Effects of three consecutive days exercise on lymphocyte DNA damage in young men

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Abstract

Intense exercise affects the immune system. This study examines effects of three consecutive days of 1 h high-intensity exercise on lymphocyte counts, oxidative DNA damage, and apoptosis in young untrained (*n*= 8, 23.8± 3.2 years; UT) and endurance-trained (*n*= 8, 21.1± 3.7 years; TR) subjects. The subjects performed cycle ergometer exercise at 75 % $\dot{V}O_2$max 1 h daily for three consecutive days (exercise session). Blood samples were collected before exercise on the first day of the exercise session (day 1, D1) and at 24 h after the session (day 4, D4). Total lymphocyte counts, a lymphocyte oxidative DNA damage index using Comet assay with human 8-oxoguanine DNA glycosylase, oxidative stress markers, and apoptosis markers were measured. Lymphocyte counts at D1 in TR were significantly lower than in UT. Lymphocyte counts in TR changed little at D4 (from 1,988± 475 to 1,854± 363 cell/μl), but the lymphocyte counts in UT decreased significantly at D4 (from 2,583± 564 to 1,911± 528 cell/μl, $P< 0.05$). Lymphocyte oxidative DNA damage increased concomitantly with exercise sessions in both groups (UT, from 31.3± 17.5 to 48.9± 15.7%; TR, from 21.9± 5.2 to 62.1± 12.5%, $P< 0.05$). Although no change was found in apoptosis markers over time, Annexin-V^+ cells decreased in TR (effect size *D*= 0.8 is large). Three consecutive days of 1 h exercise decreased lymphocyte counts with increased lymphocyte oxidative DNA

damage in UT. Lymphocyte counts remained unchanged irrespective of increased oxidative DNA damage in TR. Decreased lymphocyte apoptosis might prevent the decrease of lymphocytes in TR.

Keywords: Short-term repeated exercise, Oxidative DNA damage, Training states, Lymphocyte apoptosis

Introduction

Exercise affects the immune system. For example, pre-exercise lymphocyte counts are higher than those after a bout of high-intensity exercise (Pedersen et al. 1998). Although many researchers have examined effects of a bout of exercise on lymphocyte counts and function, few reports describe related effects of short-term repeated exercise (i.e. training camp), which is often used and needs for strength and skill improvement in athletes. However, such excessive training sometimes engenders a state of overtraining, with depressed immune function (Gleeson et al. 1995). In addition, low lymphocyte counts of athletes at rest (Green 2002; Kajiura et al. 1995) might be related to the increased risk of upper respiratory tract infection after vigorous sports activity, such as a full marathon (Peters and Bateman 1983; Peters et al. 1993). Excessive acute physiological stress degrades immune function. Therefore, it is meaningful to examine immune function after short-term intense exercise.

Acute high-intensity exercise induces oxidative damage and/or apoptosis in lymphocytes (Mooren et al. 2002; Tsai et al. 2001), resulting in lymphocytopenia (Tanimura et al. 2008). Furthermore, high-intensity repeated exercise decreases lymphocyte counts (Malm et al. 2004). Short-term repeated exercise increases oxidative stress and lymphocyte-apoptosis indices (Hsu et al. 2002; Shing et al. 2007; Tuan et al.

2007). Consequently, decreased lymphocyte counts after short-term repeated exercise might also result from increased oxidative DNA damage and/or lymphocyte apoptosis. Investigation of the kinetics needs to elucidate mechanisms of the decrease in lymphocyte. However, both the exercise-induced increase of oxidative stress and/or lymphocyte apoptosis depends on the training status (Bloomer and Fisher-Wellman 2008; Fatouros et al. 2006; Margonis et al. 2007; Mooren et al. 2004; Niess et al. 1996). Therefore, it is unclear whether training status affects the change in lymphocyte counts after short-term repeated exercise.

As described above, we hypothesized that short-term repeated exercise increases oxidative DNA damage and/or lymphocyte apoptosis, resulting in decrease of lymphocytes in untrained subjects. However, regular exercise training in athletes prevents the decrease in lymphocytes after short-term repeated exercise resulting from a smaller increase in lymphocyte oxidative DNA damage and/or apoptosis. This study examines effects of three consecutive days of 1 h high-intensity exercise on lymphocyte counts and oxidative DNA damage, and investigates apoptosis in young untrained and endurance-trained subjects.

Methods

Subjects. After approval from the University of Tsukuba Ethics Committee, all subjects were informed of the purpose of the study. They gave their signed informed consent before participation. This study conformed to the principles outlined in the Declaration of Helsinki. Subjects were recruited all year around from the University of Tsukuba by advertisements and introduction by acquaintances. Potential subjects were screened using a medical history questionnaire and a physical examination. Key inclusion criteria were the following: male, age 20–30 years, non-smoking, taking no regular medication or supplements. Eligible subjects were young healthy male volunteers: eight untrained (UT) and eight endurance-trained (TR) undergraduate or graduate students (Table 1). The untrained subjects were those who had not participated in competitive sports during the prior 2 years. The trained subjects were triathlon athletes who exercised regularly by running, cycling, and swimming for 2–3 h per day for 5 days per week. Their athletic careers were 2.5 ± 1.6 (mean ± S.D.) years. Furthermore, the trained subjects participated in this study during their off-season.

Preliminary tests. The maximal oxygen uptake ($\dot{V}O_2max$) of subjects was measured. The value of $\dot{V}O_2max$ was determined using an incremental bicycle exercise test to exhaustion, monitoring breath-by-breath oxygen uptake and carbon dioxide production on a cycle ergometer (AE280S; Minato Medical Science Co. Ltd., Osaka, Japan). This

protocol comprised 2 min of unloaded pedaling and subsequent incremental exercise. The workload was increased to 60, 80 and 100 W for 2 min each. Subsequently, the workload was increased by 30 W every 3 min until exhaustion. Objective criteria for maximal effort included at least two of the following: (1) increased workload without a corresponding increase in $\dot{V}O_2$, (2) respiratory exchange quotient equal to or greater than 1.10, (3) a pedal cadence of < 50 rpm despite maximal voluntary effort. The highest O_2 uptake over a 30-s period was defined as $\dot{V}O_2$max.

Main trial. After at least 1 week, but within 1 month of the $\dot{V}O_2$max measurement, subjects exercised on a cycle ergometer at 75 % of their $\dot{V}O_2$max 1 h daily for three consecutive days (exercise session). Blood samples were collected from each subject immediately before exercise on the first day (day 1: D1) and then 24 h after completion of the exercise session (day 4: D4) from an antecubital vein in the arms for measurement of lymphocytes, oxidative DNA damage, lipid peroxide (LPO), cortisol, lymphocyte superoxide, CD95, and Annexin-V^+. The subjects were instructed to maintain their regular diet and hydration; no dietary change was made during the experimental period. However, all subjects fasted for at least 12 h before the day of taking blood, and were provided drinking water ad libitum during exercise periods. In addition, we prohibited subjects from alcohol intake and any individual training or intense physical activity for a

day before and during the experimental period.

Analytic methods. For serum LPO and cortisol measurements, venous blood samples were collected into a tube containing clotting activators for isolation of serum. Serum samples were generated by centrifugation at 3,000 rpm for 15 min at 4 °C. They were frozen at -40 °C until analyses of LPO and cortisol measurements. For lymphocyte isolation, oxidative DNA damage, lymphocyte superoxide, CD95, and Annexin-V, venous blood samples were collected into a tube containing clotting EDTA-2Na. Lymphocytes were prepared using density-gradient centrifugation. Adding equal parts of phosphate-buffered saline (PBS) diluted the blood samples. The solution was layered carefully on Ficoll–Paque solution (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) and then centrifuged at 3,000 rpm for 30 min at 20 °C. Subsequently, the lymphocyte band between the sample layer and the Ficoll–Paque solution was removed. The lymphocyte slurry was then washed twice with PBS and centrifuged for 5 min at 3,000 rpm and 4 °C. Finally, the cellular precipitate of lymphocytes was adjusted to 1×10^5 cells ml^{-1} using a medium [5–10 % dimethyl sulphoxide, 10–20 % fetal bovine serum, and minimal essential medium buffer (Cellbanker; Juji Field Inc., Tokyo, Japan)]. Part of the cellular precipitate of lymphocytes sample was stored in Cellbanker at -80 °C for Comet assay measurement. The remainder of the sample was resuspended in RPMI 1640

(Invitrogen Corp., CA, USA.) for measurement of the lymphocyte superoxide level and Annexin-V analysis.

Serum LPO (Biotech LPO-586; Oxis International Inc., USA.) was determined using thiobarbituric acid assay. Actually, LPO is an indicator of lipoperoxidation; it is measured as an oxidative stress marker. The measurement of lymphocyte counts (Sysmex SE-9000; Sysmex Corp., Hyogo, Japan) and serum cortisol (Immunotech, Prague, Czech Republic) (Sapin et al. 1998) using the radioimmunoassay was entrusted to Mitsubishi Chemical Medicine Corporation (Tokyo, Japan). Serum cortisol and LPO concentrations were adjusted according to changes in serum volume using Dill and Costill's equation (Dill and Costill 1974). Lymphocyte counts were adjusted for changes in blood volume. All samples from the same subject were assayed in a single run. The intra-assay coefficient of variation was 11.5 % for LPO.

Oxidative DNA damage. Measurement of oxidized DNA damage was determined using the Comet assay (hOGG1 FLARE Assay Kit; Trevigen Inc., Maryland, USA) (Singh et al. 1988). Briefly, the lymphocyte slurry was thawed and then centrifuged for 10 min at 3,000 rpm and 4 °C with RPMI 1640. The lymphocyte slurry was resuspended and adjusted to 1×10^5 cells ml^{-1} using 1 ml of ice cold 1×PBS (Ca^{++} and Mg^{++} free). These suspensions (10 µl) were mixed with 100 µl low-melting point agarose (37 °C),

then 75 µl was pipetted immediately onto a slide (about 700 lymphocytes). The slide was set flat at 4 °C in the dark for 10 min until a clear ring appeared at the edge of the sample area, then it was immersed in prechilled lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, and 1 % Triton X-100) at 4 °C overnight. After cell lysis, excess buffer was tapped-off the slide and washed three times in buffer containing 10 mM HEPES-KOH (pH 7.4), 10 mM EDTA, 0.1 M KCl. Then, 100 µl of hOGG1 enzyme (Trevigen Inc., Gaithersburg, USA) was added to each sample area as an oxidation-specific enzyme solution. The slides were placed in a humidity chamber and incubated at 37 °C for 1 h. After incubation, the slides were placed in a Coplin jar in 0.3 M NaOH, 1 mM EDTA. Then, they were incubated for 30 min at room temperature in the dark. Subsequently, they were plated for electrophoresis, which was conducted at 30 V for 30 min. The slides were dipped in 70 % ethanol for 5 min. Finally, DNA was stained with addition of 50 µl of the same SYBR Green I to each slide for 5 min after SYBR Green I was prepared from SYBR Green I nucleic acid gel stain (Trevigen Inc., MD, USA) using the same prepared solution in several batches. Each slide was viewed using fluorescence microscopy (IX71; Olympus Optical Co. Ltd., Tokyo, Japan) that was equipped with an excitation filter of 460–490 nm and a barrier filter of 510 nm.

For this study, SCGE measurements were made using image analysis with analytical

software (Comet analyzer; Youworks Corp., Ibaraki, Japan). These systems can be set-up to establish the DNA migration length, image length, nuclear size, and to calculate the tail moment. This study adopted a calculated DNA damage index: the percentage DNA in tail was calculated as the sum of tail intensity× the sum of cell intensity^{-1} (Fig. 1) (Tanimura et al. 2008). The percentage DNA in tail is the most useful index available because it is not influenced by the difference of background in fluorescence intensity (Collins et al. 1996). At least 75 randomly selected cells should be analyzed per replicated slide per sample of 700 lymphocytes, as described in the manual protocol.

Measurement of lymphocyte superoxide. The isolated lymphocytes were resuspended in 10^6 cells ml^{-1} of RPMI. The lymphocytes were incubated in 495 µl of RPMI medium with 5 µl dihydroethidium working solution (DHE; final concentration, 5 µM) (D23107; Invitrogen Corp., CA, USA) for 15 min at 37 °C (Walrand et al. 2003; Wang and Huang 2005). Following incubation, the lymphocytes were washed and resuspended with RPMI medium. The lymphocyte slurry was analyzed using flow cytometry. The results were calculated as a value relative to the PRE value (Wang and Huang 2005).

Measurement of apoptosis markers. Whole blood was incubated with CD95FasR (PE, clone 7C11, Immunotech, France) and an appropriate isotype control antibody

(IgG1, clone DAK-GO1, DakoCytomation, Denmark) for 15 min at room temperature. Then, 100 µl of lysis solution (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA-2Na) was added to break up red blood cells. After 10 min of incubation, they were centrifuged for 5 min at 3,000 rpm. After the supernatant was removed, the cells were washed in PBS (0.1 % BSA, 0.1 % NaN_3) and resuspended in PBS. Cell death was evaluated using flow cytometry with Annexin-V FITC and nuclear propidium iodide (PI) (Annexin FITC kit; Immunotech, Marseilles, France) uptake to detect apoptosis and necrosis, respectively (Vermes et al. 1995). Annexin-V^+ is a sensitive and early marker of apoptotic cells because these cells lose membrane phospholipid asymmetry, which can cause exposure of phosphatidylserine at the cell surface. The isolated lymphocytes were washed with RPMI medium and centrifuged for 5 min at 3,000 rpm to remove platelets. The isolated pellets were resuspended in 5×10^6 cells ml^{-1} of binding buffer and kept on ice. Annexin-V solution (1 µl) and PI (5 µl) were added to 100 µl (5×10^5 cells) of the cell suspension for labeling. This labeling solution was incubated for 15 min in the dark on ice. The lymphocyte suspension was added to 400 µl of binding buffer and mixed gently. Lymphocytes labeled as CD95FasR and Annexin-V positive cells were analyzed using flow cytometry (Miyawaki et al. 1992).

Flow cytometry. All lymphocyte phenotype analyses were conducted using a flow

cytometer (FACSCalibur; BectonDickinson Immunocytometry Systems, CA, USA). An electronic gate was placed around the lymphocyte population in the flow cytometry forward and side scatter modes. Standard gating procedures mainly used lymphocytes (10^5 cells) with the gating technique. The negative control was always set-up with the machine voltage, whose antibody staining had no antigen control. An electronic marker was placed at the limit of negative control to quantify the percentage of lymphocyte that was positive and negative for each cell-surface antigen. All flow cytometry parameters were analyzed using CELLQuest software (BectonDickinson Immunocytometry Systems, CA, USA) and WinMDI 2.9 software (Joseph Trotter, The Scripps Research Institute, CA, USA). All samples from the same subject were assayed in a single run. The intra-assay coefficient of variation was 2.4% for FACS.

Statistical analysis. All data were presented as means with standard deviations (SD), and analyzed using software (StatView 5.0; SAS Institute Inc., NC, USA). Two-way (groups × time) analysis of variance (ANOVA) with repeated measures was used to examine differences between the two groups overtime for lymphocyte counts, percentage DNA in tail, LPO, superoxide, CD95, Annexin-V and serum cortisol. For cases in which a significant interaction was detected, post hoc pairwise comparisons were performed using Tukey's method. Unpaired *t* tests for uncorrelated data with a two-tailed hypothesis

were used to assess the physical characteristics of TR group and UT group. Paired *t* tests for correlated data with a two-tailed hypothesis were used to assess time-dependent changes in the same group. Statistical significance was inferred for $p<0.05$.

Results

All subjects completed the exercise session (cycle ergometer exercise at 75 % of their $\dot{V}O_2$max 1 h daily for three consecutive days). As presented in Table 1, a significant difference was found in the values of $\dot{V}O_2$max between TR and UT.

Differences in lymphocyte counts were found between TR and UT (Fig. 2). Lymphocyte counts at D1 were significantly lower in TR than in UT (Fig. 2). Lymphocyte counts decreased significantly from D1 to D4 in UT, but they did not change in TR (Fig. 2). Therefore, lymphocyte counts in UT were almost identical to those in TR at D4 (Fig. 2).

The percentage DNA in tail as a lymphocyte oxidative DNA damage index at D4 was significantly greater than that at D1 in either group (Fig. 3).

Changes in oxidative stress markers are presented in Table 2. Neither serum LPO concentration nor the superoxide level in lymphocyte was significantly different between groups. It did not change significantly between D1 and D4.

No significant difference was found between groups at any time point in $CD95^+$ and Annexin-V^+ lymphocyte cells (Table 3), nor was there a significant change in these cell subsets (data not shown). The Annexin-V^+ level decreased at D4 in TR insignificantly (P=0.07). However, the effect size of Cohen D was large (0.8), 95% confidence intervals

(CI); -1.0- 18.0, its statistical power was 45% (Cohen 1988).

Serum cortisol concentrations between D1 and D4 were not significantly different (Table 4). Although the cortisol concentration at D1 was greater in TR than in UT insignificantly (*P*= 0.07), the effect size of Cohen *D* was large (0.9), 95% CI; -7.5- 0.4, its statistical power was 42% (Cohen 1988).

Discussion

This study demonstrated that a significant decrease in lymphocyte counts with increase in lymphocyte oxidative DNA damage occurred in UT after high-intensity exercise during three consecutive days. However, lymphocyte counts did not change irrespective of the significant increase in oxidative DNA damage in TR. Apoptosis markers did not increase after three consecutive days of 1 h aerobic exercise in both groups. However, the Annexin-V^+ level decreased and its effect size of Cohen's *D* was large (*D*= 0.8) in TR. Therefore, the decrease in lymphocyte apoptosis might prevent a decrease in lymphocyte in TR.

High-intensity aerobic exercise of three consecutive days decreased lymphocyte counts at D4 in UT significantly, but not in TR. A bout of exercise-induced transient lymphocytopenia in both sedentary and trained men (Nieman et al. 1994; Tanimura et al. 2008). Therefore, the difference might be explained that lymphocytopenia after exercise recovers faster in TR than in UT. However, Malm et al. (2004) reported that a5-day training camp decreased lymphocyte counts at rest in athletes. The different results can be explained by the difference in the exercise load (this study, 1 h/day, 3 days; Malm's study, several hours/day, 5 days).

A lymphocyte oxidative DNA damage index, the percentage DNA in tail, was

significantly greater at D4 than at D1 in both groups. Although a few studies have investigated effects of repeated exercise on lymphocyte oxidative DNA damage (Okamura et al. 1997; Selman et al. 2002), the results of this study are not consistent with those of previous studies. Okamura et al. (1997) reported that an 8-day training camp increased uric 8-OHdG without lymphocyte 8-OHdG change. They suggested that the action of DNA repair system in lymphocytes prevented lymphocyte 8-OHdG change. The different results can be explained by the different exercise durations and the methods of detecting oxidative damage.

Although oxidative DNA damage increased significantly in both groups, serum LPO concentration and lymphocyte superoxide did not change significantly between D1 and D4 in either group. Acute exercise generally increases oxidative stress with increasing ROS after exercise (Alessio 1993; Tanimura et al. 2008). However, oxidative stress markers usually recover at 24 h after exercise (Pittaluga et al. 2006). Furthermore, Viguie et al. (1993) reported that increased lymphocyte superoxide after exercise reverted to normal levels at 1 h after exercise. These earlier studies suggest that increased ROS and oxidative stress markers usually recover at 24 h after exercise. The transient increase in oxidative stress during several hours after exercise might increase the lymphocyte oxidative DNA damage at 24 h after exercise, although we detected no evidence of such

an increase.

Time-dependent changes in $CD95^+$ and Annexin-V^+ cells were not observed in either group. However, effect sizes of Annexin-V^+ cells change in TR are large (D= 0.8): there was a tendency by which Annexin-V^+ cells in TR decreased at D4. Mooren et al. (2004) demonstrated that these markers decreased significantly at 24 h after acute exercise; this decrease was significantly great in the high-trained group. The increase in apoptosis was reportedly modified by DNA repair (Mooren et al. 2004). Therefore, our result can be explained by modification of DNA damage (Radak et al. 2003; Sato et al. 2003) acquired during exercise training (Radak et al. 2002). The decrease in lymphocyte apoptosis might prevent a decrease in lymphocyte in TR.

Lymphocyte counts at rest in TR were lower than those in UT. The increase in cortisol levels is related to the decrease in lymphocyte counts (Shephard and Shek 1996). Although the difference in serum cortisol concentrations between UT and TR at D1 was not statistically significant, its effect size was large (D= 0.9). Therefore, one reason for the decrease in lymphocyte in athletes might be a chronic increase in cortisol levels in athletes. However, it remains unclear whether cortisol levels completely explain the low level of lymphocyte in TR or not.

There is the limitation of this present study. We had carried out an experiment on the

assumption that the change of lymphocyte oxidative DNA damage affect on lymphocyte counts after exercise. Thereby, we used Comet assay with enzyme to evaluate lymphocyte oxidative DNA damage. However, we did not measure DNA repair system (antioxidant, DNA repair enzyme), because Comet assay did not evaluate DNA repair system. This point is the limitation of this study. Further studies need to measure DNA repair system.

We evaluated samples obtained before and 24 h after exercise session in this study because we sought to evaluate lymphocyte change and oxidative DNA damage without the effect of acute exercise and circadian rhythm on these changes. The exercise protocol in this study was adopted as one that is likely to induce lymphocytopenia after a bout of exercise for untrained subjects (Tanimura et al. 2008).

The results described herein suggest that regular exercise training affects resistance to short-term repeated exercise stress. Additional studies must identify the appropriate exercise loads that can preserve immune functions while supporting good conditioning in athletes.

In conclusion, three consecutive days of 1 h high-intensity aerobic exercise decreased lymphocyte counts with increased lymphocyte oxidative DNA damage in young untrained men. Lymphocyte counts did not change, irrespective of increased

oxidative DNA damage in young trained men. Decreased lymphocyte apoptosis might prevent a decrease in lymphocytes in trained men.

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Figure legends

Figure 1. Normal lymphocyte (a) and DNA-damaged lymphocyte (b). Percentage DNA in tail was calculated by the sum of tail intensity/sum of cell intensity as DNA damage index. Sum of tail intensity: brightness total of the tail, +sum of cell intensity: brightness total of the whole cell (Tanimura et al. 2008).

Figure 2. Effect of exercise session on total lymphocyte counts in untrained and trained subjects.

Values are mean ± SD.

* Significantly different from D1 in each group, $P< 0.05$.

† Significantly different from UT group, $P< 0.05$.

Figure 3. Effects of exercise session on oxidative DNA damage in untrained and trained subjects.

Values are mean ± SD.

* Significantly different from D1 in each group, $P< 0.05$.

Table 1. Physical characteristics

	Untrained	Trained
Age (years)	23.8 ± 3.2	21.1 ± 3.7
Height (cm)	170.7 ± 3.7	170.5 ± 3.4
Weight (kg)	63.5 ± 6.7	63.6 ± 3.7
Body mass index (kg/m^2)	21.9 ± 2.6	22.0 ± 1.2
Percentage body fat (%)	16.2 ± 4.4	14.1 ± 1.2
$\dot{V}O_2$ max (ml/ min/ kg)	40.7 ± 3.8	53.5 ± 3.5[a]

Values are represented as mean ± SD.

[a] Significantly different from untrained groups, $P< 0.05$.

Table 2. Effects of exercise sessions on oxidative stress markers of untrained and trained subjects

	D1	D4
Serum lipid peroxidation concentration (μM)		
Untrained	1.45± 0.91	1.32± 0.60
Trained	1.05± 0.50	0.93± 0.31
Superoxide in lymphocyte (%)		
Untrained	100	96± 67
Trained	100	97± 35

Values are represented as mean ± SD.

Table 3. Effects of exercise sessions on apoptosis markers of untrained and trained subjects

	D1	D4
CD 95-positive cell (%)		
Untrained	12.0± 8.7	10.5± 7.0
Trained	15.5± 15.2	13.2± 8.8
Annexin-V positive cell (%)		
Untrained	21.0± 6.4	21.0± 9.6
Trained	22.3± 9.8	13.8± 5.9

Values are represented as mean ± SD.

Table 4. Effects of exercise sessions on serum cortisol concentrations of untrained and trained subjects

	D1	D4
Cortisol (μg/dl)		
Untrained	16.1± 4.2	15.4± 3.0
Trained	19.6± 3.1	19.2± 2.6

Values are represented as mean ± SD.

Figure 1
(Tanimura et al.)

A

B

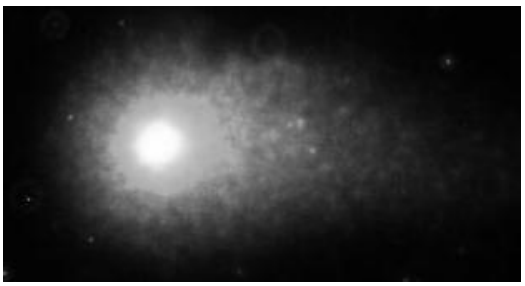

Figure 2
(Tanimura et al.)

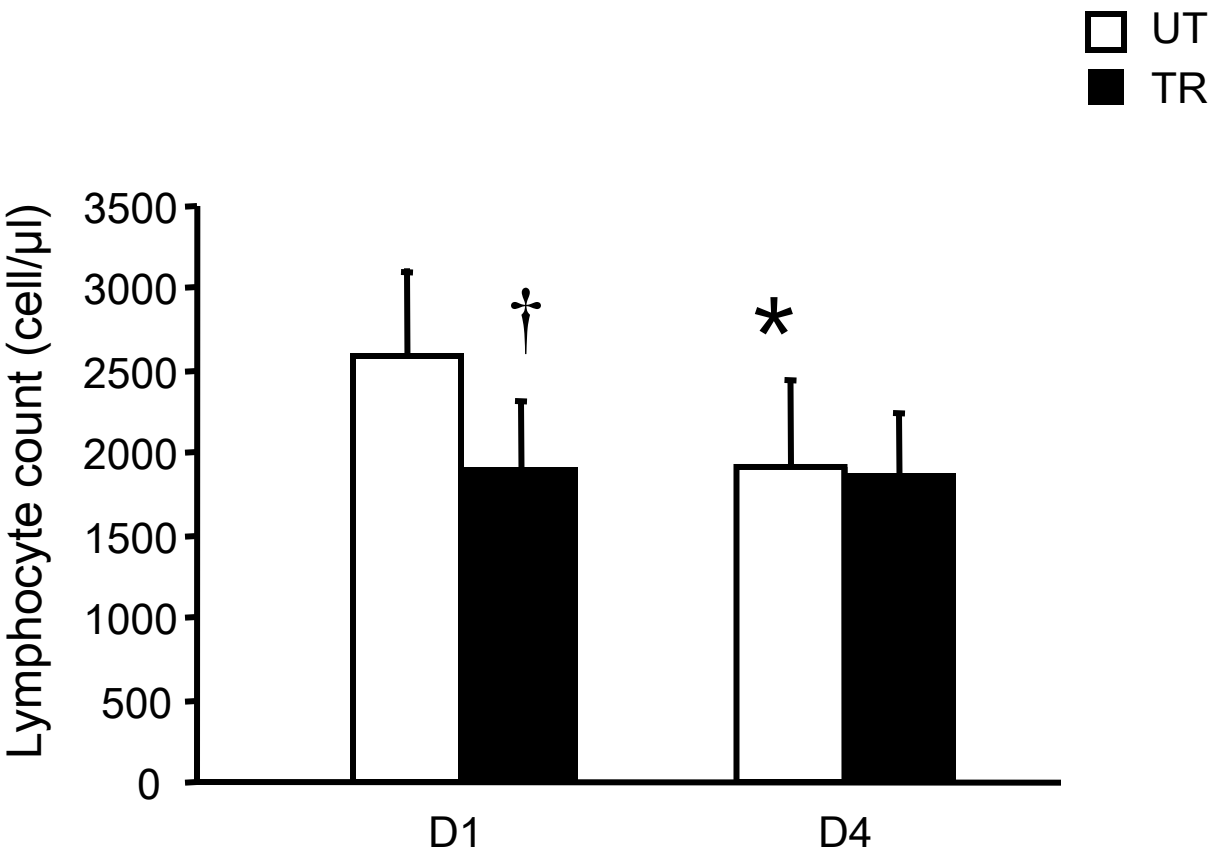

Figure 3
(Tanimura et al.)