Effect of Tai Chi Exercise on Immune Function in Middle-aged and Elderly Women

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Abstract

Objective: The purpose of this study was to investigate the effect of Tai Chi (TC) exercise on immune functions among middle-aged and elderly women, and to evaluate the relationship between immune modulation of Natural Killer T (NKT) cells and Dendritic Cells (DCs) and T Helper (Th) 1/Th2 immune response.

Methods: Sixty healthy middle-aged and elderly women were randomly assigned into 2 groups: Tai Chi (TC) group (n=30) and control (CON) group (n=30). Subjects in TC group participated in TC exercise for 6 months, 60 minutes a day, four times a week. Meanwhile, subjects in CON group maintained their normal physical activity levels during the whole study period. Peripheral blood samples were collected right before, and after 4 and 6 months of the exercise program, and the samples were analyzed within 24 hours after collection.

Results: After the 6-month TC exercise program, the percentage of CD4+ T lymphocytes, the CD4+CD8+ ratio, and the percentage of NK and NKT cells in TC group significantly increased (p<0.05). The percentage of interferon-γ (IFN-γ) producing T cells increased significantly after 4 months (p<0.01) and 6 months (p<0.05) of exercise. The percentage of interferon-4 (IL-4) producing T lymphocytes also demonstrated an increase after 4 months (p<0.05) and 6 months (p>0.05) of exercise. The percentage of CD123+ DCs and CD11c+ DCs also significantly increased after the 6-month program (p<0.01), with the percentage of CD11c+ cells increasing much more dramatically than CD123+ DCs. However, the CON group did not show any significant changes in these parameters.

Conclusion: Regular TC exercise favors the development of Th1 immune responses in middle-aged and elderly women. TC-induced changes in Th1 and Th2 immune responses are associated with the immune modulation of NKT cells and DCs and their reciprocal interactions.

Keywords: Physical activity; Immunology; Immunosenescence; Aging; Female

Introduction

Immunosenescence is the steady degeneration of the immune system that occurs with age in humans and animals [1]. Multiple aspects of the immune response are severely disregulated by aging, among which the T cell immune response is the most dramatically affected. T cell-mediated production of cytokines Interleukin (IL)-2 and Interferon (IFN)-γ decreases significantly with age. The amount of IFN-γ decreases along with the activity of T Helper 1 (Th1) cells and the amount of IL-4 and IL-10 increases along with the activity of T Helper 2 (Th2) cells [2,3], resulting in a decreasing ratio of Th1/Th2 in elderly people. The decrease in the numbers of Natural Killer (NK) cells and Natural Killer T (NKT) cells contributes to the deleterious immune response in the elderly, and the number of myeloid Dendritic Cells (DCs) also progressively declines with age [4].

Aging is also associated with decreased physical activity. Exercise can profoundly influence health, and there is mounting evidence that the mechanisms behind these effects are related, in part, to the impact of exercise on immune function [5]. It has been shown that the numbers of circulating T cells and their functions are influenced by brief periods of exercise [6,7]. Hinton et al. [8] reported significant changes in the composition of the total lymphocyte cultures immediately post-exercise: increased numbers of CD56+ NK cells and CD8+ T cells and decreased numbers of T-helper cells (CD4+), in male endurance-trained runners after an interval running session of 15x1-min intervals at 95% VO2 max. A decrease in mitogen-stimulated T cell proliferation and T cell production of IL-2 and IFN-γ was reported immediately after acute, intensive exercise [9]. In fact, the volume of exercise has been shown to be a critical element in inducing a positive or negative effect on the immune response [5,10]. It has been shown that moderate exercise enhances T cell function and decreases respiratory infections [11]. Although many components of the immune system are known to exhibit various changes depending on the stage and type of exercise, the long-term effects of regular moderate exercise on immune responses remain incompletely understood.

Tai Chi (TC) is a traditional Chinese martial art with a long history. It usually consists of TC Chuan and TC Weapon (for example, TC Sword, TC Spear, TC Broadsword). TC has gained popularity in Western countries in recent years for its recognized positive effects on health in the elderly [12]. TC incorporates slow-moving, gentle physical activity, balance, and weight shifting, with meditation, relaxation, deep
breathing, and imagination. Reported benefits of TC include increased balance and decreased incidence of falls, increased strength and flexibility, reduced pain and anxiety, improved self-efficacy, improved sleep, and enhanced cardiopulmonary function [12]. TC has also been shown to significantly increase the ratio of CD4+ vs. CD8+, significantly increase CD4+/CD25+ regulatory T cell number [13] reduce the levels of HbA1c in type II diabetes and enhance the Th1 response [14] and increase varicella-zoster virus specific cell-mediated immunity [15], significantly increased the numbers and activity of natural killer cells in the peripheral blood [3]. To our knowledge, there haven't been any studies as to whether TC could promote the amelioration of the age-induced Th1/Th2 immune imbalance.

The purpose of this study was to investigate the effect of TC on the subsets of T cells, NK cells, NKT cells and DCs, and the cytokines IFN-γ and IL-4 producing T cells, in aged 50–65 women who had practiced TC for six months. Our hypothesis was that TC induces a Th1-type immune response in elderly people, ameliorating the age-induced immune imbalance. By evaluating the relationships between TC exercise and T lymphocyte immune function, we sought to elucidate the role that TC exercise plays in reversing the age-associated imunosenescence.

**Materials and Methods**

**Subjects**

Sixty women, aged 50–65, were recruited by advertising for the study, and randomly divided into 2 groups: Tai Chi (TC) exercise group (n=30) and non-exercise control (CON) group (n=30). Table 1 shows the physical characteristics of the study subjects, and both groups were of similar age and body composition. Body mass and Body Mass Index (BMI) did not demonstrate any significant changes during the whole study period in either TC group or CON group. Exclusion criteria included angiocardiopathy (cardiopulmonary illness), autoimmune disorder, metabolic disorder, malignancies actively being treated with chemotherapy, hormone replacements (such as the use of corticosteroids), and acute illness from infection, and major surgery during the preceding 6 months. All subjects had no TC training experience before. Written informed consent was obtained from all participants upon enrollment, and the protocol was reviewed and approved by the institutional review board of Shanghai University of Sport. The subjects in the CON group were instructed not to participate in any formal exercise but simply to maintain their normal levels of physical activity as usual for the whole 6-month study period, and they kept diaries to record their daily activities.

**TC exercise program**

All subjects in TC group participated in TC Chuan (24 forms;
Supplementary figure 1 for TC techniques) and TC Sword (42 forms) exercise for 6 months, 4 sessions a week. They were supervised and instructed by an experienced TC teacher who has practiced TC for 18 years. Each TC session lasted 60 minutes consisting of a 10 minutes warm up, a 40 minutes practice, and a 10 minutes cool down. The subjects in TC group spent 3 months in learning and practicing all forms of TC Chuan and TC Sword, and then continued to practice for another 3 months. All TC subjects kept diaries to record details.

Blood sampling and analysis

**Blood sampling:** At the onset, 4-month, and 6-month during the study period, peripheral venous blood (5 ml) of each subject in both groups was collected in an anticoagulant heparin tube. Blood samples were analyzed within 24 hours of collection. Blood was collected between 7 am–9 am in the morning after a 12 hours overnight fast (only water was allowed). On the day of blood collection, subjects did not carry out any TC exercise. All subjects refrained from any exercise for at least 24 hours before the blood collection/sampling.

**Analysis of T cell subset, NKT cells, and NK cells**

**Flow cytometric analysis for T cells:** Peripheral blood (100 µl) was incubated with anti-human CD4-fluorescein isothiocyanate (FITC), anti-human CD8-phycocerythrin (PE), as well as anti-human CD3-peridinin chlorophyll protein (PerCP)-Cy5 antibodies (Immunotech Co.) in the dark for 15 minutes. Then haemolysis fixative agent (0.5 ml) (Beckman Coulter) was added for another 10 minutes, followed by the addition of Isoton II diluent (0.5 ml) (Beckman Coulter). CD3+, CD4+, and CD8+ cells were measured using fluorescence detection by flow cytometry.

**Flow cytometric analysis for NK/NKT cells:** Peripheral blood (100 µl) was stained with anti-human CD3-FITC, anti-human CD16-PE, and anti-human CD56-PE antibodies (Beckman Coulter, California, USA). After staining, red blood cells were lysed with OptiLyse C Lysing Solution (Beckman Coulter). Finally, cells were analyzed using a Coulter EPICS XL Flow Cytometer (Beckman Coulter) with System IITM software. NK cells were defined as CD3 CD16 CD56 cells, and NKT as CD3 CD16 CD56 cells [16].

**Analysis of IFN-γ and IL-4 producing CD3+CD4+ T cells**

Ten µl of PMA (1 µg/ml), 8 µl of ionomycin (50 µg/ml) and 6.8 µl of monensin fluid (0.1 mg/ml; Sigma Chemical Co.) were added to peripheral blood (200 µl) along with RPMI-1640 media (200 µl), and incubated for 4 to 6 hours at 37°C in a 5% CO2 incubator. Monoclonal anti-CD3-PerCP (20 µl) and anti-CD4-FITC antibodies (20 µl; Beckman Coulter) were then added and the mixture was incubated for another 15 minutes. After the incubation, cell suspension of each sample was divided into three 100-l aliquots, and all samples were treated with fixation/permeabilization reagents according to the manufacturer's instructions (Beckman Coulter). Intracellular cytokines in each aliquot were stained using PE-conjugated mAbs against IL-4, IFN-γ, or an isotype control (Beckman Coulter) for 30 minutes. The samples were washed with PBS and the supernatant was discarded. The cell pellet was resuspended in PBS and analyzed by flow cytometry. All incubations (except PMA-activation and incubation) were performed at room temperature in the dark.

**Analysis of CD123+ and CD11c+ dendritic cells by direct immunofluorescence staining of whole blood**

**Monoclonal antibodies:** PE-conjugated anti–IL-3 receptor α chain (CD123), PE-conjugated anti–CD11c, PerCP-conjugated anti–HLA-DR, and FITC-conjugated lineage cocktail 1 (lin 1), as well as PE- and PerCP-conjugated isotype control murine mAbs were obtained from BD (San Jose, CA). The lin 1 contains monoclonal antibody clones against CD3 (T cells), CD14 (monocytes/macrophages), CD16 (natural killer cells), CD19 (B cells), CD20 (B cells), and CD56 (natural killer cells). Sensitive detection of DCs was achieved by exclusion of cells positive for CD3, CD14, CD16, CD19, CD20, or CD56 using Lineage Cocktail 1 (FITC-conjugated Ab mixture against CD3, CD14, CD16, CD19, CD20, and CD56). Thereafter, myeloid and lymphoid DCs (DC1s and DC2s, respectively) were detected using PE-conjugated Ab against CD11c and CD123, respectively.

**Flow cytometric analysis:** Peripheral blood cells obtained from the subjects were analyzed by three-color flow cytometry. To minimize selective cell loss during the preparation procedure, the cells were first stained with mAbs followed by lysing the erythrocytes. Briefly, aliquots (100 µl) of peripheral blood were incubated with a mixture of anti-lin mAbs (BD Biosciences), a PerCP-conjugated anti–HLA-DR mAb, and either a PE-conjugated anti-CD11c mAb to detect myeloid DC or a PE-conjugated anti-CD123 mAb to detect plasmacytoid DC, or a PE-conjugated isotype control (BD Biosciences) in the dark at room temperature for 15 min (Supplementary table 1). Then 2 ml of FACS lysing solution (BD) was added, vortexed, and incubated for 10 minutes to lyse the erythrocytes. The mixture was centrifuged at 300×g for 5 minutes, and the supernatant was discarded. The mixture was then vortexed gently with 1 ml of wash buffer, centrifuged again as described above, and the supernatant was discarded. Thereafter, the mixture was vortexed and resuspended in 300 µl of 1% paraformaldehyde for 10 min, and then washed and analyzed on a flow cytometer. Identical flow cytometric settings were used for the acquisition of all samples enabling the expression of cell surface molecules on different samples to be directly compared. All incubations were performed at room temperature in the dark. DCs were defined as the cells positive for PerCP-conjugated anti–HLA-DR mAb and negative for FITC-conjugated lin 1. Anti-CD11c or anti-CD123 mAb conjugated with PE was used for further identification of the mDC and pDC subsets.

**Statistical analysis**

All data were expressed as means ± SEMs, and p<0.05 was set as the criterion for significance. The Statistical Package for Social Sciences (SPSS Inc, USA; version 12.0) was used for statistical analysis. Data were analyzed using one-way Analysis Of Variance (ANOVA), and the independent samples t-tests were applied to assess differences between control group and TC group before exercise program. Paired-samples t-tests were only used to assess differences of variables between pre-exercise and 4-month, as well as between pre-exercise and 6-month.

**Results**

**Effect of TC exercise on T lymphocytes and their subsets**

As shown in Figure 1, the percentages of T lymphocytes (CD3+) and their subsets (CD4+ and CD8+) in the peripheral blood before exercise program (at onset) did not show any significant inter-group differences between CON and TC groups. Six-month TC exercise program significantly increased the percentage of CD4+ T (Th) lymphocytes and the CD4+:CD8+ ratio (p<0.05; Figures 1B and 1D), while no significant change in the percentages of CD3+ or CD8+ T cells was observed (Figures 1A and 1C). Within the TC group, the difference was significant in the CD4+:CD8+ ratio at 6-month of TC exercise compared with the ratio at the onset (p<0.05; Figure 1D). There was also a significant difference in the percentage of CD4+ T lymphocytes at
6-month compared with at 4-month of TC exercise \((p<0.05; \text{Figure 1B})\). However, the percentages of T lymphocytes (CD3\(^+\)) and their subsets (CD4\(^+\) and CD8\(^+\)) in CON group did not demonstrate any changes during the whole study period (onset; 4 months; and 6 months).

**Effect of TC exercise on NK and NKT cells**

Figure 2 shows the changes in the percentages of NK cells and NKT cells in the peripheral blood in both CON and TC groups. The percentages of NK cells and NKT cells before exercise program (at onset) did not show any inter-group differences between CON and TC groups. However, after the 6 months of TC exercise program, the percentage of both CD123\(^+\) (plasmacytoid) DCs and CD11c\(^+\) (myeloid) DCs significantly increased (Figure 4). There was significant increase in the percentages of CD11c\(^+\) DCs \((p<0.01; \text{Figure 4 A})\) and CD123\(^+\) DCs \((p<0.01; \text{Figure 4B})\) at 6 months of TC exercise compared with at the onset. There was also significant increase in the percentage of CD11c\(^+\) DCs \((p<0.01; \text{Figure 4 A})\) and CD123\(^+\) DCs \((p<0.01; \text{Figure 4B})\) at 6-month compared with at 4-month of TC exercise. The number of CD11c\(^+\) DCs (myeloid) (Figure 4A) increased much more dramatically than that of CD123\(^+\) DCs (plasmacytoid) (Figure 4B). The percentages of both CD123\(^+\) (plasmacytoid) DCs and CD11c\(^+\) (myeloid) DCs in CON group did not show any changes during the study period (onset; 4 months; and 6 months).

**Effect of TC exercise on cytokines IL-4 and IFN-γ producing CD4\(^+\) T cells**

Figure 3 shows the changes in the percentages of IFN-γ producing CD4\(^+\) T cells (Th1) and IL-4 producing CD4\(^+\) T cells (Th2) in both CON and TC groups. The percentages of both IFN-γ and IL-4 producing CD4\(^+\) T cells before exercise program (at onset) were not significantly different between CON and TC groups (Figure 3). However, TC exercise program significantly increased both IFN-γ and IL-4 producing CD4\(^+\) T cells (Figure 3). There was significant difference in the proportion of IFN-γ \((p<0.01; \text{Figure 3A})\) and IL-4 \((p<0.05; \text{Figure 3B})\) producing T cells at 4-month of TC exercise compared with at the onset. IFN-γ producing T cells also demonstrated a significantly higher percentage at 6-month than at the onset \((p<0.05; \text{Figure 3A})\). The percentages of IFN-γ and IL-4 producing CD4\(^+\) T cells in CON group did not show any significant changes during the whole study period (onset; 4 months; and 6 months).

**Effect of TC exercise on CD123\(^+\) DCs and CD11c\(^+\) dendritic cells**

As shown in figure 4, the percentages of both CD123\(^+\) (plasmacytoid) DCs and CD11c\(^+\) (myeloid) DCs before TC exercise program (at onset) were not significantly different between CON and TC groups. However, after the 6 months of TC exercise program, the percentage of both CD123\(^+\) (plasmacytoid) DCs and CD11c\(^+\) (myeloid) DCs significantly increased (Figure 4). There was significant increase in the percentages of CD11c\(^+\) DCs \((p<0.01; \text{Figure 4 A})\) and CD123\(^+\) DCs \((p<0.01; \text{Figure 4B})\) at 6 months of TC exercise compared with at the onset. There was also significant increase in the percentage of CD11c\(^+\) DCs \((p<0.01; \text{Figure 4 A})\) and CD123\(^+\) DCs \((p<0.01; \text{Figure 4B})\) at 6-month compared with at 4-month of TC exercise. The number of CD11c\(^+\) DCs (myeloid) (Figure 4A) increased much more dramatically than that of CD123\(^+\) DCs (plasmacytoid) (Figure 4B). The percentages of both CD123\(^+\) (plasmacytoid) DCs and CD11c\(^+\) (myeloid) DCs in CON group did not show any changes during the study period (onset; 4 months; and 6 months).
Discussion

The human immune system demonstrates degenerative changes with advancing age [17], especially T cells which demonstrate prominent age-related alterations in distribution and function, such as declined number of circulating Th cells, NK cells, and DCs, decreased production of Th1 cytokines (IFN-γ) and increased production of Th2 cytokines (IL-4) with aging [2,4,3]. However, the findings of our present study demonstrated that after a 6-month TC exercise program, the percentages of CD4+ T cells decreased while the number of CD8+ T cells significantly increased in middle-aged and elderly women.

Many studies have reported exercise-induced changes in the immune function [5]. Changes in exercise-induced immune functions are associated with the exercise type, intensity, and the duration of both the exercise and any intermissions [5]. It was observed that the number of CD4+ T cells decreased while the number of CD8+ T cells significantly increased, resulting in a decreased CD4+/CD8+ ratio, in seven middle- and long-distance runners undergoing four-week intensive training after 36 hours of rest [18]. After a bout of strenuous and prolonged exercise (>1.5 h), the number of circulating T lymphocytes decreased below pre-exercise levels for several hours after exercise, with a decreased CD4+/CD8+ ratio [19]. Excessive training can cause significant drop in NK cell number, leading to immune suppression [20]. However, our present work and some previously published studies showed that moderate physical exercise improved immune function [21,22]. A 6-month supervised exercise training program led to nominal increases in some aspects of immune function in previously sedentary elderly [22]. While others reported that NK cell activity increased by 33% after moderate intensity training for 16 weeks in the elderly population [23], and increased by 57% in a group of middle-aged women after six weeks of moderate intensity exercise training (60% VO2 max) [24]. Yeh et al. [14] reported that after 12 weeks of TC exercise, transcription factor T-bet as well as expression of Th1/Th2/T regulatory (Treg) cells significantly increased in type 2 diabetes patients. So far, there have been no studies reporting changes in the age-induced Th1/Th2 immune imbalance after regular long-term Tai Chi exercise. The results of our present study demonstrated for the first time that regular TC exercise appears to favor improvement of Th1 immune responses in middle-aged and elderly women and the percentage of NKT cells and CD11c+ DCs significantly increased after 6-month regular TC exercise as compared to the control group.

Th1 cytokines, of which IFN-γ is typical, support cell-mediated inflammatory reactions by activating cytotoxic and inflammatory functions; while Th2 cytokines, of which IL-4 is typical, support humoral immune responses [25]. Th1 and Th2 cytokines are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype [26]. IFN-γ selectively inhibits proliferation of Th2 cells, and IL-4 and IL-10 inhibit cytokine synthesis by Th1 cells [26]. A number of studies have shown that physical exercise affects Th1 and Th2 immune responses as well as the balance between these responses. Excessive or exhaustive exercise has been reported to induce exercise-related immunosuppression via altering the Th1/Th2 balance by decreasing the percentage of circulating Th1 lymphocytes, with increase or no change in Th2 cells; and also by decreasing IFN-γ production and increasing IL-4 production resulting in a decreased IFN-γ/IL-4 ratio [6,27-29]. However, 2-month endurance training of moderate intensity promoted IFN-γ production in moderately trained athletes [30], and 6 months of moderate combined (endurance and resistance) training increased spontaneously CD28 expressing Th1 cells and mitogen-stimulated IFN-γ producing Th1 cells in elderly subjects [31]. Consistent with these findings, our present study showed that after 6-month regular TC exercise, the percentages of IL-4 and IFN-γ producing T cells significantly increased, and IFN-γ producing T cells were upmodulated more dramatically than IL-4 producing T cells. These results suggest that long-term TC exercise can up-regulate Th1 cell-mediated immune responses and promote the switch of the focus of the immune system from humoral immunity to cell immunity, resulting in a higher Th1-type immune response (Th1 dominance) and therefore enhancing antiviral function. However, the mechanism by which TC modulates the Th immune responses and the Th1/Th2 balance remains unclear.

CD1d-restricted NKT cells expressing both invariant T-cell receptors and NK cell receptors are an important immunoregulatory subset of T lymphocytes, and they play an important role not only in immune defense and surveillance, but also in regulating the immune balance [16]. Activated NKT cells are involved in immune differentiation of Th1 and Th2 cells, and in the switch of the immune response in the opposite direction, i.e., towards a Th1-type response [16]. Most recently, we investigated the relationship between NKT cells and the Th1/Th2 lymphocyte imbalance after overtraining and excessive exercise, and showed that α-galactosylceramide played an important modulatory role in the exercise-induced Th1/Th2 lymphocyte imbalance, which may correlate with NKT immunoregulatory cells [32]. It was reported that not only the NKT cell function depends on stimulation and the microenvironment in the initial stages of the immune response, but also the NKT activation signal is transmitted by the specific type of APCs, such as DCs [33].

DCs are the most important and potent APCs, priming the primary
immune response [34]. Two distinct DC subsets, myeloid DCs (mDC, also called Type 1 DCs, DC1), and plasmacytoid DCs (pDC, also called Type 2 DCs, DC2) that may produce different outcomes after their interaction with T lymphocytes, have been identified in humans [34,35]. DC1 (identified as CD11c+CD123-) subsets induce Th1 cell differentiation by the secretion of IL-12. DC2 (identified as CD11c+CD123+) subsets secrete mainly IL-10, which induces initial T cells to differentiate into Th2 cells, resulting in immune tolerance. On the basis of these distinct properties of the DC subtypes, mDCs and pDCs are considered to be specialized APCs inducing a Th1 and Th2 response, respectively. Exercise has been reported to induce a significant increase in DC numbers and modulate myeloid DC differentiation and maturation in rats [36,37]. The findings of our present study demonstrated that TC exercise promotes CD11c+ DCs and CD123+ DCs, with the change in CD11c+ DCs being more dramatic, suggesting that TC exercise facilitates the induction of Th1 cell differentiation.

As the most powerful APCs, DCs can activate resting NKT cells in vivo, present antigen peptide and provide a co-stimulus signal for NKT cell sensitization, activation and amplification [38]. It was shown that DCs produce high levels of co-stimulatory molecules to activate T cells, after combining DCs and T cells to mediate the Th1 response by secreting large amounts of IL-12, helping to eliminate tumors [39]. Others reported that exogenous or endogenous IL-12 induces a progressive decrease in the number of liver NKT cells producing IL-4, thus suppressing the development of Th1 immune responses [38]. However, immature DCs do not induce Th cell differentiation, and IFN-γ mainly induces immature DC differentiation to DC1 cells. The increased percentage of NKT cells enhanced IFN-γ secretion, promoting DC1 production and possibly favoring the development of Th1 immune responses. Therefore, NKT cells and DCs demonstrate reciprocal interactions and cooperatively regulate Th immune differentiation. Our results suggested that TC-induced changes in the Th1 and Th2 immune responses were related to immune modulation of NKT cells and DCs, and their reciprocal interactions with each other.

In conclusion, our present study has shown that a six-month regular TC exercise program significantly improved immune function in middle-aged and elderly women. The CD4+/CD8 ratio showed a significant increase, and the percentages of NKT cells, CD11c+ DCs and CD123+ DCs, as well as the percentages of cytokine IFN-γ producing T cells were also significantly increased. The percentage of CD11c+ DC was increased more dramatically than that of CD123+ DC. We conclude that regular TC exercise can improve age-associated immune imbalance and promote the development of Th1 immune responses, and these changes may be related to the immune modulation of NKT cells and DCs and their reciprocal interactions with each other. Therefore, regular TC exercise has beneficial effects of reversing the age-associated immunosenescence in middle-aged and elderly people.

Acknowledgements

The authors thank Dr. Lai Wei (National Eye Institute, and National Center for Complementary and Alternative Medicine, National Institutes of Health, USA) for critically reading the manuscript. This study was supported by grants from the Shanghai Pujiang Program, the Shanghai Natural Science Foundation (No. 07ZR14103) and the Shanghai Leading Academic Discipline Project (No. T0902). Appreciation is also extended to all participating staff and subjects, whose cooperation made this study possible.

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