced immune response (5). However, both hypotheses lack sufficient evidence.

The abnormal activation of immune cells in Kawasaki disease is similar to the highly activated immune system mediated by infection. The upregulation of immune response in Kawasaki disease affects multiple organ systems, but is particularly centered in the cardiovascular system. Increased macrophages, cell activation and cytokine release in the blood vessels result in inflammation that damages the vessels. Recent studies have found that children with Kawasaki disease develop excessive activation of immune function in the acute phase. Activated cells not only stimulate cloned cells to secrete multiple immunoglobulins, including autoantibodies by releasing lymphokines, but also induce further proliferation and activation of monocytes, macrophages and lymphocytes. These cells also release a variety of cytokines and immune-active molecules, further promoting inflammatory vascular injury (6).

T-cell activation was recently shown to be an important characteristic of Kawasaki disease pathogenesis. The ITPKC

Correspondence to: Dr Mao-Ping Chu, Department of Pediatrics, Heart Center, the First Affiliated Hospital of Wenzhou Medical College, No. 2 Fuxuexiang, Wenzhou, Zhejiang 325000, P.R. China
E-mail: chumpwz@126.com

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gene, a negative regulator of T-cell activation, is known to be associated with susceptibility to and severity of the disease (7). A certain type of T cells, $CD4^+CD25^+$ regulatory T cells (Tregs), are particularly significant to this disease. These cells have low immunoregulatory reactivity and immunosuppressive effects and are important for maintaining immune homeostasis (8). Recent studies have confirmed that $CD4^+CD25^+$ Tregs are important in autoimmune diseases by inhibiting cell activation and promoting the secretion of certain cytokines to inhibit the immune response of self-reactive cells (9).

While an understanding of the disease pathogenesis is undeniably important, there also exists a need for better treatment options. One compound, pachyman, a polysaccharide extracted from wild mushrooms, is commonly used in traditional Chinese medicine as an anti-inflammatory agent. Due to this apparent ability to modulate inflammation, pachyman is a potential new therapy for Kawasaki disease.

To investigate the role of $CD4^+CD25^+$ Tregs in Kawasaki disease pathogenesis, the cells in a mouse model of Kawasaki disease were assessed. Serum levels of interleukin (IL)-4 and interferon (IFN)-γ, two cytokines regulated by these cells, were also profiled. Additionally, we administered pachyman treatment to affected mice to determine its ability to alter $CD4^+CD25^+$ Tregs as well as IL-4 and IFN-γ levels.

Materials and methods

Establishment of mouse model of Kawasaki disease. Sixty female BALB/c mice, weighing 24-30 g (mean 26.3±4.6), were provided by the Wenzhou Medical College Experimental Animal Center. *Lactobacillus casei* cell wall extracts (LCWE) were prepared in 1 mg/ml PBS, according to Duong's methods (10), from dried strains (China Industrial Microbial Culture Preservation Center) commonly used in the domestic fermentation industry. Forty BALB/c mice were administered 0.5 ml LCWC by intravenous (i.v.) injection of LCWC and, 7 days later, were randomly divided into an experimental and a model group (n=20 each group). Mice in the experimental group were treated with 200 mg/kg/day pachyman (Ningbo Dekang Biological Product Co., Ltd.) via gastric lavage, whereas mice in the model group were given an equivalent volume of normal saline via gastric lavage. The remaining 20 mice (no LCWE, no pachyman) were used as controls. Fourteen days later, blood was collected retro-orbitally and treated with EDTA anticoagulant.

Determination of percentage of $CD4^+CD25^+$ Treg cells. Density gradient centrifugation was used to isolate mononuclear cells from peripheral blood, which were then diluted to a cell concentration of $1x10^6$ cells/ml. For each sample, one aliquot was placed into a positive and another into a negative control tube. PE-labeled rat anti-mouse CD4mAb (2 μl) (anti-mouse CD4-PEmAb) and FITC-labeled rat anti-mouse CD25mAb (2 μl) (anti-mouse CD25-FITCmAb; both from Becton-Dickinson, Franklin Lakes, NJ, USA), were added to the positive tube. Mononuclear cell suspension (100 μl) was then mixed in and tubes were incubated for 20 min in the dark. Subsequently, 2-3 ml normal saline was added prior to centrifugation at 1,500 rpm for 5 min. Supernatant was discarded and 100 μl cells suspended in PBS were added. Flow cytometry was used to detect the cells. Negative control tubes were detected first to determine positive and negative boundaries. Positive tubes were then detected to calculate the percentage of $CD4^+CD25^+$ Treg among total $CD4^+$ cells.

Determination of IL-4 and IFN-γ levels. Serum was isolated and ELISA was used to determine IL-4 and IFN-γ levels, according to the manufacturer's instructions (eBioscience, Germany).

Statistical analysis. SPSS 13.0 statistical software was used for statistical analysis. Data are shown as the means ± standard deviation ($\bar{\chi} \pm s$). Single-factor analysis of variance was used to compare $CD4^+CD25^+$ Treg percentages and serum IL-4 and IFN-γ levels among groups, as was the inter-group pairwise comparison (SNK). Pearson's correlation test was used to analyze the relationship between $CD4^+CD25^+$ Treg and IL-4 and IFN-γ levels in the serum. Tests were two-sided, with α level of 0.05, and $P<0.05$ was considered to indicate a statistically significant result.

Results

Altered percentages of $CD4^+CD25^+$ Treg cells. Mononuclear cells were isolated from the blood samples of control mice (disease and treatment-free) or those with Kawasaki disease (model: saline-only treatment; experimental: pachyman treatment). Flow cytometry of isolated mononuclear cells detected differences in the percentages of $CD4^+CD25^+$ Treg cells (Fig. 1). Mice from the model group had an average of 4.77±1.11%, while mice from the experimental group had an average of 6.84±1.31%, and mice from the control group had an average of 7.85±1.71% (Fig. 2). These differences were statistically significant among the groups ($F=25.290$, $P=0.001$). The $CD4^+CD25^+$ Treg percentage in the model group was found to be significantly lower compared to the experimental group ($P<0.05$), while the $CD4^+CD25^+$ Treg percentage in the experimental group was significantly lower than that in the control group ($P<0.05$).

Altered serum IL-4 and IFN-γ levels. Since $CD4^+CD25^+$ Treg cells are involved in immunity and, therefore, regulate certain cytokines, we investigated whether the altered proportion of $CD4^+CD25^+$ Treg cells in mice with Kawasaki disease, and particularly those treated with pachyman, also corresponded to an altered expression of cytokines IL-4 and IFN-γ. Using ELISA, we detected serum levels of the two cytokines in the 3 groups. Mean serum IL-4 levels were 16.93±4.04 pg/ml in the model group, 13.76±3.19 pg/ml in the experimental group and 7.84±1.99 pg/ml in the control group (Fig. 3). Similarly, serum IFN-γ levels were 27.06±7.19, 20.89±6.43 and 13.61±2.80 pg/ml for the model, experimental and control groups, respectively (Fig. 4). These differences in serum IL-4 and IFN-γ levels among the groups were statistically significant ($F=42.001$, $P=0.001$; $F=26.895$, $P=0.001$, respectively). As expected, serum IL-4 and IFN-γ levels were significantly lower in the model group compared to the experimental group ($P<0.05$); similarly, serum IL-4 and IFN-γ levels in the experimental group were significantly lower than those in the control group ($P<0.05$).

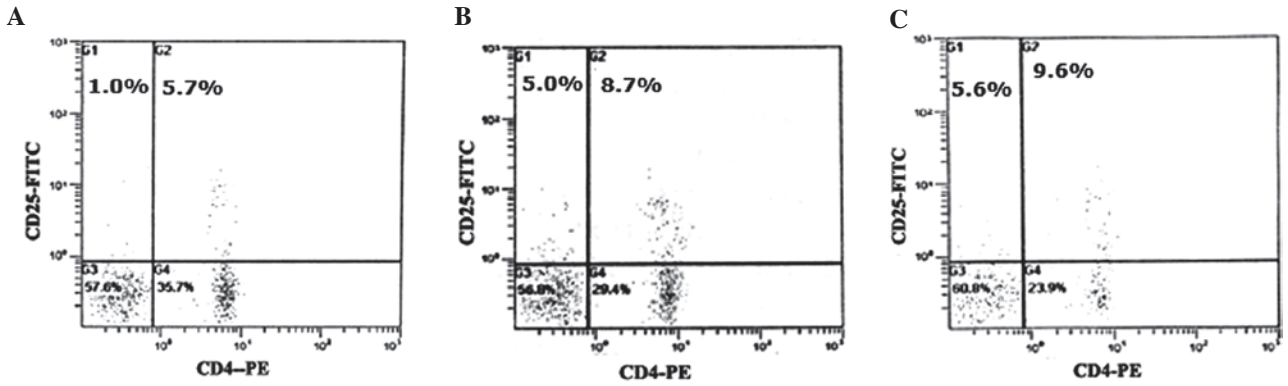

Figure 1. Identification of $CD4^+CD25^+$ Treg cells in mouse models of Kawasaki disease by flow cytometry. (A) Model group; (B) experimental group; (C) control group.

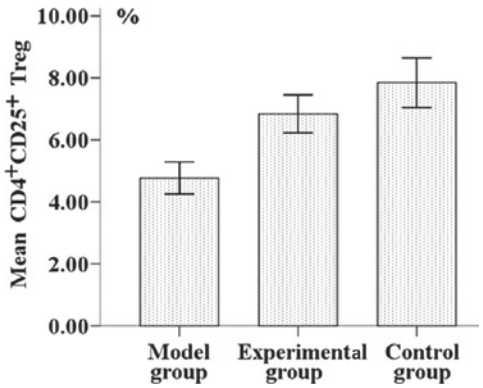

Figure 2. Percentage of $CD4^+CD25^+$ Treg cells from total $CD4^+$ cells in blood of mouse models of Kawasaki disease.

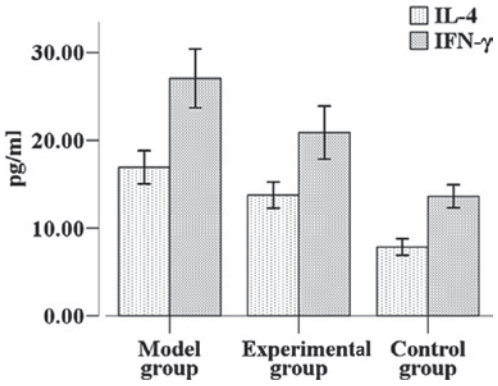

Figure 3. Serum IL-4 and IFN-γ levels detected by ELISA in mouse models of Kawasaki disease.

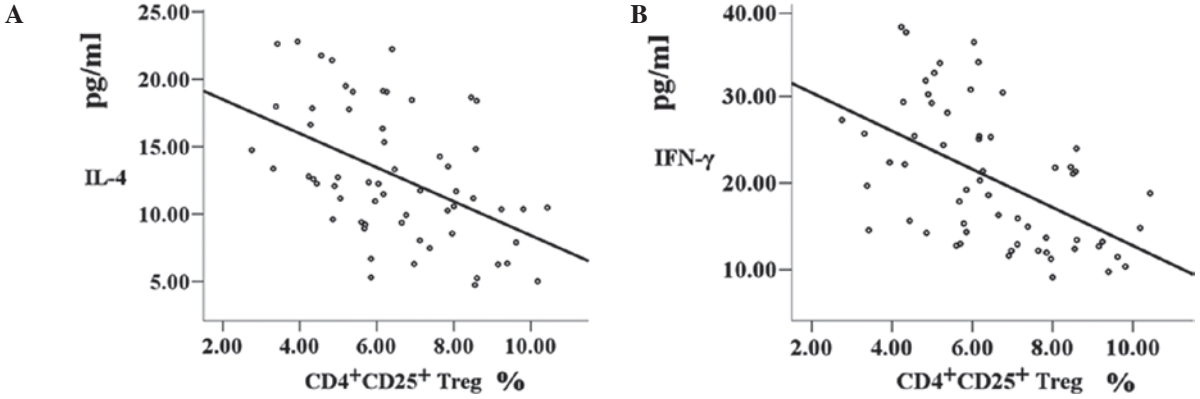

Figure 4. Correlation between the percentage of $CD4^+CD25^+$ Treg cells and (A) IL-4 and (B) IFN-γ levels.

Percentage of $CD4^+CD25^+$ Treg cells correlates with IL-4 and IFN-γ levels. To determine the relationship between the altered percentages of $CD4^+CD25^+$ Treg cells and changes in cytokine expression, we analyzed the correlation between these phenomena. The $CD4^+CD25^+$ Treg percentage in the blood of the mice was 6.48±1.89%, and the serum IL-4 and IFN-γ levels were 12.84±4.92 and 20.52±7.95 pg/ml, respectively. Statistical analysis revealed a negative correlation between blood $CD4^+CD25^+$ Treg cells and serum IL-4 and IFN-γ levels, with correlation coefficients (r) of -0.483 and -0.524, respectively ($P<0.05$ for both).

Discussion

$CD4^+CD25^+$ Tregs are a subset of T cells with immunoregulatory, immune incompetence and immunosuppressive activities. These cells regulate immune responses and are important in maintaining a stable internal environment in the body.

Furthermore, $CD4^+CD25^+$ Treg cells are involved in autoimmune diseases, organ transplant rejection, cancer, allergy and microbial infection (11-14). In the present study, we showed that the percentage of $CD4^+CD25^+$ Treg cells in the blood of mice with Kawasaki disease is significantly lower than that in the blood of healthy mice. This finding indicates an abnormality in the number of $CD4^+CD25^+$ Tregs produced in Kawasaki disease, consistent with altered $CD4^+CD25^+$ Treg cells in the peripheral blood of patients with Kawasaki disease (15).

Previous studies have reported that patients with Kawasaki disease exhibit significantly higher serum levels of IL-4 and IFN-γ, Th_1 and Th_2 cytokines. These levels decrease in the recovery period, possibly resulting from activated Th_1 and Th_2 (16,17). Similarly, we found that serum IL-4 and IFN-γ levels in a mouse model of Kawasaki disease are significantly higher compared to those of healthy mice. This finding suggests dysfunction of Th_1 and Th_2 cells in Kawasaki disease, leading to abnormal secretion of cytokines. The potential mechanism behind this dysfunction is as follows: $CD4^+CD25^+$ Treg may inhibit the differentiation and function of Th_1 and Th_2 cells by cell contact approaches, or inhibit proliferation of effector T cells and secrete cytokines through direct cell-cell contact and/or cytokine function (18). Our data showed a negative correlation between the two parameters; the percentage of $CD4^+$ cells that are $CD4^+CD25^+$ Treg cells is negatively correlated with serum IL-4 and IFN-γ levels, consistent with the hypothesis.

Pachyman is the main chemical ingredient of the traditional Chinese medicine Poria cocos, accounting for 70-90% of its weight, and is mainly composed of linear β (1-3)-D-glucan (19). Pachyman has various biological activities, including the ability to improve non-specific immune system function, significantly enhance phagocytic function of mouse peritoneal macrophages and increase the killing activity of NK cells. This compound also improves specific immune system function, enhances mouse immunoreactivity to body fluid that can sensitize erythrocytes, and promotes proliferation of spleen lymphocytes and the mixed lymphocyte reaction in mice. In this study, we investigated whether the immune properties of pachyman altered the immune response in a mouse model of Kawasaki disease. Administration of pachyman via gastric lavage significantly increased the percentage of $CD4^+CD25^+$ Tregs in the blood of mice with Kawasaki disease compared to those with the disease, but treated with saline only. Furthermore, serum IL-4 and IFN-γ levels significantly decreased, trending toward the levels detected in healthy controls. These changes may stem from pachyman activating $CD4^+CD25^+$ Treg cells, thereby inhibiting the secretion of IL-4, IFN-γ and possibly other cytokines by Th_1 and Th_2 cells. Expression of MHC II molecules may also be regulated, thus enhancing the body's immunocompetence and improving immune function.

In conclusion, $CD4^+CD25^+$ Treg cells is potentially important in the development of Kawasaki disease, by regulating the state of Th_1 and Th_2 cells. This regulation appears to result in an abnormal secretion of the cytokines, indicating a significant possible approach to a better understanding of the pathogenesis of Kawasaki disease. Treatment with pachyman improves blood $CD4^+CD25^+$ Treg percentages in mice with Kawasaki disease, inhibiting secretion of Th_1 and Th_2 cytokines and improving immune function.

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