Original article

Cycling and Tai Chi Chuan exercises exert greater immunomodulatory effect on surface antigen expression of human hepatitis B virus

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Background Both athletes with intensive exercise and aged people may have weakened immunity against virus infection. This study aimed to evaluate whether people undergoing aerobic exercises including competitive cyclists with moderate training (CMT) and middle-aged people practicing Tai Chi Chuan (TCC) exercise have higher immunity against hepatitis B virus than age-matched sedentary controls including college students (CSC) and middle-aged people (MSC).

Methods Human peripheral blood mononuclear cells from competitive cyclists and sedentary controls were stimulated by phytohemagglutinin (PHA) to prepare conditioned medium (MNC-CM) for the assessment of inhibitory effects on hepatitis B surface antigen (HBsAg) expression in human hepatoma Hep3B cells.

Results The inhibitory effects on the relative HBsAg expression of CMT's and TCC's MNC-CM were greater than those of the controls. The CMT's MNC-CM prepared from 5 μg/ml PHA decreased HBsAg expression to 61.5%, whereas that of CSC remained at 83.8%. Similarly, this expression by treatment of TCC group' MNC-CM was 68.4% whereas that of MSC group was 84.3%. The levels of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IFN-α and interleukin-1β (IL-1β) in the MNC-CM from the CMT and TCC groups were greater than those in the controls. Antibody neutralization of CMT's MNC-CM and addition of recombinant cytokines into CSC's MNC-CM indicated that IFN-γ, TNF-α and IFN-α had synergistic effects against HBsAg expression. Similar blocking effect was noted in TCC versus MSC groups.

Conclusion These results suggest that the immunomodulatory response to suppress HBsAg expression in CMT and TCC with moderate aerobic exercise is greater than that in age-matched sedentary controls.

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The prevalence of chronic hepatitis B, with more than 350 million patients in the world and 3 million in Taiwan, China, is progressively increasing worldwide.[1,2] Moreover, hepatitis B virus (HBV) is highly correlated to the formation of hepatocellular carcinoma.[3] The clinical outcome of hepatocellular carcinoma is poor, especially in patients with unresectable disease at diagnosis. Clearly, it is important to develop better strategies against HBV infection.

People who exercise regularly are often thought to have resistance to many diseases, including virus infection. However, some epidemiological investigations have shown higher incidences of upper respiratory infection after prolonged, strenuous exercise during competition season.[4,5] In contrast, moderate exercise resulted in a decreased upper respiratory tract infection rate.[6] Tai Chi Chuan (TCC), without marked change in heart rate during exercise,[7] is regarded as a light or moderate exercise. TCC is a traditional Chinese martial art practised for many centuries. TCC, exhibits the characteristics of relaxation and deep conditioned breathing, progressively developed into a modern healthy exercise. The attributes about mild and unhurried movements make it a popular exercise for the middle-aged to the elderly. It is always defined to be light or moderate aerobic exercise. The stimulating effects on the immune system against virus can be enhanced by exercise with moderate intensity. For example, after moderate exercise , elderly subjects who were immunized with trivalent influenza vaccine responded with a higher antibody titer to influenza in their blood than that seen in controls.[8] The resistance to infection of herpes simplex virus type 1 was also

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proposed to be associated with moderate exercise in mice.[9]

Some cytokines are deeply involved in anti-viral immunity, particularly those against the HBV. The direct inhibitory effect of interferons (IFNs) on viral replication has been well documented.[10,11] Other studies have reported that physical stress including exercise can alter IFN production.[12-14] It is well-known that cytokines such as tumor necrosis factor-alpha (TNF-α) and IFN-gamma (IFN-γ) can mediate the antiviral immunity. IFN-γ secreted by human peripheral blood mononuclear cells (PBMNCs) exhibits an enormous effect on HBV expression.[15] The TNF-α may contribute to cell-mediated antiviral immune response to HBV. After undertaking moderate exercise, plasma concentrations of TNF-α in healthy untrained men peaked at the end of exercise.[16]

The anti-viral response is exerted by the interaction between cytokines secreted from various immune cells. The T cells induced by the HBV vaccine may reduce the replication of HBV in serum by producing IFN-γ and TNF-α.[17] The anti-viral activity of the immune system exerted by IFN-α has been well studied. IFN-α is a naturally occurring cytokine by immunomodulatory response to promote antiviral properties.[18] It has been licensed for the treatment of chronic hepatitis B in the 1990s. However, the adverse effects of human recombinant IFN-α were too severe to be widely used in clinical practice. Therefore, to promote the endogenous production of cytokines including IFN-α by immunomodulation might be a better antiviral strategy.

We examined the difference from immune reaction against HBV activity in the *ex vivo* system between cyclists with moderate training and middle-aged people with aerobic TCC exercise and age-matched sedentary controls. PBMNCs were isolated and stimulated with different concentrations of phytohemagglutinin (PHA), mimicking the immune reaction induced *in vivo*. We proposed that moderate aerobic exercise may have a greater immunomodulatory response to inhibit gene expression of HBV in competitive cyclists with moderate training and middle-aged people with TCC exercise. Whether this effect was exerted by the secreted cytokines from PBMNC was also tested.

METHODS

Subjects with moderate cycling training and age-matched controls

The study was approved by the Human Ethics Committee of Chinese Culture University. Twelve competitive cyclists with moderate training (CMT) and fourteen sedentary controls from college students (CSC) were recruited into this study while giving informed consent. They were male with a mean age of (22.9±2.7) years (range 19 to 26 years), a mean height of (173±4) cm, and a body mass of (65.2±3.4) kg. There was no significant difference in these parameters between the cyclist group and controls (Table 1). During the experimental period, no subject participated in any competitions or intensive training programs. The CMT was no more than 120 km per week for 4 months before the study, and lasted for 4 weeks before blood collection. They exercised on a bicycle ergometer with work intensity at an individually estimated VO_{2max} of 65% for 60 minutes every day between 8 am and 11 am at an average room temperature of 24ºC. Sixty minutes of exercise were divided into four 15-minute intervals with 5 minutes of rest in each interval. During the experimental period, the CSC group subjects stayed in the same place and were seated to watch a video. The subjects were provided with a breakfast consisting 240 ml of orange juice and milk separately, one egg and six slices of white toast 1 hour before the moderate training program. All subjects refrained from ingesting any drugs, alcoholic drinks, caffeinated beverages, smoking and vegetarian diet for 4 weeks prior to the study and during the experimental period. The subjects had no clinical illnesses or surgical treatment during the 4 weeks before blood collection and the period of experiment. All of the subjects were neither HBV carriers nor hepatitis B surface antigen (HBsAg) positive. Each fasting blood sample was taken between 8 am and 9 am after the subjects had rested quietly for at least 48 hours. Serum albumin concentrations were determined by the Bromcresol Green method.

Table 1. Basic physiologic characteristics of competitive cyclists with moderate training and controls

Variables	CSC (*n*=14)	CMT (*n*=12)
Age (year)	23.2±2.9	22.9±2.3
Height (cm)	172±3	173±3
Body mass (kg)	64.6±3.6	65.9±4.1
Resting heart rate (beats/min)	71.3±4.2	50.2±2.6
Max. heart rate (beats/min)	187.5±3.2	182.4±3.0
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	38.7±4.5	59.3±2.6
Albumin (g/dl) (pre-experiment)	4.1±0.3	4.5±0.2

The cyclist athletes with moderate training group were designated as (CMT) and the sedentary control group as (CSC). Results are expressed as mean ± SE. VO_{2max}: maximal oxygen uptake.

Subjects with habitual TCC practising and age-matched controls

Twenty-four middle aged people recruited provided with informed consent, and the study was approved by the Human Ethics Committee of Chinese Culture University. Six females and 6 males, who keep TCC practice at least 3 times a week for more than 3 years, served as a TCC group. Other twelve subjects (6 males and 6 females) were enrolled as a sedentary control group. They have no other habitual exercises before the study. All of them have no history of cardiovascular, pulmonary, musculoskeletal illness or surgery, which was determined clinically within 4 months prior to blood collection and the period of study. Before blood collection, the TCC group fulfilled a TCC exercise program for 3 months. To ensure the same duration, the subjects made each posture following a prerecorded video tape every day between 8 am and 11 am in an air conditioned room at 24ºC. At the same time,

the controls were seated to watch the same video at a same location. Each process consisted of warming up (low back and hamstrings stretching, gentle calisthenics and balance training) for 20 minutes, TCC practice for 24 minutes, and cooling down for 10 minutes. Each set of TCC practice included 108 postures, with some repeated sequences. The subjects were guided by a Tai-Chi instructor to perform similar motions and postures at a same speed while practicing TCC. Fasting blood was taken between 8 and 9 a.m. after they rested for at least 48 hours. The subjects in both groups showed a mean age of (55.2 ± 4.8) years (range 48 to 66 years old), a mean height of (162.5 ± 7.8) cm, and a mean weight of about (60.6 ± 9.2) kg. These parameters were not significantly different (Table 1). Neither HBV carriers nor patients with HBsAg positive were were found in all subjects.

Measurements of cardiopulmonary fitness

Oxygen uptake (VO_{2max}) was determined two weeks before the experiment using an initial incremental maximal exercise test. In brief, maximal oxygen uptake and ventilation ability were measured by a system for cardiopulmonary exercise testing (Q-plus IW/Corival 400, Seattle, WA, USA) while the subjects were exercising on their own bicycles with an electronically braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA, USA). The subjects warmed up by loading at 75 W for 2 minutes and then began to increase loading at 25 W every 2 minutes until volitional fatigue. A polar pacer heart rate monitor was used to record the heart rate.

Preparation of mononuclear cell-conditioned medium (MNC-CM)

Human MNCs were taken from the peripheral blood of each healthy volunteer who submitted the sheet of informed consent. The cells were separated by density centrifugation (400 × *g*, 30 minutes) in a Ficoll-Hypaque solution (1.077 g/ml) as described.[19] MNCs were washed three times in phosphate-buffered saline, suspended in RPMI-1640 medium (Gibco/BRL, Grand Island, NY, USA) containing 10% heat-inactivated autoserum, and seeded on autoserum-coated culture plates. The cells were cultured at an initial concentration of 1×10^6 cells/ml in 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) with 50 mg/L penicillin (Gibco/BRL), 100 mg/L streptomycin (Gibco/BRL), 1000 μmol/L L-glutamine (Gibco/BRL), and RPMI 1640 medium containing a series of levels (0, 1.25, 2.5 and 5 μg/ml) of PHA (Difco Lab., Detroit, MI, USA) at 37°C in a fully humidified incubator with 5% CO_2. After cultivation for 24 hours, the collected aliquot was filtered through a 0.45 membrane to remove MNC and then stored at –80°C until use.[20] This was termed as conditioned medium (CM). Subsequently, PHA-MNC-CM prepared with 5 μg/ml PHA for 24 hours was used to observe the relative HBsAg expression in Hep3B cells using the method described below. For cytokine assay, MNC-CM prepared with 5 μg/ml PHA was collected. Hep3B cell medium, in which a volume of only 30% PBS with 5 μg/ml PHA replaced the MNC-CM, was defined as the untreated control.

PHA is a natural mitogen of T lymphocytes isolated from plants. It has been used as an immune stimulant in various studies regarding immune response and effect. In our studies, for example, we stimulated PBMNC with gradient concentrations of PHA to mimic varying degree of immune reaction for evaluation of drug-induced,[21,22] exercise-mediated immunomodulation.[19,23] The basis of this model is that PHA stimulates production and release of various cytokines from PBMNC including those related to anti-viral and anti-tumor immunity. Among these cytokines, IFN-γ, TNF-α and IFN-α have been shown as effector molecules acting on the anti-HBV immunity.[15,24,25] We used PHA as an immune stimulant and cytokine levels in PHA-MNC-CM as a measure of anti-HBV effectors.

Hepatoma cell cultures

Hep3B/C16 (Hep3B), a moderately differentiated human hepatocellular carcinoma cell line, which is integrated in the HBV genome in its chromosome and stably produces HBsAg, was taken as the cell model for studying HBV replication.[26,27] The Hep3B cells were cultured at an initial concentration of 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco/BRL), 1×10^5 IU/L penicillin, 100 mg/L streptomycin, and 1000 μmol/l L-glutamine, in a humidified 5% CO_2 incubator at 37°C. The 30% volumes of conditioned medium from MNC and fresh DMEM plus 10% fetal calf serum were added and incubated for 3 days in a humidified 5% CO_2 incubator at 37°C. Our pre-test experiment revealed that the addition of 30% CM was optimal for Hep3B cell to grow. The final concentration of PHA was 0 – 1.5 μg/ml in the MNC-CM, which had no direct effect on the relative HBsAg expression of Hep3B cells tested in our preliminary study. The cell viability was assessed by a tetrazolium dye colorimetric MTT test[28] and expressed as follows: MTT value of the experimental group/MTT value of the untreated control group.

Assay for relative HBsAg expression

Hep3B cells cultured in DMEM with 10% fetal bovine serum for 24 hours were transferred to serum-free DMEM with or without 30% (v/v) MNC-CM and incubated for 48 hours. The secreted HBsAg in the culture medium was measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (General Biological, Taipei, Taiwan, China). The determined optical density (OD) values of the ELISA kits during measurement were normalized with cell numbers. PHA did not interfere with the HBsAg assay. The detection of HBsAg in serum of patients indicated a HBV infection and the risk for developing compensated cirrhosis and hepatocellular carcinoma.[29-31] Thus, HBsAg is a useful index to evaluate the viral activity.[32] The relative HBsAg expression was determined by the

following formula: (HBsAg/MTT) from PHA-MNC-CM/ (HBsAg/MTT) from the culture medium of the untreated control group. The HBsAg/MTT from the culture medium of the untreated control group was treated as 100% expression. The measurement of secreted viral antigens from HBV host cells such as HBsAg secretion from Hep3B cells has been used in various investigations to evaluate the effect of cytokine[33] and drugs[34,35] on HBV activity.

Assay for cytokines

To detect the effects of cytokines on the reduction of relative HBsAg expression in Hep3B incubated with PHA-MNC-CM, commercial ELISA kits for IL-1β, TNF-α, IFN-α and IFN-γ (R&D Systems, Minneapolis, MN, USA) were used and determined at a wavelength of 450 nm using the method described by Wang et al.[20] The correlation coefficients (r^2) for the standard curves of three cytokines were between 0.998–0.999. Three separate experiments were performed in duplicate.

Antibody neutralization

PHA-MNC-CM from the cyclists with moderate training (termed as CMT-PHA-MNC-CM) was pre-incubated with various cytokine-neutralizing antibodies including anti-IFN-γ (30.0 μg/ml, is 10 folds more over than the concentration as near 100% of neutralization), anti-TNF-α (2.4 μg/ml), anti-IFN-α (1.0 μg/ml) and anti-IL-1β (5.1 μg/ml) in combination or alone at 37°C for 90 minutes. PHA-MNC-CM from the controls was termed as CSC-PHA-MNC-CM. Viable cells were counted after 48 hours of incubation with added antibodies of the cytokines. Three separate experiments were performed in duplicate.

Addition of IFN-γ, TNF-α and IFN-α into CSC-PHA-MNC-CM

CSC-PHA-MNC-CM was pre-incubated with various cytokines including IL-1β (500 pg/ml), TNF-α (1200 pg/ml), IFN-α (700 pg/ml) and IFN-γ (1000 pg/ml) or in combination at 37°C for 24 hours.

Statistical analysis

Results were presented as mean ± standard error (SE). Differences between the treatment groups, which consisted of matched samples, were assessed by Student's *t* test. A confidence level of 5% (P <0.05) was considered statistically significant.

RESULTS

Maximal oxygen uptake (VO_{2max}) of CMT and CSC

The mean VO_{2max} of the control group and the CMT group was (38.7±4.5) ml·kg^{-1}·min^{-1} (range 35.6–42.1) and (59.3±2.6) ml·kg^{-1}·min^{-1} (range 56.8–62.2) respectively, showing a significant difference (P <0.001) (Table 1). There was no significant difference in age, height and body mass between the two groups (P >0.05). No significant changes were observed in mean VO_{2max} (data not shown) or albumin level in the CMT group after the experiment.

Anthropometric measurement and peak oxygen uptake (VO_2 peak) of TCC and MSC

The anthropometric measurements of the two groups are shown in Table 2. In the MSC group the mean age was (54.8±5.4) years (range 50 to 63 years), whereas in the TCC group the mean body weight was (58.7±9.6) kg (range 43 to 73 kg), the mean age was (55.3±5.3) years (range 48 to 66 years), and the mean body length was (161.7±9.7) cm (range 140 to 175 cm). There was no significant difference in these measurements between the MSC group and TCC group (P >0.05). The VO_2 peak observed in the TCC group was (27.9±6.1) ml·kg^{-1}·min^{-1}, which was similar to (25.2±5.6) ml·kg^{-1}·min^{-1} (P >0.05) in the MSC group. RER values were not significantly different before and after TCC exercise (Table 3). During TCC exercise, the concentration of blood lactate (from 1.95 to 1.76 mmol/L) was not obviously changed. The data on HR_{max} (at (48.35±7.61)% to (65.66±6.23)%) and VO_2 peak (at (23.15±14.85)% to (47.17±13.39)%) showed that the intensity of TCC exercise increased slightly.

Table 2. The anthropometric measurement of middle-aged people with Tai Chi Chuan and sedentary controls

Variables	TCC (n=12)	MSC (n=12)	t value	P value
Age (years)	55.3±5.3	54.8±5.4	0.438	>0.05
Height (cm)	161.7±9.7	163.6±5.4	0.391	>0.05
Weight (kg)	58.7±9.6	63.8±8.4	0.133	>0.05
Body fat (%)	26.4±6.1	29.2±4.9	0.124	>0.05
VO_2 peak (ml· kg^{-1}· min^{-1})	27.9±6.1	25.2±5.6	0.136	>0.05

The middle-aged people with Tai Chi Chuan were designated as (TCC) and the sedentary control group as (MSC). VO_2 peak: peak oxygen uptake. Results are expressed as mean ± SE.

Table 3. The blood lactate concentration and energy expenditure before and after Tai Chi Chuan exercise

Variables	Before TCC exercise	After TCC exercise
Blood lactate (mmol/L)	1.95±0.36	1.76±0.42
HR_{max} (%)	48.35±7.61	65.66±6.23
VO_2 peak (%)	23.15±14.85	47.17±13.39
METS	1.4±0.9	3.9±1.2
RER	0.75±0.07	0.78±0.09

The middle-aged people with Tai Chi Chuan were designated as (TCC). HR_{max}: percentage of exercise intensity of maximal heart rate achieve during incremental exhaustive exercise; VO_2 peak: percentage of exercise intensity of peak oxygen uptake. METS: metabolic equivalents. RER: respiratory exhange ratio.

Reduction of relative HBsAg expression in Hep3B cells stimulated by PHA-MNC-CM

The relative HBsAg expression in Hep3B cells after stimulation with PHA-MNC-CM decreased with increasing doses of PHA, and a dose-response effect was more obvious in the CMT group. Compared with the CSC group, there was a much lower relative HBsAg expression of 61.5% of HBV in Hep3B cells incubated with CMT-PHA-MNC-CM prepared by stimulation with PHA at the same concentration of 5 μg/ml (Figure 1B). No obvious cytotoxcity to Hep3B cells was observed in both groups when the concentration of PHA was lower than 5 μg/ml. However, relative HBsAg expression was

not significantly reduced in Hep3B in the CSC group (Figure 1A).

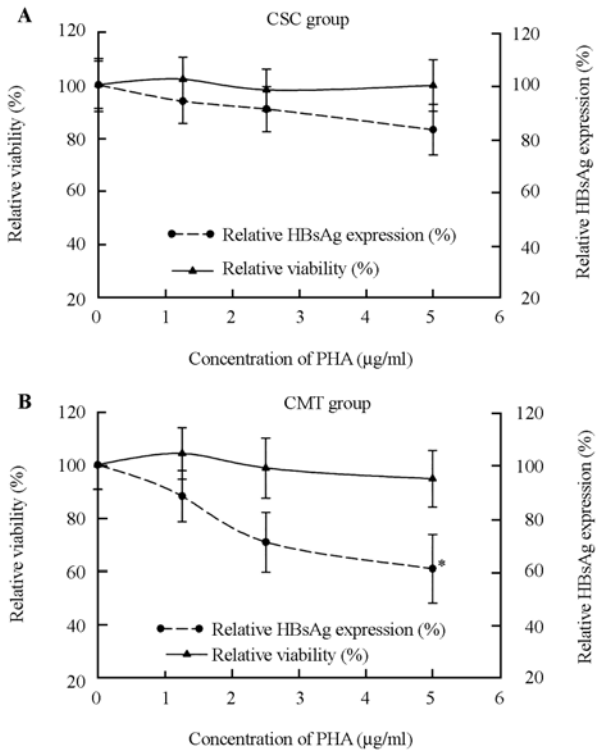

Figure 1. The relative HBsAg expression in Hep3B treated with PHA-MNC-CM from the CMT and CSC groups. **A:** CSC-PHA-MNC-CM, conditioned media prepared from sedentary control blood mononuclear cells that were stimulated by phytohemagglutinin. **B:** CMT-PHA-MNC-CM, conditioned media prepared from cyclist blood mononuclear cells that were stimulated by phytohemagglutinin. The concentrations of PHA (μg/ml) were the final concentrations during incubation of blood mononuclear cells. *Significant difference (P <0.01).

Relative HBsAg expression was 68.4% of HBV in Hep3B cells incubated with TCC-PHA-MNC-CM, which was lower than 84.3% of that in Hep3B cells incubated with MSC-PHA-MNC-CM (Table 4).

Table 4. Effects of PHA-MNC-CMs and cytokine neutralization antibody on the relative HBsAg expression of middle-aged people with Tai Chi Chuan and controls

Groups	Relative HBsAg expression (%)	MTT (%)
Untreated control	100	100
MSC-PHA-MNC-CM	84.3±11.7	99.3±13.4
TCC-PHA-MNC-CM	68.4±13.3	96.1±11.2
+Anti-IL-1β	71.3±11.4	98.9±12.1
+Anti-IFN-γ	81.3±13.2	96.4±13.1
+Anti-IFN-α	77.5±11.4	96.7±14.1
+Anti-TNF-α	78.7±12.7	97.4±12.4
+Anti-TNF-α + Anti-IFN-α + Anti-IFN-γ	86.3±9.7	96.7±12.8

Aliquots of TCC-PHA-MNC-CM were pre-incubated with or without two cytokine-neutralizing antibodies anti-TNF-α (anti-tumor necrosis factor α, 2.4 μg/ml), anti-IFN-α (anti-interferon-α, 1.0 μg/ml), anti-IL-1β (anti-interleukin-1β, 5.1 μg/ml) and anti-IFN-γ (anti-interferon-γ, 30.0 μg/ml) at 37°C for 90 minutes before addition to Hep3B cell culture. Triplicated data from separate experiments are expressed as mean ± SE.

After stimulation by PHA at 5 μg/ml for 24 hours, the amounts of IFN-γ, IL-1β, TNF-α and IFN-α in CMT-PHA-MNC-CM were increased markedly ((1345±196) pg/ml, (830±189) pg/ml, (1937±314) pg/ml and (947±175) pg/ml respectively), compared with those in CSC-PHA-MNC-CM ((425±146) pg/ml, (352±103) pg/ml, (756±238) pg/ml and (326±118) pg/ml, respectively).

The significant augmentation of secreted IFN-γ from (58±41) to (1345±196) pg/ml occurred as the amount of PHA increased from 0 μg/ml to 5 μg/ml in CMT-PHA-MNC-CM (Figure 2A). The production of IFN-γ in the CMT-PHA-MNC-CM prepared by stimulation with PHA at 5 μg/ml was about 3 folds higher than that in the CSC-PHA-MNC-CM. Although the increment of TNF-α production was much higher than that of IFN-γ, the trend of increment was similar. The concentration of TNF-α in CM from the CMT group increased exponentially from (369±122) to (1937±314) pg/ml (Figure 2B). The TNF-α production in CMT-PHA-MNC-CM was about 2 folds higher than that in CSC-PHA-MNC-CM. The concentrations of IFN-α the in CMT-PHA-MNC-CM increased exponentially from (80±59) to (947±175) pg/ml (Figure 2C). With a similar trend, the concentration of IL-1β in the CMT-PHA-MNC-CM increased exponentially from about (225±67) to (830±189) pg/ml (Figure 2D).

The amounts of secreted cytokines such as TNF-α, IFN-γ and IFN-α were greater in the MNC-CM in the TCC group than those in the MSC group (Table 5).

Table 5. The cytokines secreted in PHA-MNC-CMs of middle-aged people with Tai Chi Chuan and controls (pg/ml)

Cytokines	MSC-PHA-MNC-CM	TCC-PHA-MNC-CM
IL-1β	282±107	626±167
IFN-α	317±132	832±215
TNF-α	512±244	1186±274
IFN-γ	335±103	665±160

The middle-aged people with Tai Chi Chuan were designated as (TCC). TNF-α: tumor necrosis factor α; IFN-α: interferon-α, IL-1β: interleukin-1β and anti-IFN-γ: interferon-γ. Triplicated data from separate experiments are expressed as mean ± SE.

Effects of anti-cytokine antibody neutralization against HBsAg expression in Hep3B cells

To investigate the effects of cytokines on the reduction of relative HBsAg expression in Hep3B cells, aliquots of CMT-PHA-MNC-CM were pre-incubated with one or more cytokine-neutralizing antibodies including anti-IFN-γ, anti-TNF-α, anti-IFN-α, and anti-IL-1β for 90 minutes prior to cultivation of Hep3B cells. The relative HBsAg expression in Hep3B cells elevated to (75.4±11.9)% from (61.5±12.9)% in the presence of 500 NU/ml anti-IFN-γ antibodies and to (73.2±11.2)% in the presence of 500 NU/ml anti-TNF-α antibodies (Table 6). The relative HBsAg expression in Hep3B cells elevated to (68.6±10.4)% in the presence of anti-IFN-α antibody. The relative HBsAg expression in Hep3B cells came to the lowest level ((64.7±10.6)%) in the presence of anti-IL-1β antibody. Although there was a statistically significant difference when compared to CMT-PHA-MNC-CM alone, relative HBsAg expression was elevated

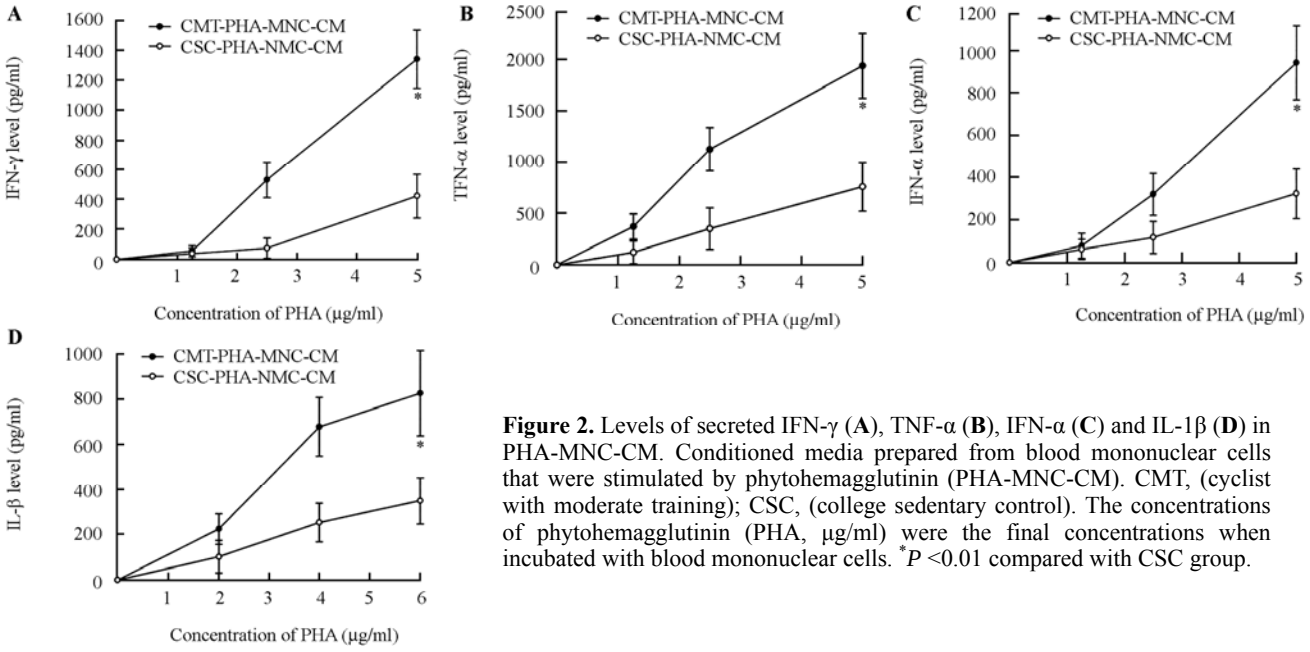

Figure 2. Levels of secreted IFN-γ (**A**), TNF-α (**B**), IFN-α (**C**) and IL-1β (**D**) in PHA-MNC-CM. Conditioned media prepared from blood mononuclear cells that were stimulated by phytohemagglutinin (PHA-MNC-CM). CMT, (cyclist with moderate training); CSC, (college sedentary control). The concentrations of phytohemagglutinin (PHA, μg/ml) were the final concentrations when incubated with blood mononuclear cells. $^{*}P$ <0.01 compared with CSC group.

Table 6. Effects of PHA-MNC-CMs, cytokine neutralization antibody and recombinant cytokine addition on the relative HBsAg expression

Groups	Relative HBsAg expression (%)	MTT (%)
Untreated-control	100	100
CMT-PHA-MNC-CM	61.5±12.9	95.2±10.6
+Anti-IL-1β	64.7±10.6	103.0±11.7
+Anti-IFN-γ	75.4±11.9	97.3±10.7
+Anti-IFN-α	68.6±10.4	97.5±9.9
+Anti-TNF-α	73.2±11.2	102.3±10.9
+Anti-IFN-γ + Anti-TNF-α	84.9±11.8	98.2±9.6
+Anti-IFN-γ + Anti-IFN-α	80.6±10.5	101.5±11.8
+Anti-TNF-α + Anti-IFN-α	76.4±11.1	98.4±10.6
+Anti-TNF-α + Anti-IFN-α + Anti-IFN-γ	89.4±9.1	97.7±9.6
CSC-PHA-MNC-CM	83.8±9.5	100.3±9.7
+IL-1β	80.2±9.8	98.6±10.7
+IFN-γ	70.2±8.4	97.8±9.2
+IFN-α	80.3±10.2	103.2±10.1
+TNF-α	75.2±9.7	98.1±11.6
+IFN-γ + TNF-α	62.3±9.9	102.6±8.7
+IFN-γ + IFN-α	66.5±10.2	100.3±9.7
+TNF-α + IFN-α	69.5±9.1	97.5±10.8
+TNF-α + IFN-α + IFN-γ	66.6±10.5	102.5± 9.9

Aliquots of CMT-PHA-MNC-CM were pre-incubated with or without two cytokine-neutralizing antibodies anti-TNF-α (2.4 μg/ml), anti-IFN-α (1.0 μg/ml), anti-IL-1β (5.1 μg/ml) and (anti-IFN-γ 30.0 μg/ml) at 37°C for 90 minutes before addition to Hep3B cell culture. Aliquots of CSC-PHA-MNC-CM were pre-incubated with cytokines, IL-1β (500 pg/ml) alone or TNF-α (1200 pg/ml), IFN-α (700 pg/ml), and IFN-γ (1000 pg/ml), at 37 °C for 24 hours before addition to Hep3B cell culture. Triplicated data from separate experiments are expressed as mean ± SE.

a little in Hep3B cells. Notably, the reduction of relative HBsAg expression in Hep3B cells was further elevated to a higher level when every other two antibodies were combined. Compared to relative HBsAg expression in Hep3B cells incubated with CMT-PHA-MNC-CM without adding any antibodies, the effect of combined anti-TNF-α and anti-IFN-γ reached a higher level in this study ((84.9±11.8)%), followed by anti-IFN-α and anti-IFN-γ ((80.6±10.5)%) and anti-TNF-α and anti-IFN-α ((76.4±11.1)%). The highest reduction effect on the relative HBsAg expression in Hep3B cells was about (89.4±9.1)% with a combination of all three cytokine antibodies. The inhibitory activity against relative HBsAg expression was lost partially when TCC-PHA-MNC-CM was neutralized by anti-IFN-γ, anti-TNF-α and/or anti-IFN-α antibodies (except anti-IL-1β) (Table 4). This finding suggests the importance of TNF-α, IFN-α and IFN-γ in reducing the relative HBsAg expression in Hep3B cells.

Effects of addition of IFN-γ, TNF-α and IFN-α into CSC-PHA-MNC-CM against HBsAg expression in Hep3B cells

Combined IL-1β (480 pg/ml) and CSC-PHA-MNC-CM did not promote its effect on the relative HBsAg expression ((80.2±9.8)%) compared with CSC-PHA-MNC-CM alone ((83.8±9.5)%) (Table 6). Adding combined IL-1β (500 pg/ml), TNF-α (1200 pg/ml), IFN-α (700 pg/ml), and IFN-γ (1000 pg/ml) in CSC-PHA-MNC-CM obviously decreased the relative HBsAg expression to (66.6±10.5)%. The inhibitory effect of added cytokines further confirmed the role of the tested cytokines against HBsAg expression.

DISCUSSION

In this study, PBMNC isolated from competitive cyclists with moderate training and middle-aged people practising TCC had a greater immunomodulatory effect on HBsAg expression. This effect may be attributed to the secretion of cytokines. The exercise intensity of cyclists and middle-aged people practicing TCC was moderate. The percentage of HR_{max} after TCC exercise or 65.66% was defined as moderate exercise. The percentage of VO_2 peak after TCC exercise or 47.17% was also a strong evidence showing TCC as moderate intensity exercise. The exercise intensity of TCC was neither high nor low. The relative HBsAg expression of HBV was lower in

Hep3B cells incubated with CMT-PHA-MNC-CM than with CSC-PHA-MNC-CM, and in Hep3B cells incubated with TCC-PHA-MNC-CM than with MSC-PHA-MNC-CM. Lowder et al[36] found that moderate exercise of TCC inhibited activity of influenza virus *in vivo*. Thus, moderate intensity exercise of TCC might be a suitable way to enhance anti-viral immunity.

In this study CM was prepared by removal of MNC after stimulation by various concentrations of PHA, suggesting that there may be soluble mediators produced by MNC capable of inhibiting HBsAg expression in Hep3B cells. Antibody neutralization and cytokines supplement showed that IFN-γ, TNF-α, IFN-α, but IL-1β, were soluble mediators contributing to the inhibitory activity of CMT-MNC-CM. In our study moderate exercise of CMT and TCC was shown to be safe for cultured MNC because there were no cytotoxicity to MNC (data not shown).

IFN-γ was found to exert HBsAg inhibitory effects.[37,38] In active experienced older runners, moderate exercise increased the concentration of blood IFN-γ after stimulation with PHA.[39] The higher concentration of IFN-γ in CMT-PHA-MNC-CM and lower relative HBsAg expression with anti-IFN-γ antibody neutralization showed the important role of IFN-γ. The reduction of cell cytoplasmic HBV DNA is highly correlated with the secretion of IFN-γ in the supernatants of PBMNC medium.[40,41] TNF-α is considered as an activator of antiviral effects in the immune system. TNF-α disrupts the formation of cytoplasmic viral capsids to cause noncytopathic suppression of hepatitis B virus DNA replication in hepatocytes.[42] When TNF-α is added to Hep3B cells, a significant reduction of HBV replication is noted.[43] The higher concentration of TNF-α and lower relative HBsAg expression with anti-TNF-α antibody neutralization in CMT-PHA-MNC-CM and TCC-PHA-MNC-CM have shown the critical role of TNF-α. The anti-virus activity associated with increased secretion of IFN-α has been observed in other similar experimental models. In cultured MNCs combined with antibody of IFN-α in herpes simplex viruses (HSV)-infected human embryo lung fibroblasts, the level of viral suppression is reduced.[44] Despite the greater secretion of cytokines accompanied by reduced expression of HBsAg, there was no cytotoxic effect of CMT-PHA-MNC-CM and TCC-PHA-MNC-CM on the host HBV-harboring Hep3B cells in this study. This indicated that the inhibitory activity of CMT-MNC-CM and TCC-PHA-MNC-CM against HBsAg expression may not come from the cytotoxicity to Hep3B cells. HBV-specific cytotoxic T lymphocytes are reported to secrete both TNF-α and IFN-γ to abolish HBV gene expression and replication without cytopathy.[29] The synergistic reduction of the inhibitory effect on HBsAg expression in CMT-PHA-MNC-CM by administration of anti-TNF-α and anti-IFN-γ antibodies demonstrates that the interaction between them also attributes to the anti-viral activity. Compared with anti-TNF-α, anti-IFN-α and anti-IFN-γ exhibit the greatest blocking effect on inhibition of relative HBsAg expression in CMT-PHA-MNC-CM, suggesting that these cytokines play an immunomodulatory anti-viral role during moderate exercise. A similar phenomenon can be observed in TCC-PHA-MNC-CM compared to MSC-PHA-MNC-CM.

Whether HBV-specific cytotoxic T lymphocytes play a role in MNCs remains to be elucidated. Why we used MNC, not single isolated cell lineage, is that the various kinds of cells in MNC may represent a better simulation for the immune cell interaction inside human body.

In conclusion, competitive cyclists with moderate training and middle-aged people practising TCC, a moderate intensity aerobic exercise, have a greater immunomodulatory effect on HBsAg expression of HBV than age-matched sedentary controls. This effect may be attributed to the secretion of such cytokines as IFN-γ, TNF-α and IFN-α from human peripheral blood MNC. The real *in vivo* effects need further investigation.

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