

Effect of 6- day intense Kendo training on lymphocyte counts and its expression of CD95

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Abstract

This study examines the effects of 6-day intensive training on lymphocyte counts and their expression of CD95. Eight healthy Kendo athletes underwent 6-day Kendo training of about 310 min each day. Blood samples were collected at 2 weeks before (PRE), the first day (Day 1), third-day (Day 3), fifth-day (Day 5), and 1 week after the training period (POST) to determine lymphocyte counts and CD95 expression on CD95 lymphocytes (CD4⁺, CD8⁺) using flow cytometry. The total lymphocyte counts were significantly lower at Day 3 than at PRE. The CD8⁺ cell counts were significantly lower at Day 3 than at PRE. The percentage of CD95⁺ lymphocytes was significantly higher at Day 1 and Day 3 than at PRE. The percentage of CD8⁺CD95⁺ cells did not change significantly. The total lymphocyte counts decreased and a concomitant increase of CD95⁺ lymphocyte was observed, whereas the decrease in CD8⁺ cell counts was not associated with the increase in CD8⁺CD95⁺ cells. Therefore, short-term high-intensity exercise induced a decrease in the T lymphocyte counts without increasing in CD95⁺ expression.

Introduction

Short-term high-intensity repeated exercise (intense training) is necessary for athletes. However, high-intensity exercise induces lymphocytopenia (Malm et al. 2004; Pedersen and Ullum 1994). Indeed, previous studies reported that lymphocyte counts at rest are lower in athletes (Ferry et al. 1990; Keen et al. 1995). Increased risk of developing upper respiratory tract infection (URTI) following intensive training or competition schedules has been reported in athletes (Lancaster et al. 2004). URTI is influenced by natural killer cell activity (NKCA) and lymphocyte proliferative response (T lymphocyte function) (Nieman 1997). The decrease in NKCA reduces host protection against virus and bacteria for a short time period (Nieman et al. 1995). In addition, the suppression of lymphocyte proliferative response after high-intensity exercise can be explained in part by a decrease in CD4⁺ cell counts (Tvede et al. 1989), and the lowered CD4⁺/CD8⁺ ratio can be related to URTI.

Recently, previous studies demonstrated the relation between athlete health and T lymphocyte function (Green 2002; Krzywkowski et al. 2001). Green et al. found that the detrimental effect on T lymphocyte function of acute exercise might be mediated by increased lymphocyte apoptosis (Green et al. 2003; Green and Rowbottom 2003).

Lymphocyte apoptosis is a potential mechanism of exercise-induced lymphocytopenia

(Mars et al. 1998), and cortisol mediates the induction of apoptosis (Riccardi et al. 2000). Although some reports have described that a bout of high-intensity exercise induces lymphocyte apoptosis (Mooren et al. 2002, 2004; Timmons and Bar-Or 2007), other studies have demonstrated that it does not induce lymphocyte apoptosis (Steensberg et al. 2002; Tanimura et al. 2008). Mooren et al. reported basal (pre-exercise) lymphocyte apoptosis was higher in the highly trained ($\dot{V}O_{2max}$ more than 60 ml/min/kg) group than in the less trained ($\dot{V}O_{2max}$ less than 55 ml/min/kg) group (Mooren et al. 2004). In addition, exhaustive exercise induces peripheral blood lymphocytes apoptosis whereas moderate exercise does not (Mooren et al. 2002). Thereby, the alteration of lymphocyte apoptosis may depend on the exercise intensity and the trained status of subjects.

Furthermore, Simpson et al. (2007b) reported that lymphocyte apoptosis did not contribute to lymphocytopenia after a bout of high-intensity exercise. Previous reports have described that short-term high-intensity repeated exercise increased lymphocyte apoptosis indices (Hsu et al. 2002; Tuan et al. 2007). However, those studies did not examine lymphocyte count alteration. Therefore, it remains unclear whether lymphocyte apoptosis contributes to lymphocytopenia after short-term high-intensity repeated exercise.

A type I transmembrane receptor, CD95, is expressed on peripheral lymphocytes. Although CD95 measurement itself is not a marker of apoptotic cells, it plays a regulatory role in immune homeostasis by initiating the apoptotic pathway leading to cell death. The CD95⁺ T lymphocyte might increase significantly after a bout of high-intensity exercise (Mars et al. 1998; Mooren et al. 2002, 2004), but the CD95⁺ B lymphocyte is altered only slightly (Timmons and Bar-Or 2007). Furthermore, CD95 expressions of CD4⁺ (helper T lymphocyte) cells and CD8⁺ (cytotoxic T lymphocyte) cells show different responses after a bout of high-intensity exercise (Krzywkowski et al. 2001; Simpson et al. 2007b). However, the response of CD95⁺ lymphocytes to short-term high-intensity repeated exercise remains unknown.

Consequently, this study was undertaken to examine the effects of 6-day intense Kendo training on lymphocytes and CD4⁺ and CD8⁺ cell counts, and on their expressions of CD95 in young Kendo athletes.

Methods

Subjects

Eight healthy university Kendo athletes participated in 6-day Kendo training. Table 1 presents their physical characteristics. Subjects were non-smokers; they were not taking regular medications. The regular training workload of the subjects is 2 h/day for 6 days/week. A previous study reported that the averaged Japanese Kendo athlete's maximum oxygen consumption was 46.8 ± 3.4 ml/min/kg (Hayashi et al. 1993). This study was approved by the Ethics Committee of the Graduate School of Comprehensive Human Sciences of the University of Tsukuba.

Exercise protocol

Kendo is a Japanese traditional martial art resembling western fencing, using a bamboo sword. The Kendo training was conducted for 310 min (9:00-11:30 a.m.; 14:30-17:30 p.m.) per day for 6 days; all subjects took the same diet during training. The training included *Suburi* (swing practice, 20 min), *Kirikaeshi* (40 min), *Kakarigeiko* (15 min), break (10 min), *Gokakugeiko* (60 min), and cooling down (5 min) during the AM practice, and *Suburi* (20 min), Games (100 min), break (10 min), *Gokakugeiko* (45 min), and cooling down (5 min) during the PM practice. The first day's training included only the PM practice. Exercise intensity during the training was estimated at

77–88% of HR_{max} (Akimoto et al. 1998).

Blood samples and lymphocyte counts.

Venous blood samples were drawn from a forearm vein in a resting condition (between 13:30 and 14:15) at 2 weeks prior (PRE), during training period (Day 1, Day 3 and Day 5, respectively, D1, D3, and D5), and 1 week after training period (POST). Leukocyte counts were made using a cell counter (Sysmex SE-9000, Sysmex Corp., Hyogo, Japan) according to direct current detecting method. Lymphocytes were counted using microscopy. The lymphocyte counts were adjusted for changes in blood volume using the equation of Dill and Costill (1974).

Flow cytometry

One hundred microliters of whole blood was incubated for three-color immunophenotyping, using appropriate combinations of monoclonal antibodies (Beckman Coulter Inc., CA, USA) conjugated to fluorescein isothiocyanate (CD8⁺FITC), phycoerythrin (CD95⁺PE), and allophycocyanin (CD4⁺APC). Samples with isotypic control antibodies (IgG1[FITC]/IgG1[PE]/IgG[PCy-5]) were run in parallel with each sample. All lymphocyte phenotype analyses were conducted using a flow cytometer (FACSCalibur, Becton Dickinson Immunocytometry System, CA, USA). An electronic gate was placed around the lymphocyte population in the flow cytometry

forward-scatter and side-scatter modes. Standard gating procedures used mainly lymphocytes (10^5 cells) by the gating technique. An electronic marker was placed at the limit of negative control to quantify the percentage of lymphocyte subsets that were positive and negative for each cell surface antigen. All parameters obtained using flow cytometry were analyzed using software (Cell Quest, Becton Dickinson Immunocytometry System, CA, USA. and Win MDI 2.9, Joseph Trotter, The Scripps Research Institute, CA, USA). Figure 1 depicts an example of flow cytometric analysis of CD95⁺ lymphocytes in whole blood. An electronic marker was placed at the limit of the negative control to quantify the percentage of lymphocytes and lymphocyte subsets that were positive and negative for each cell surface antigen. Absolute quantities of lymphocytes that were positive for each cell surface antigen were determined by multiplying the percentage values by the corresponding total lymphocyte count.

Serum cortisol concentrations were analyzed using the radioimmunoassay with a cortisol RIA kit (Immunotech, Prague, Czech Republic) (Sapin et al. 1998).

Statistical analysis

All data are presented as means \pm SD. Changes over time were tested using analysis of variance for repeated measurements (ANOVA). In ANOVA, a value of $p < 0.05$ was inferred as statistically significant. The significant difference location was determined

using Bonferroni/Dunn post hoc test set at $p < 0.005$ if significance was indicated.

Calculations were performed using software (StatView 5.0; SAS Institute Inc., North Carolina, USA).

Results

The total lymphocyte counts were significantly lower at D3 than at PRE and recovered thereafter (Table 2). The CD8⁺ cell counts were significantly lower at D3 than at PRE, but CD4⁺ cell counts did not change significantly during the training period (Table 2).

Figure 2 shows the CD95⁺ lymphocyte counts and its percentage of total lymphocytes. The CD95⁺ lymphocyte counts were significantly higher at D3 than at PRE. The percentages of CD95⁺ lymphocytes were significantly higher at D1 and D3 than at PRE. Both the counts and the percentage of CD95⁺ lymphocytes recovered at D5 and POST.

Counts of CD4⁺ and CD8⁺ lymphocyte expressing CD95⁺ are presented in Fig. 3. Although the counts of CD4⁺CD95⁺ lymphocytes were significantly higher at D3, the counts of CD8⁺ CD95⁺ lymphocytes were significantly lower at D3 than at PRE.

Figure 4 shows the percentage of CD4⁺ and CD8⁺ lymphocyte expressing CD95⁺. The percentage of CD4⁺ CD95⁺ was significantly higher at D3 than at PRE. No change in the percentage of CD8⁺CD95⁺ was found during the training.

Figure 5 shows the serum cortisol concentrations. The serum cortisol concentration at D3 was significantly higher than at PRE.

Discussion

Results of this study demonstrated that the short-term high-intensity repeated exercise caused a significant transient decrease in total lymphocyte counts. In T lymphocytes, CD8⁺ cell counts decreased significantly, but CD4⁺ cell counts did not. Although total lymphocyte expression of CD95 increased significantly, CD8⁺CD95⁺ cell counts decreased significantly: no change in the percentage of CD8⁺CD95⁺ was found during the training.

A bout of high-intensity exercise induced transient lymphocytopenia (including CD4⁺ and CD8⁺ cells) after exercise (Simpson et al. 2007a; Vider et al. 2001). Moreover, repetition of high-intensity exercise decreased lymphocyte counts (including CD4⁺ and CD8⁺ cells) at rest (Hoffman-Goetz et al. 1990; Malm et al. 2004). Although total lymphocytes and CD8⁺ cell counts had decreased significantly at D3, the CD4⁺ cell counts did not change significantly during the training period in the present study. This result is partly consistent with that of a previous study that 5-day intense training decreased total lymphocytes and CD8⁺ cell counts (Malm et al. 2004). However, our study is not consistent with the study of Malm et al. as for the CD4⁺ cell counts alteration. We took blood samples at resting time after the morning exercise. Thereby, our results may be influenced by not only the effect of repeated exercises but also the

effect of the acute exercise. We showed significant decrease of CD8⁺ cells and insignificant decrease of CD4⁺ cells. Acute exercise induces more marked CD8⁺ cell counts decline than CD4⁺ cells (Gleeson and Bishop 2005). Therefore, the discrepancy between the study of Malm et al. (2004) and this study may be explained by the difference of timing of taking blood. Because Malm et al. measured resting time in the early morning before exercise.

The underlying mechanisms for the change of lymphocyte counts following exercise are yet to be fully determined. Alterations in the concentration of circulating stress hormones appear to play a key role in the redistribution of circulating lymphocytes related to exercise. Short-term high-intensity exercise increases cortisol levels (Aizawa et al. 2006). Cortisol influences the response of lymphocyte counts to exercise (Okutsu et al. 2005; Shinkai et al. 1996). Increased serum cortisol concentrations results in decreased circulating lymphocyte counts by inhibiting their entry into and facilitating their egress from the circulation (Fauci 1975).

Catecholamine induces mobilization of cell attached to the vascular endothelium by the expression of cell adhesion molecules. Lymphocytes expressed a high intensity of β_2 -adrenergic receptors and the density of these receptors increases with both exercise and exposure to catecholamines (Shephard 2003). The expression of these receptors is

higher density on the CD8⁺ cell than CD4⁺ cell (Shephard 2003). The differences in the β_2 -adrenergic receptor expression between lymphocyte subpopulations also help to explain the greater relative changes in the increase of CD8⁺ cell following exercise compared with the increase of CD4⁺ cell. Because this increased CD8⁺ cell may decrease by down-regulation, CD8⁺ cell might decrease in this study.

To our knowledge, this is the first report of T lymphocyte expression of CD95 after short-term high-intensity repeated exercise. This study demonstrated the increase of CD95⁺ on total lymphocytes. However, the isolated CD95 measurement is not a marker of apoptotic cells. A few prior reports described that short-term high-intensity repeated exercise induced lymphocyte apoptosis assessed by Annexin V or DNA fragmentation (Hsu et al. 2002; Tuan et al. 2007). The alteration of apoptosis may depend on the exercise intensity and the trained status of subjects (Hsu et al. 2002; Mooren et al. 2002). A previous study reported Annexin V after marathon was substantially enhanced in the low oxygen uptake group than in the high oxygen uptake group; however, CD95 receptor expression significantly increased in the both groups and did not show the difference between the both groups (Mooren et al. 2004).

Simpson et al. (2007b) reported that the increased number of CD95⁺ lymphocyte after one-bout of exercise was accounted for by an increase in the CD8⁺CD95⁺, not by the

numbers of CD4⁺CD95⁺. This study also demonstrated the different changes of expression CD95⁺ between CD4⁺ cells and CD8⁺ cells during short-term high-intensity repeated exercise. However, the repeated exercise induced to increase CD4⁺CD95⁺, and decrease CD8⁺CD95⁺.

The serum cortisol concentration at D3 was significantly higher than at PRE. Cortisol mediates the induction of CD95⁺ expression (Riccardi et al. 2000). Therefore, the increase of cortisol level at D3 might induce increased CD4⁺CD95⁺ cell at D3 in this study. However, increase in CD4⁺CD95⁺ cell at D3 did not result in decrease in CD4⁺ lymphocyte counts probably responsible for some mechanisms of inhibiting progression of apoptosis. On the other hand, CD8⁺ cells decreased without increased CD95 expression in lymphocyte. Therefore, the exercise-induced transient decrease in CD8⁺ cell counts might result from other mechanisms, e.g., inhibiting lymphocyte entry into the blood and promoting lymphocyte movement into the tissues. Thereby, the decrease in lymphocyte counts at D3 might relate to the increase of cortisol level at D3 in this study.

Furthermore, this study showed that decreased lymphocyte counts tended to recover at D5. Although the exact reasons of attenuated effect of exercise on lymphocyte counts remain unclear, some adaptation mechanisms to repeated exercise might act. In this

study, the increase in serum cortisol concentration was recovered at D5, possibly by the reduced stress for accustomed to exercise, and it might influence the recovery of lymphocyte at D5. Another reason for the change of lymphocyte counts in spite of ongoing exercise might be the attenuated sensitivity to increased cortisol (Bishop et al. 2005; DeRijk et al. 1996; Lancaster et al. 2004). Furthermore, the increased expression of CD95 on total lymphocytes and CD4⁺ cells recovered at D5. This may be explained by recovered cortisol level. These results agree with the study of Ivanova et al. (2007).

Several limitations exist in this study. First, this study is an uncontrolled study. Second, it is necessary for evaluating lymphocyte apoptosis to measure both the expression of CD95 receptor and Fas ligand. Because CD95 is a membrane receptor that initiates apoptosis after ligation with Fas ligand which is emphasized by the coincidence of Annexin V (Mooren et al. 2004). The isolated CD95 measurement is not a marker of apoptotic cells. Thus, we need to measure Annexin V. Finally, further study needs the evaluation that excludes a possible “hang-over” effect of the acute training session on total lymphocyte, CD4⁺ and CD8⁺ cell counts.

In conclusion, 6-day intense Kendo training induced a transient decrease in total lymphocyte counts with a decrease in CD8⁺ cells and without CD4⁺ cell counts. Total lymphocyte counts decrease and concomitant increase of CD95 expression in

lymphocyte was observed, whereas decreased CD8⁺ cells did not associate with increased CD8⁺CD95⁺ cells. Therefore, short-term high-intensity exercise induced decrease in the T lymphocyte counts without increase in CD95⁺ expression.

Figure legends

Fig. 1. Flow cytometric analysis of CD95⁺ lymphocytes in whole blood.

- a. Gated lymphocyte population based on forward-scatter (FSC) *versus* side-scatter (SSC) characteristics.
- b. Expression of CD95 on lymphocyte within the lymphocyte gate. The dashed vertical line represents isotype control. *MI*: gated CD95 positive lymphocyte is counted at the right side from the *dashed vertical line*. *PE* phycoerythrin.
- c. Representative profiles of two-color immunofluorescence for identification of CD95 expression in CD4⁺ T cells. *APC* allophycocyanin.
- d. Representative profiles of two-color immunofluorescence for identification of CD95 expression in CD8⁺ T cells. *FITC* fluorescein isothiocyanate.

Fig. 2. Effects of training on CD95⁺ lymphocytes.

- a. Total counts of lymphocytes expressing CD95⁺.
- b. Percentage of CD95⁺ lymphocytes.

Percentages were calculated using the ratio of CD95⁺ lymphocyte counts and total lymphocyte counts. Values are represented as means \pm SD. *signifies a statistically significant difference versus PRE by post hoc test ($p < 0.005$).

Fig. 3. Effects of the training on CD4⁺ CD95⁺ and CD8⁺ CD95⁺ lymphocyte counts.

Values are represented as means \pm SD. *signifies a statistically significant difference versus PRE by post hoc test ($p < 0.005$).

Fig. 4. Effects of training on the percentages of CD4⁺ CD95⁺ lymphocyte and CD8⁺ CD95⁺ lymphocyte.

The percentages were calculated using the ratio of CD4⁺ (CD8⁺) CD95⁺ lymphocyte counts and total lymphocyte counts. Values are represented as means \pm SD. *signifies a statistically significant difference versus PRE by post hoc test ($p < 0.005$).

Fig. 5. Effect of training on serum cortisol concentration.

Values are represented as the means \pm SD.

*signifies a statistically a significant difference versus PRE by post hoc test ($p < 0.005$).

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Table1. Physical characteristics

Characteristic	Values
Age (years)	19.6 ± 0.9
Height (m)	1.73 ± 0.05
Body mass (kg)	71.9 ± 8.3
Body mass index (kg/m ²)	24.0 ± 1.7
Kendo career (years)	12.5 ± 1.8

Values are represented as means ± SD.

Table 2. Changes of total lymphocyte and T lymphocyte subset counts (cell/ μ l) with training.

	PRE	Day 1	Day 3	Day 5	POST
Lymphocytes	1,919 \pm 583	1,650 \pm 420	1,494 \pm 506*	1,729 \pm 465	1,749 \pm 366
CD4 ⁺ cells	731 \pm 218	632 \pm 156	613 \pm 216	713 \pm 146	693 \pm 142
CD8 ⁺ cells	598 \pm 204	520 \pm 130	390 \pm 104*	502 \pm 124	534 \pm 158

Values are represented as means \pm SD.

* $p < 0.05$: statistically significant different versus PRE values.

Fig. 1

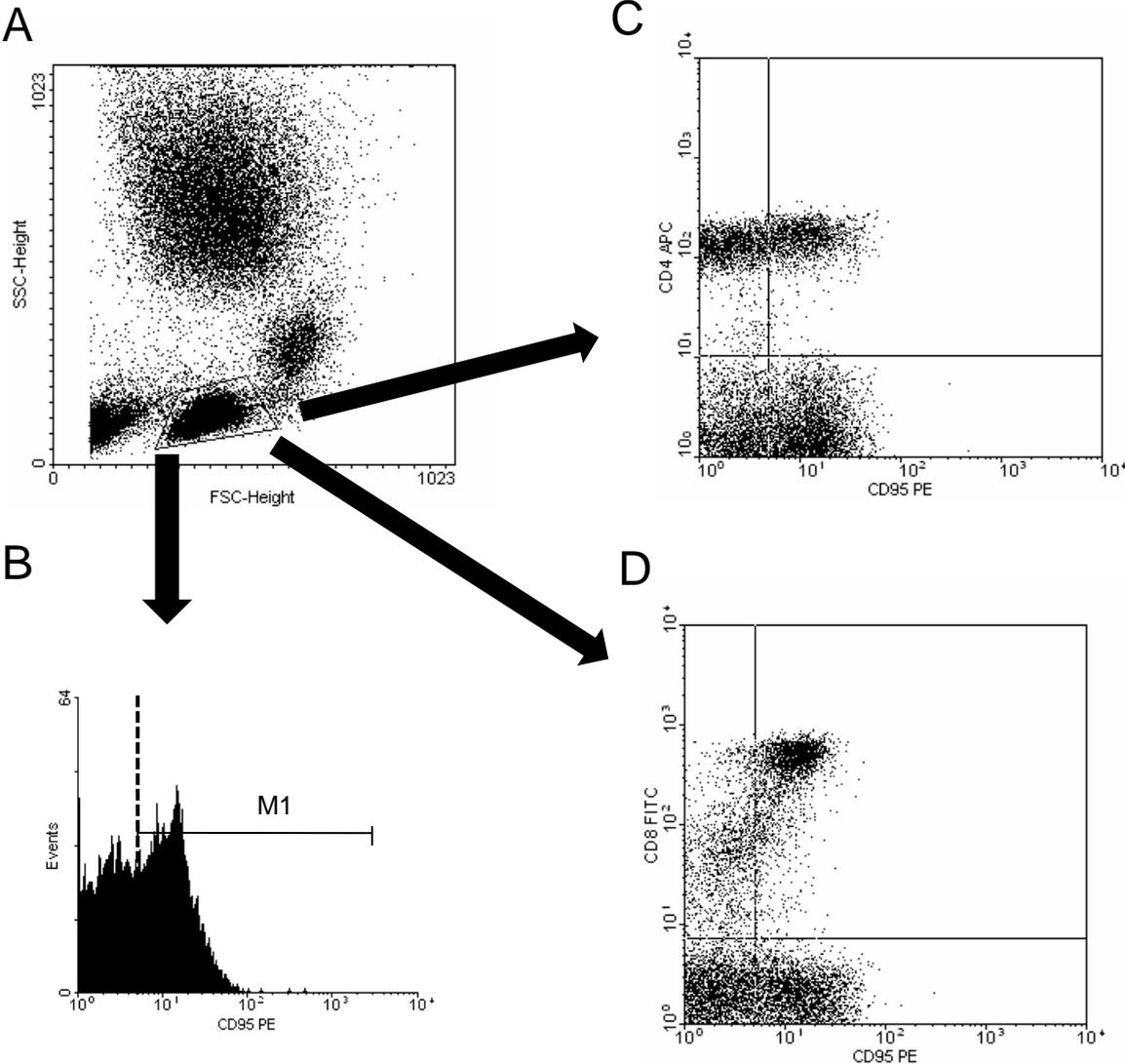


Fig. 2

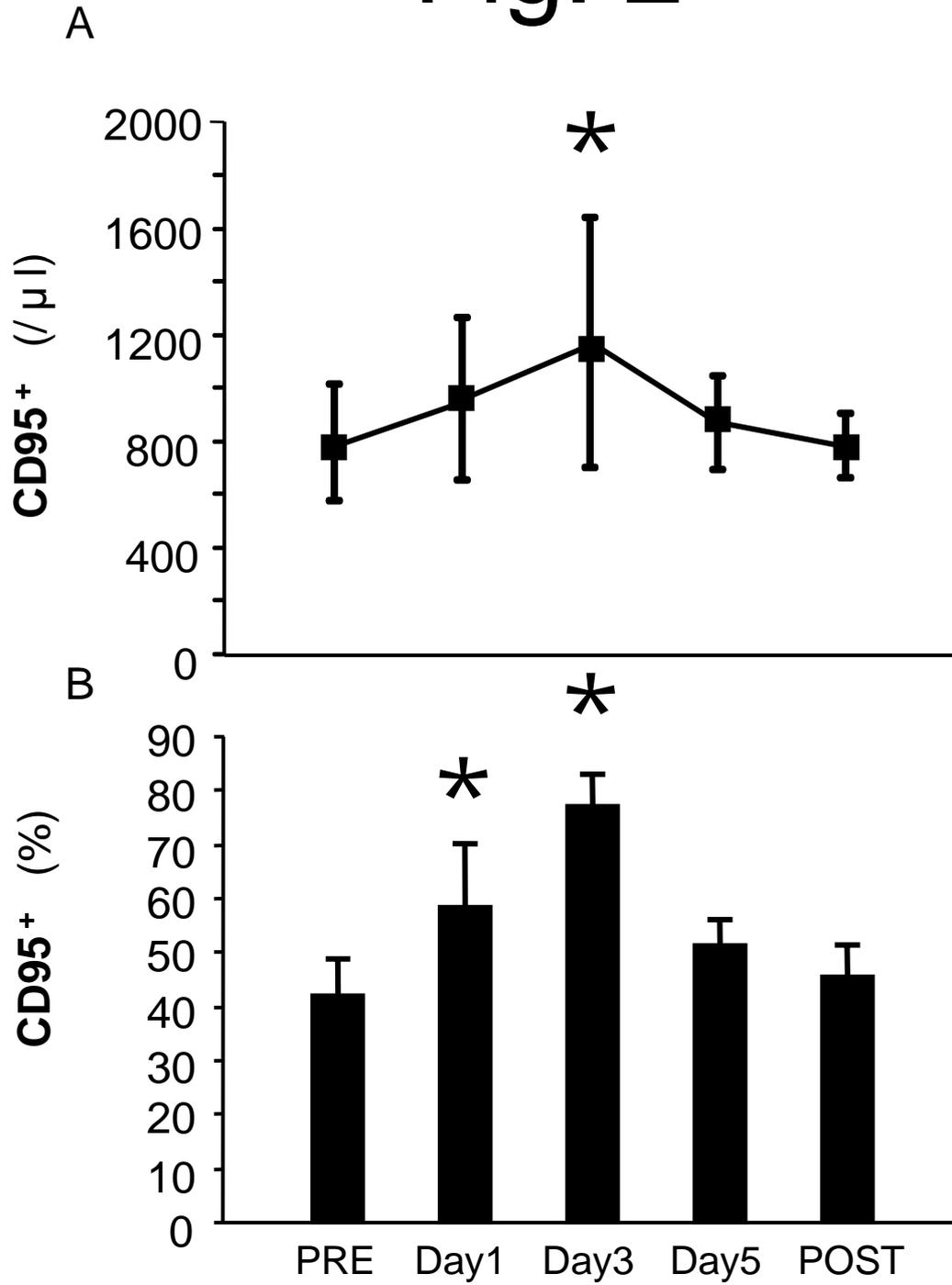


Fig. 3

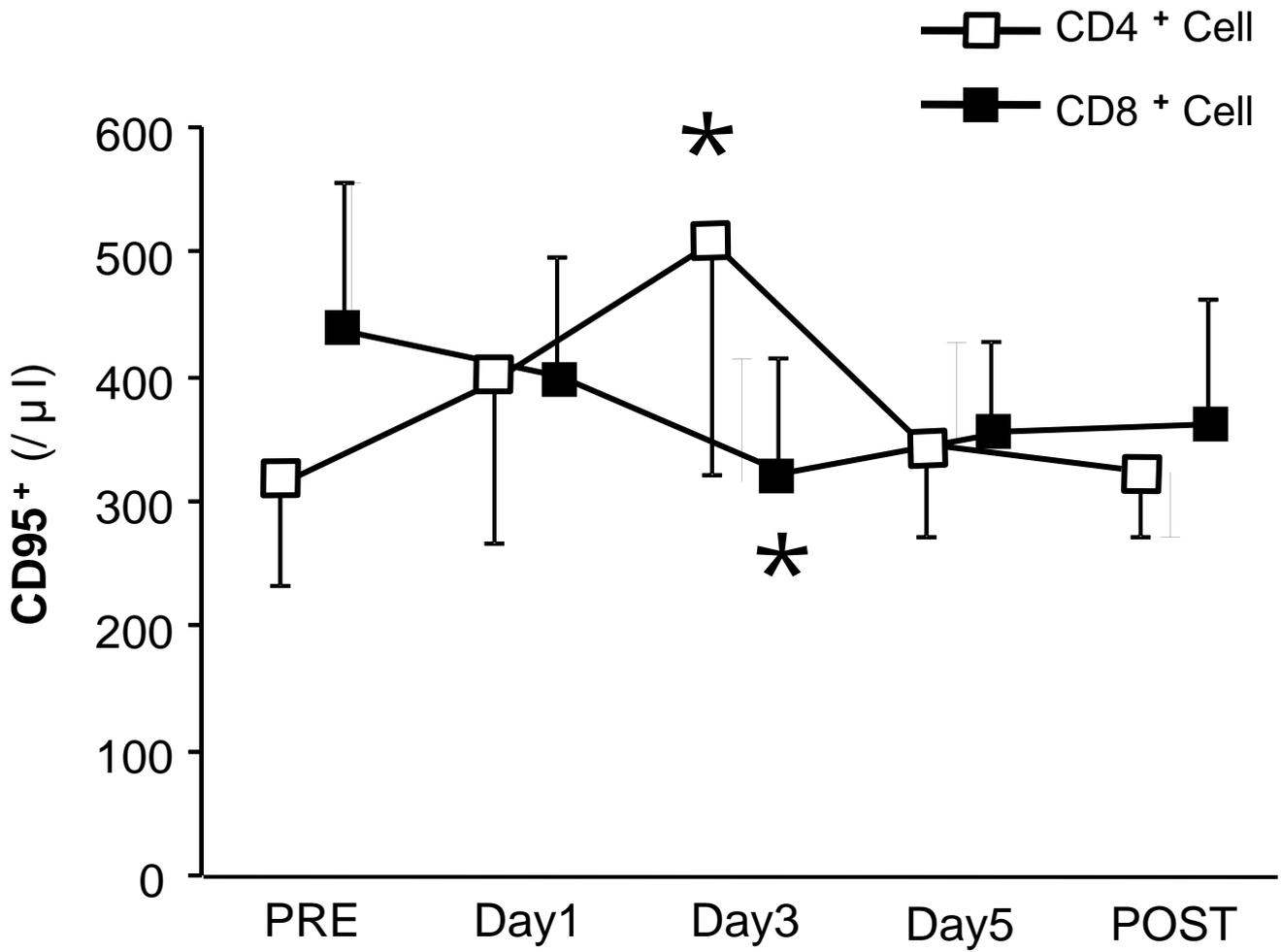


Fig. 4

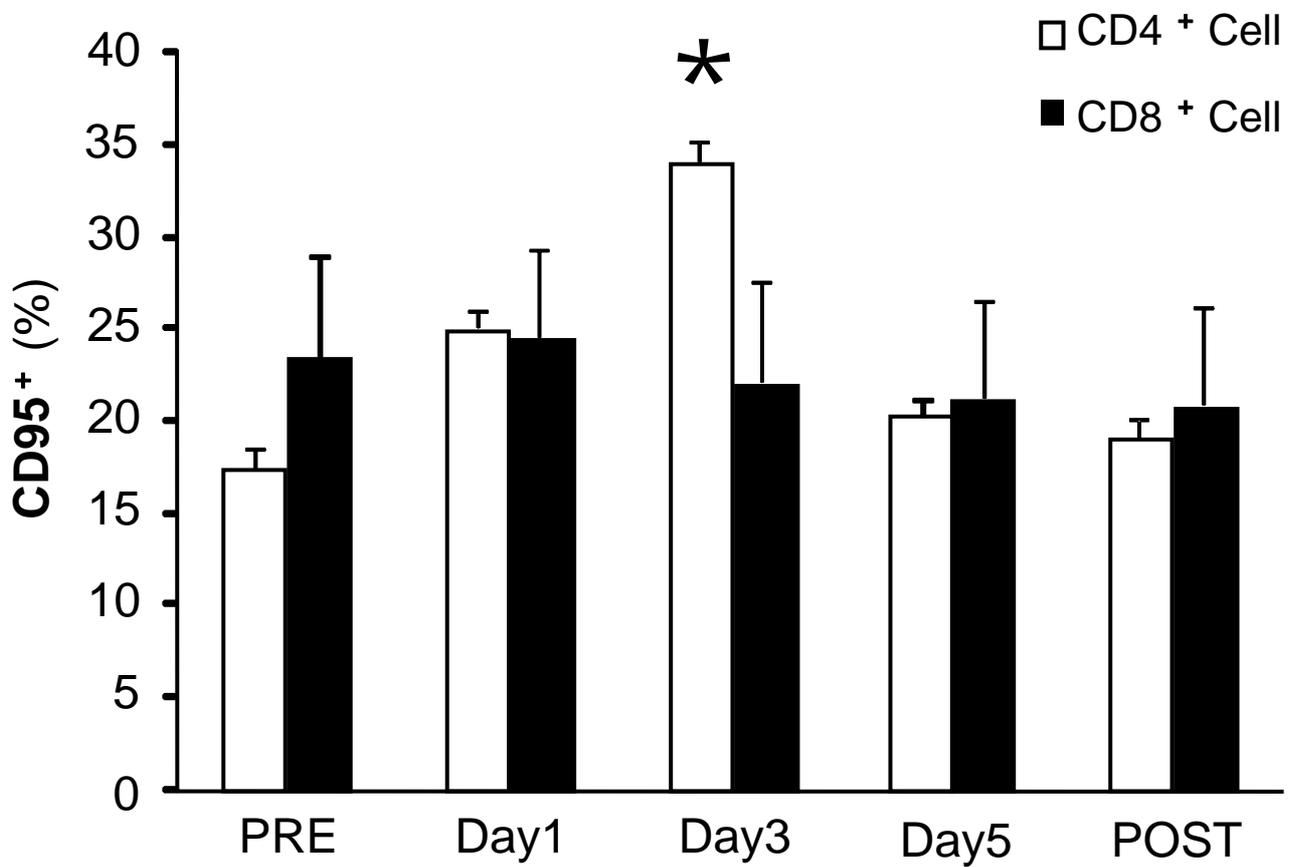


Fig. 5

