A single bout of dynamic exercise enhances the expansion of MAGE-A4 and PRAME-specific cytotoxic T-cells from healthy adults

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ABSTRACT

The ex vivo expansion of tumor-associated-antigen (TAA)-specific cytotoxic T-cells (CTLs) from healthy donors for adoptive transfer to cancer patients is now providing additional treatment options for patients. Many studies have shown that adoptive transfer of expanded CTLs can reduce the risk of relapse in cancer patients following hematopoietic stem cell transplantation (HSCT). However, the procedure can be limited by difficulties in priming and expanding sufficient numbers of TAA-specific-CTLs. Because acute dynamic exercise mobilizes large numbers of T-cells to peripheral blood, we hypothesized that a single bout of exercise would augment the ex vivo expansion of TAA-specific-CTLs.We therefore collected lymphocytes from blood donated by healthy adults at rest and after brief maximal dynamic exercise.TAA-specific CTLs were expanded using autologous monocyte-derived-dendritic cells pulsed with melanoma-associated antigen 4 (MAGE-A4), with preferentially expressed antigen in melanoma (PRAME), and with Wilms' tumor protein (WT-1). Post exercise, 84% of the participants had a greater number of CTLs specific for at least one of the three TAA.Cells expanded from post exercise blood yielded a greater number of MAGE-A4 and PRAME-specific-cells in 70% and 61% of participants, respectively. In the 'exercise-responsive' participants (defined as participants with at least a 10% increase in TAA-specific-CTLs post-exercise), MAGE-A4- and PRAME-specific-CTLs increased 3.4-fold and 6.2-fold respectively. Moreover, expanded TAA-specific CTLs retained their antigen-specific cytotoxic activity. No phenotype differences were observed between expanded cells donated at rest and postexercise. We conclude that exercise can enhance the ex vivo expansion of TAA-specific-CTLs from healthy adults without compromising cytotoxic function. Hence, this study has implications for immunotherapy using adoptive T-cell transfer of donor-derived T-cells after allogeneic HSCT.

Key words: Immunotherapy, CTLs, physical activity, adoptive transfer, tumor-associated-antigens

INTRODUCTION

Immunotherapy-based cancer treatments are providing a new wave of treatment options in conjugation with more traditional approaches, including chemotherapy and hematopoietic stem cell transplantation (3). The adoptive transfer of *ex vivo* expanded tumor-associated-antigen (TAA)-specific cytotoxic T-cells (CTLs) has been shown to be a potentially potent approach for the treatment of various types of cancer, including metastatic melanoma (26), lymphoma (5), neuroblastoma (29), and lymphocytic and myeloid leukemia (12, 17). However, there are challenges that must be overcome before the treatment can be used more broadly in cancer patients with various malignancies. Techniques for manufacturing antigen-specific CTLs using GMP compliant methodologies for patients after allogeneic HSCT have arisen from studies using virus-specific T-cells, where products can now be rapidly manufactured in less than 10 days (16, 31, 40). A key factor of this technique is the selection of target antigens where a natural occurrence of T-cells specific for that antigen can be found. However, non-viral TAA are often self-antigens, which in healthy donors are infrequent and usually also of low avidity, as most self-reactive T-cells are anergized (13). Therefore, extensive cell stimulationsare required to expand sufficient numbers of TAA-specific CTLs for adoptive transfer. Further, results are often marred by the concomitant expansion of CTLs that are reactive to healthy cells (13, 23, 25, 42). Despite recent advances that have allowed researchers to overcome difficulties in adoptive transfer immunotherapy, such as avoiding mechanisms of tumor escape (7, 13, 23), the limitation of expanding sufficient numbers of tumor-reactive CTLs from healthy donors remains, even if an apheresis procedure is performed.

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A single bout of dynamic physical exercise (e.g., running or cycling) markedly increases the numbers of leukocyte subtypes in peripheral blood and alters their composition. This occurs due to mechanisms such as shear stress and increases in catecholamines, glucocorticoids, and other hormones that have direct impact on blood leukocytes (41). A single bout of exercise causes a 2-4 fold increase in the number of circulating blood monocytes and lymphocytes, the magnitude of which is related to the intensity and duration of the exercise (1, 22, 39, 41). Exercise specifically mobilizes CTLs with a phenotype consistent with tissue migration and enhanced cytotoxicity, and also increases the expression of molecules associated with T-cell activation (15, 19). The redeployment of these activated cells by exercise may provide a means to overcome the difficulty in obtaining sufficient numbers of functional TAA-specific CTLs from healthy donors.

The aim of this study was to determine if a single bout of dynamic exercise would enhance the *ex vivo* expansion of TAA-specific CTLs. Three leukemia- and lymphoma-associated antigens were selected as representative targets: the cancer-testis antigen melanoma-associated antigen 4 (MAGE-A4), and the antigens overexpressed by malignant cells preferentially-expressed antigen of melanoma (PRAME) and Wilm's Tumor 1 (WT-1).The robust expansion of MAGE-A4-specific CTLs for clinical use would improve treatment options for Hodgkin's Lymphoma patients with EBV-negative tumors (32),while enhancing the expansion of PRAME (38)- and WT-1 (8)-specific CTLs would offer an important T-cell therapeutic to patients with acute leukemias after allogeneic HSCT. We demonstrate here that a single bout of dynamic exercise increases the yield of MAGE-A4 and PRAME-specific CTLs in the majority of the healthy adult participants. These findings indicate that exercise is a simple yet effective- and economical approach to enhance the expansion of TAA-specific CTLs for adoptive T-cell therapy post allogeneic HSCT.

METHODS

Experimental Design

This was a within-subjects repeated measure study of 19 healthy adults (10 women) designed to assess the effects of brief dynamic exercise on the expansion of TAA-specific T-cells. Standard sample size calculations using an estimated effect size (d) of 0.6 and α=0.05 indicated that 19 participants were expected to yield a power of 0.8 to detect differences between resting and post exercise cells. Unstimulated and expanded cells donated at rest and post exercise were analyzed using flow cytometry, IFN-γ ELISPOT assays, and ^{51}CR release cytotoxicity assay (expanded cells only).

Participants

Healthy adult volunteers were recruited in Houston, TX for this study. Written informed consent and medical history were obtained from each participant after the procedures, benefits, and risks were explained verbally and provided in writing. Institutional review boards at the University of Houston and at Baylor College of Medicine granted ethical approval for the study. Participants were instructed to avoid alcohol, nonprescription drugs, and strenuous exercise 24h prior to each laboratory visit.

Exercise trials and blood sampling

Participants visited the laboratory between 7am and 10am on two occasions separated by 7 days. During Visit 1, participants were asked to ascend 260 stairs (10 floors) as quickly as possible. Completion time, maximal heart rate (Polar Electro, USA), and circulating lactate values were recorded in 11 participants. Earlobe capillary blood samples were drawn using heparin-lined microcapillary tubes at rest and post exercise, and analyzed in duplicate for lactate concentration using an automated lactate analyzer (Analox P-GM7 Micro Stat, Analox, UK). A 3ml venous blood sample was collected at rest and immediately post exercise in vacutainer tubes treated with ethylene-diamine-tetra-acetic acid (EDTA) (Becton-Dickenson, USA) for differential leukocyte cell counts (BC3200, Mindray North America, Mahwah, NJ). A 40ml venous blood sample was collected at rest and post exercise in vacutainer tubes treated with sodium heparin (Becton-Dickenson) for monocyte-derived (mo)-dendritic cell (DC) generation and CTL expansion. An additional blood sample was taken at rest and post exercise into a 6ml serum gel tube (Becton-Dickenson). Blood was processed within 4 hours of being drawn. Blood serum was frozen at -80°C until analysis. During Visit 2, participants donated a resting 40ml blood sample collected in sodium heparin vacutainer tubes. At the second visit, 13 participants also completed the Bruce Maximal Exercise Test (9) on a treadmill (Woodway Desmo, WoodwayUSA Inc, Waukesha, WI) until volitional exhaustion. The speed and incline increased at three-minute intervals to increase the intensity. Heart rate, ventilation, and oxygen consumption were measured throughout the test using an automated metabolic cart (Cosmed Quark CPET, Rome, Italy).

Generation of DCs from blood

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Lymphoprep; Nycomed) density gradient separation. Mo-DCs were generated by plate adherence of PBMCs. PBMCs were incubated for 2 hours in DC medium (CellGenix media with 2mM L-glutamine (GlutaMAX; Invitogen)) at a concentration of 10 x 10^6 cells/well in a 6-well plate (Costar). Nonadherent cells were removed by gentle washing with PBS (Sigma), and cryopreserved at -80°C for later stimulation with mature mo-DCs. Adherent cells were cultured in DC media with 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 400U/ml interleukin-4 (IL-4) (both R&D Systems) for 5 days at 37° C in a humidified CO_2 incubator. On day 5, immature mo-DCs were harvested by gentle rinsing and resuspended at 0.5 x 10^6 cells/ml in DC medium and matured in a cytokine cocktail of GM-CSF (800 U/ml), IL-4 (400 U/ml), IL-1β (10ng/ml), IL-6 (10ng/ml), tumor necrosis factor-α (TNF-α; 10ng/ml, all R&D), and prostaglandin E2 (PGE2; 1μg/mL; Sigma-Aldrich). On day 7, mature mo-DCs were harvested and used as antigen presenting cells.

Expansion of tumor-associated-antigen-specific T-cells

Mature mo-DC were incubated with each TAA, usingthe peptide libraries of MAGE-A4, PRAME, and WT-1 (100 ng/PepMix; JPT Peptide Technologies), in 10 μl PBS for 1 hour at

37° C. Autologous, cryopreserved, non-adherent PBMCs were thawed and stimulated with the TAA-pulsed mo-DCs at a stimulator-to-effector ratio of 1:10. Non-adherent PBMCs isolated from resting blood were stimulated with mo-DCs generated from resting blood, and non-adherent PBMCs isolated from post exercise blood were stimulated with mo-DCs generated from post exercise blood. Cells were cultured 7 days at 1 x 10^6 cells/ml in 24 well plates (Costar) in 2ml RPMI-1640 supplemented with 45% Clicks media (Irvine Scientific), 5% human AB serum, and 2mmol/L GlutaMax, and recombinant human IL-7 (10ng/ml), IL-12 (10ng/ml), and IL-15 (5ng/ml)(all Peprotech). After 7 days, cells were harvested and restimulated with autologous TAA-pulsed mo-DCs at a ratio of 1 to 4 for a further 7 days. All mo-DCs for the second stimulation were generated from resting blood. Cells were split and fed with fresh media containing IL-7, IL-12, and IL-15 on days 5 and 12. Due to limitations in the number of available cells, all three TAA-specific cell lines were not expanded for each participant; MAGE-A4 specific cells were expanded from 17 participants, PRAME specific cells were expanded from 18 participants, and WT-1 specific cells were expanded from 13 participants.

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) analysis was conducted on PBMCs and twice-stimulated (expanded) cells using IFN-γ ELISPOT multiscreen IP plates (Millipore, USA). Cells were stimulated overnight with MAGE-A4, PRAME, and WT-1 PepMixes (1 μg/ml) at a concentration of 150000 cells/well (PBMCs) or 100000 cells/well (expanded cells) in a 37° C humidified CO_2 incubator. Cells stimulated with staphylococcal enterotoxin B (1μg/ml; Sigma-Aldrich) served as a positive control, and cells stimulated with Ad-penton (JPT Peptide Technologies) or incubated in media only served as negative controls. Each condition was measured in triplicate. Plates were evaluated by Zellnet Consulting. Spot-forming cells (SFCs) were enumerated to obtain the frequency of TAA-reactive cells.

Cytotoxicity assay

The cytotoxic specificity of TAA-specific T-cells expanded from resting and post exercise blood were analyzed in a standard 4h ^{51}Cr release assay using effector:target ratios of 40:1, 20:1, 10:1, and 5:1. Target cells were autologous Phytohemagglutinin(PHA)-blasts pulsed with each TAA pepmix, and were generated from non-adherent cells cultured for one week in the presence of IL-2 (100U/mL; Chiron) and PHA (Sigma, 5μg/mL) and fed every other day. A portion of the resultant PHA-blasts were pulsed for 1h with TAA-PepMix (0.2μg) and used as autologous target cells. The remaining PHA-blasts were pulsed with irrelevant (Ad-penton) peptide and used as controls to measure non-specific T-cell cytotoxicity. All PHA-blasts were pulsed with 10μl ^{51}Cr for 1 h. ^{51}Cr – labeled target cells were mixed with effector cells at doubling dilutions to produce the desired ratios. Target cells incubated in complete medium or 5% Triton X-100 (Sigma Aldrich) were used to determine spontaneous and maximum ^{51}Cr release respectively. Supernatants were collected after 4h and radioactivity was measured on a gamma counter. The mean percentage of specific lysis was calculated as: 100 x (experimental release – spontaneous release)/(maximal release – spontaneous release. Each condition was measured in triplicate.

Flow cytometry

To document exercise-induced shifts in leukocyte subsets in peripheral blood, PBMCs from rest and post exercise were labeled with FITC-conjugated anti-CD45RA (IgG2b, clone HI100), anti-CD28 (clone CD28.2) or Alexa488-conjugated anti-KLRG1 (clone 13D12F2), PE-conjugated anti-CD57 (clone TB01), anti-CD62L(clone DREG-56), or anti-CD27 (IgG1, clone O323), PerCP-Cy5.5-conjugated anti-CD4 (IgG2b, clone OKT-4), anti-CD8 (IgG1, clone RPA-T8), or anti-CD56 (IgG1 Clone CMSSB), and APC-conjugated anti-CD3 (IgG1, Clone UCHT1) in a four-color direct immunofluorescence procedure. All monoclonal antibodies were previously titrated to determine optimal dilutions. Cells were incubated with 50 μl of each pre-diluted mAb for 30 minutes in the dark at room temperature. Antibodies were purchased from eBioscience Inc (San Diego CA, USA), except anti-CD57 (Abcam, Cambridge, UK) and the anti-KLRG1, which was kindly provided by Hans Peter Pircher of the University of Freiburg, Germany (21). To document the phenotypes of the expanded cells, aliquots of twice-stimulated cells were labeled with the above antibody panel in a four-color direct immunofluorescence procedure.

Cell phenotypes were assessed on a BD Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI, USA) equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wave length of 640 nm. Lymphocytes were identified by forward and side scatter characteristics and gated electronically using Accuri C6 (CFlow software v1). Single color tubes were used for compensation. A minimum of 20,000 events in the lymphocyte gate were collected. Naïve $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-cells were identified as $CD45RA^+CD62L^+$, central memory (CM) cells were identified as $CD45RA^-CD62L^+$, effector memory (EM) cells were identified as $CD45RA^-CD62L^-$ and the $CD45RA^+$ highly differentiated effector memory (EMRA) cells as $CD45RA^+CD62L^-$(30).

Measurement of blood hormones and determination of viral serostatus

ELISA kits were used to determine serum levels of epinephrine and norepinephrine (2-CAT ELISA, LDN, Nordhorn, Germany), cortisol (Abcam, Cambridge, MA), and neopterin (IBL International, Toronto ON, Canada), and to detect IgG antibodies against CMV (Genway Biotech, San Diego, CA) and EBV (EBV-VCA, IBL International, Toronto ON, Canada). Assays were performed according to manufacturers' directions using a 96 well microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

Data were screened for normality and transformed (logarithm or square root) when required. Paired T-tests were used to compare the number and phenotype of PBMCs and CTLs and the number of SFCs from blood donated at rest and post exercise. Independent T-tests were used to compare participant characteristics between exercise-responders and exercise-non-responders; Fisher's exact test was used to compare categori-

cal variables and group. Restricted maximum likelihood linear mixed models were used to compare physiological responses to exercise, as well as the number and phenotype of PBMCs and CTLs from rest and post exercise, among the exercise-responders and exercise non-responders. The models included main effects of exercise-time (resting and post exercise) and group assignments (MAGE-A4- and PRAME- exercise-responders and exercise-non-responders), and interaction effects between exercise-time and each group. Analyses were performed using Statistical Package for the Social Sciences version 17 software (SPSS, Chicago, IL, USA). Statistical significance was set at $p<0.05$.

RESULTS

Exercise increases leukocyte number for expansion

We first evaluated the effect of exercise on absolute white cell numbers. As shown in **Table 1**, brief exercise (i.e. rapidly ascending 260 steps, led to a significant increase in the number of leukocytes, lymphocytes, and monocytes in whole blood, and increased the numbers of T-cells in the peripheral blood mononuclear cell (PBMC) fraction. To determine whether CTLs could be expanded from both resting and post exercise blood obtained from healthy donors, PBMCs isolated from blood at rest and post exercise were stimulated with autologous TAA-pulsed mo-DCs in the presence of interleukin- (IL-)7, IL-12, and IL-15. While a greater number of PBMCs were stimulated post exercise **(Table 2)**, the numbers of T-cells expanded from resting versus post exercise blood samples following the second week of stimulation were the same **(Table 2)**. Further, the fold- expansion of the CTLs calculated from the total number of input PBMCs did not differ with exercise **(Fig. 1A)**; however, when the fold-expansion was calculated relative to the number of input naïve ($CD45RA^+CD62L^+$) T-cells, the rate of expansion was significantly greater post exercise (mean ±SD: day 7 resting: 9.90 ±4.58, post exercise: 17.07 ±9.84; t(10)=-3.793, p=0.004;day 14 resting: 71.35 ±55.36, post exercise: 115.0 ±101.41; t(10)=-2.683, p=0.023) **(Fig. 1B)**.

Table 1. Cell numbers at rest and post exercise. Data are displayed as mean ±SEM (range). Significant differences from resting are indicated by ** (p<0.01) and * (p<0.05). PBMCs: Peripheral blood mononuclear cells.

Source	Cell type	Resting	Post exercise
Whole blood	Leukocytes ($x10^3$ cells/µl	5.26 ±2.0 (3.6-10.7)	9.28 ±2.8 ** (6.8-17)
	Lymphocytes ($x10^3$ cells/µl)	1.54 ±0.29 (1.1-2.1)	3.56 ±0.72 ** (2.6-5.3)
	Monocytes ($x10^3$ cells/µl)	0.39 ±0.12 (0.25-0.60)	1.04 ±0.31 ** (0.50-1.70)
	Granulocytes ($x10^3$ cells/µl)	3.33 ±1.69 (1.8-8.0)	4.67 ±1.99 ** (2.8-10.0)
PBMCs	CD3+T-cells ($x10^3$ cells/µl)	1.10 ±0.29 (0.66-1.63)	1.80 ±0. 51** (1.07-2.91)
	CD45RA+CD62L+ T-cell ($x10^3$ cells/µl)	0.53 ±0.18 (0.27-0.81)	0.65 ±0.22** (0.30-1.01)
	CD45RA-CD62L+ T-cell ($x10^3$ cells/µl)	0.31 ±0.10 (0.18-0.46)	0.44 ±0.16** (0.23-0.80)
	CD45RA-CD62L- T-cell ($x10^3$ cells/µl)	0.19 ±0.06 (0.08-0.34)	0.44 ±0.23** (0.16-0.98)
	CD45RA+CD62L- T-cell ($x10^3$ cells/µl)	0.04 ±0.03 (0.01-0.12)	0.16 ±0.12** (0.04-0.37)

Table 2. The numbers of monocyte-derived dendritic cells (mo-DCs), PBMCs, and once and twice stimulated CTLs in resting and post exercise blood. Data are displayed as mean ±SEM (range). Significant differences from resting are indicated by ** (p<0.01) and * (p<0.05).

Cell type	Resting	Post exercise
Mo-DCs (Day 0) ($x10^6$ cells)	0.94 ±0.48 (0.4-1.8)	1.56 ±0.78* (0.6-2.9)
PBMCs (Day 0) ($x10^6$ cells)	10.25 ±5.08 (4-18)	15.38 ±7.31 * (6-24)
Once-stimulated CTLs (Day 7) ($x10^6$ cells)	16.74 ±11.79 (4.78-48.90)	22.16 ±12.70* (6.27-49.05)
Twice-stimulated CTLs (Day 14) ($x10^6$ cells)	80.44 ±75.13 (5.04-329.58)	90.61 ±69.03 (11.57-262.08)

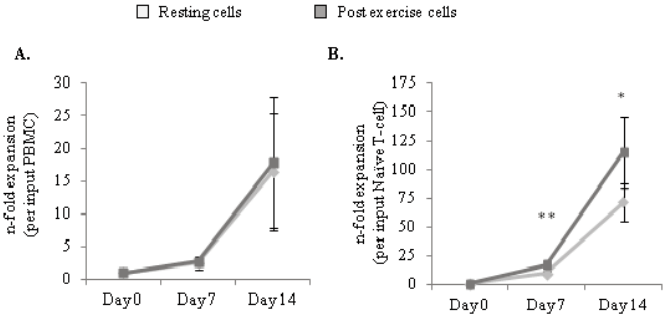

Figure 1. The expansion of the CTL lines. **A**) *n*-fold expansion of CTLs per input PBMC and **B**) *n*-fold expansion of CTLs per input naïve (CD45RA+CD62L+) T-cell. Cell counts were assessed at end of each stimulation cycle on days 7 and 14. Significant difference between cells expanded from blood donated at rest and post exercise is indicated by ** (p<0.01) and * (p<0.05). Mean ±S.D. is shown, data are from 12 (**A**) and 11 (**B**) participants.

CTLs expanded from rest and post exercise have similar phenotypes

Although exercise increased the proportion of natural killer (NK) cells and later-differentiated T-cells in blood (data not shown), resting and post exercise CTLs no longer differed in the proportions of NK-cells, T-cells, and most T-cell subsets after two stimulations. CTLs expanded post exercise contained a greater proportion of low differentiated ($CD45RA^+CD62L^+$) $CD4^+$ T-cells (resting: 18.2±7.5 %, post exercise: 21.9± 8.1%; t(7)=-2.613, p=0.035) and a smaller proportion of later differentiated ($CD45RA^-CD62L^-$) $CD4^+$ T-cells (resting: 29.1 ±7.4 %, postexercise: 24.8 ± 7.6%; t(7)=3.903, p=0.006) **(Fig. 2A)**. The T-cell subsets that exhibited the greatest increase in cell number in both resting and post exercise cells during expansion were the central memory ($CD45RA^-CD62L^+$) $CD4^+$ and $CD8^+$ T-cells **(Fig. 2B)**. Similar results were obtained when T-cell subsets were identified based on combinations of CD27 and CD28 surface molecules (data not shown).

Most participants are 'exercise-responders' for one or more TAA

To determine whether the twice stimulated T-cells expanded from rest and post exercise were specific for each TAA, we measured interferon-γ (IFN-γ) secretion after stimulating CTLs overnight with MAGE-A4, PRAME, and WT-1 pepmixes in an ELISPOT assay. All of the *ex vivo* expanded T-

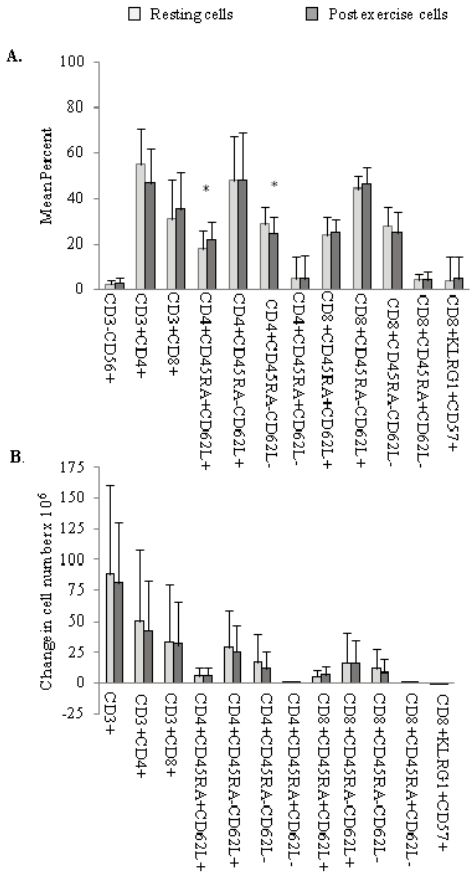

Figure 2. The phenotype of the CTL lines. **A**)The percentage of the indicated T-cell subsets within CTLs expanded by two weeks of stimulation with autologous TAA-presenting mo-DC. **B**) The absolute change in number of the expanded CTL subsets from the number of T-cells in each subset at day 0. Significant difference between cells expanded from blood donated at rest and post exercise is indicated by * ($p<0.05$). Mean ±S.D. is shown, data are from 8 participants.

cells showed TAA recognition; however we observed a large variation among the participants with respect to the difference in the number of IFN-γ-secreting cells (spot-forming cells; SFCs) between TAA-specific T-cells expanded from rest versus post exercise. **(Table 3)**. We therefore classified participants as 'exercise-responders' (greater than 10% increase in SFC post exercise) or 'exercise-non-responders' (less than 10% increase in SFCs post exercise) for each TAA-specific CTL line expanded. 84% of the participants (16 of 19) were exercise-responders for at least one TAA-specific CTL line **(Fig. 3A)**. For MAGE-A4-specific CTLs, 70% of participants were exercise-responsive, for PRAME-specific CTLs, 61% of participants were exercise-responsive, and for WT-1-specific CTLs, 38% of participants were exercise-responsive **(Fig. 3B).**

Table 3. The total number of CTLs (x 10^3) recognizing the indicated TAA expanded from rest and post exercise by two stimulations with autologous mo-DC. CTLs were enumerated by IFN-γ ELISPOT. Results for each participant are shown.

	MAGE-A4		PRAME		WT-1	
Participant	Rest	Post exercise	Rest	Post exercise	Rest	Post exercise
1	2.8	10.8	10.9	15.4	6.4	17.8
2	5.9	12.9	85.6	0.7	209.1	8
3	45.2	12.8	25.5	8.8	28.7	10.7
4	2.1	5	0.9	23.1		
5	0.7	2.8	5.2	4.6		
6	33	37.7	16.8	20		
7	5.2	1.2	9.3	3.8	2.9	1.2
8	0.4	2.2	3.8	3.3	0.8	0.2
9	11.5	10.6	93.9	37.5	24.1	12.8
10	1.1	6.1	2.2	4.7	7.7	4.7
11	22.1	44.6	84.5	137.2	100.1	90
12	10.8	18.6	11.3	1.0	3.6	41.2
13					13.1	122.8
14	4	10	2.1	12.6	1.4	7.9
15	2.5	6.5	0.8	5.2	0.6	0.5
16	0.2	1.6	1.7	2		
17	0.4	0.3	0.5	8.1		
18	19.4	10.9	25.5	31.1	15.5	16.9
19			0.2	4.0		

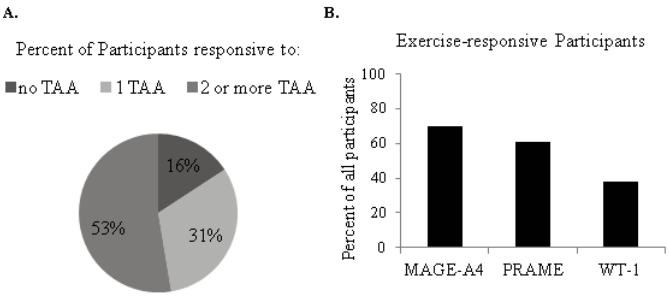

Figure 3. Exercise increases the number of TAA-specific CTLs in some, but not all, participants. **A**) The percent of participants that were exercise-responders for none of the TAA, for 1of the TAA, or for 2 or more TAA. **B**) The percent of participants who had at least a 10% increase in TAA-specific CTLs post exercise (exercise-responders) for the MAGE-A4, PRAME, and WT-1 specific-cell lines.

Exercise increases the number of TAA-specific CTLs among 'exercise-responders'

Exercise-responders had a significantly greater number of MAGE-A4, PRAME- and/or WT-1-specific CTLs post exercise ($p<0.05$) **(Fig. 4A)**. The 12 (of 17) participants who were MAGE-A4-exercise-responders had a mean increase of 6118 (SD: 5629) MAGE-A4-specific CTLs post exercise, compared to resting cells (median: 4825, range: 1489 to 22492; $t(11)=-6.307$, $p=0.000$) (corresponding to a mean percent increase of 254% in SFCs among the 12 participants). The 11 (of 18 participants) PRAME-exercise-responders had a mean increase of 10671 (SD: 15124) PRAME-specific CTLs post exercise (median: 4590, range: 220 to 52690; $t(10)=-2.902$, $p=0.016$) (mean increase of 523%). The 5 (of 13 participants) WT-1-exercise-responders had a mean increase of 33332 (SD: 44927) WT-1-specific CTLs post exercise (median: 11426, range: 1419 to 109728; $t(4)=-3.497$ $p=0.025$) (a mean increase of 511%). Exercise-responders also had a marked increase in SFCs without stimulation (CTL alone) following exercise; however the number of SFCs resulting from TAA-stimulation was significantly greater than without stimulation at rest and post exercise ($p<0.05$).

Exercise-non-responders had no significant difference between blood donated at rest versus post exercise with respect to the number of T-cells recognizing MAGE-A4 and PRAME ($p>0.05$). However, there was a trend for a decreased frequency of MAGE-A4- and PRAME-specific T-cells expanded from post exercise samples versus samples obtained at rest (mean decrease±SD: 45%±30% and 57%± 34%, respectively). WT-1-exercise-non-responders had significantly fewer WT-1-specific T-cells expanded from post

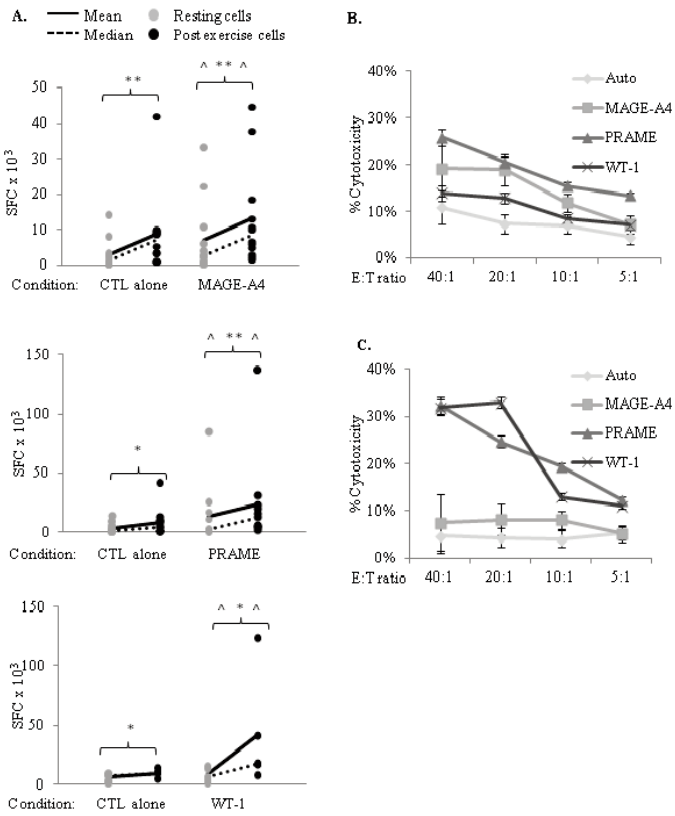

Figure 4. TAA-specific responses after two weeks of stimulation among exercise-responders. **A**) The total number of IFN-γ-secreting cells (SFCs) among cells expanded from blood donated at rest (grey) and postexercise (black) following overnight stimulation with the indicated TAA, or without stimulation (CTL alone). Results for the exercise-responsive participants are shown; each spot represents an individual participant. Significant difference between cells expanded from blood donated at rest and post exercise is indicated by ** ($p<0.01$) and * ($p<0.05$), ^indicates significant difference from CTL alone condition; $p<0.05$. ^{51}Cr release at 4 hours after coincubation of representative CTL lines expanded from resting cells (**B**) and post exercise cells (**C**) with autologous PHA blasts pulsed with MAGE-A4, PRAME, WT-1, or unpulsed PHA blasts (auto). The data are the mean ±SD percentage lysis at effector: target ratios of 40:1, 20:1, 10:1, and 5:1. Each condition was measured in triplicate.

exercise blood, with a mean decrease of 30721 (SD: 69130) WT-1-specific CTLs post exercise (median: -6514, range: -87 to -201093;$t(7)=2.742$, $p=0.029$) (data not shown).

The cytolytic activity of the CTL lines was measured using a 4h Cr^{51} release assay. Data from the cytotoxicity assays confirmed that the TAA-specific T-cells were functional, as both resting and post exercise CTLs exhibited antigen-specific killing of TAApeptide-pulsed autologous target cells **(Fig. 4B and C)**.

PRAME exercise-responders and non-responders differ in T-cell phenotype

We sought to identify other parameters in which exercise-responders and exercise-non-responders differed. Although exercise-responders appeared to have fewer MAGE-A4-, PRAME-, and WT-1-specific CTLs at rest compared to non-responders, this did not reach statistical significance **(Table 4)**. Exercise-responders and non-responders did not differ in the number of SFCs in the CTL alone condition or in response to SEB (data not shown). Further, using data collected in 11 of the 19 participants, we compared demographic characteristics and physiological responses to exercise between the exercise-responders and non-responders to the MAGE-A4- and PRAME antigens **(Table 5)**. WT-1-specific-CTL lines were not examined in this context as only two of these 11 participants were exercise-responders. While

Table 4. The total number of CTLs recognizing the indicated TAA from blood donated at rest and post exercise and expanded by two stimulations with autologous mo-DCs in exercise-responsive and exercise-non-responsive participants. CTLs were enumerated as spot-forming cells (SFCs) by IFN-γ ELISPOT. Groups did not differ in the number of SFCs within each time point. Mean ±S.D. (range) is shown. Significant difference from cells expanded from rest is indicated by ** ($p<0.01$) and * ($p<0.05$).

	MAGE-A4 cell line		PRAME cell line		WT-1 cell line	
	responders N=12 (7 female)	non-responders N=5 (2 female)	responders N=11 (7 female)	non-responders N=7 (2 female)	responders N=5 (4 female)	non-responders N=8 (3 female)
Resting SFC (x 10^3)	7.1 ±10.3 (0.2-33)	16.3 ±17.3 (0.4-45)	13.3 ±25.0 (0.4-84.4)	33.5 ±39.1 (3.8-93.9)	8.0 ±6.1 (1.4-15.5)	46.7 ±73.4 (0.6-209.1)
Post exercise SFC (x 10^3)	13.2 ±14.0 (1.6-44.6)**	7.2 ±5.9 (0.3-12.8)	23.9 ±38.6 (2.0-137.1)**	8.5 ±13 (0.7-37.5)	41.3±47.2 (7.9-122.8)*	16.0 ±30.2 (0.2-90.0)*

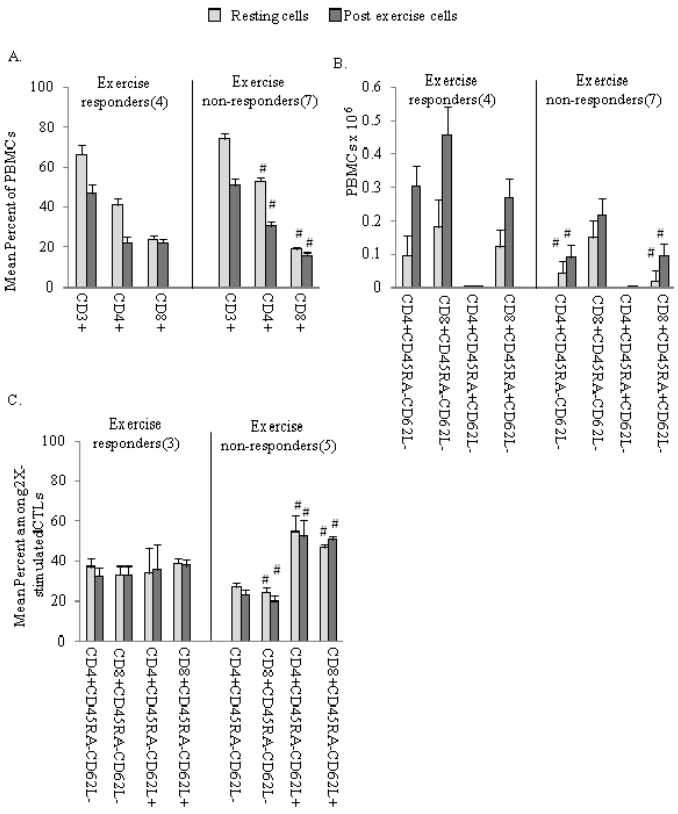

Figure 5. PRAME exercise responders and exercise non-responders differ in the phenotype of T-cell subsets at rest and post exercise in unstimulated and expanded cells. **A**) The percent of total T-cells and $CD4^+$ and $CD8^+$ T-cells in PBMCs (day 0) at rest and post exercise. **B**) The number of medium and late differentiated $CD4^+$ and $CD8^+$ T-cells in PBMCs at rest and post exercise. **C**) The percent of medium and late differentiated $CD4^+$ and $CD8^+$ T-cells among CTLs (day 14) at rest and post exercise. Data are displayed as means ± SEM of 11 or 8 participants, # indicates significant difference between PRAME exercise-responders and exercise-non-responders ($p<0.05$).

Table 5. Characteristics and exercise performance measures of representative participants from MAGE-A4 and PRAME exercise-responders and exercise-non-responders. Mean ±SD (range) from 11 participants (5 female) are shown. BMI: body mass index, PA: self-reported physical activity level (1=infrequent, 7=>3h/week of vigorous activity) #indicates significant difference from non-responder ($p<0.05$),* indicates significant difference from resting ($p<0.05$).

		MAGE-A4 cell line		PRAME cell line	
		responders N=8 (4 female)	non-responders N=3 (1 female)	responders N=4 (3 female)	non-responders N=7 (2 female)
Age (yrs)		27.4 ±1.8 (25-30)#	24.3 ±2.5 (22-27)	27.0 ±2.4 (25-30)	26.3 ±2.4 (22-29)
BMI (kg/m^2)		23.6 ±3.3 (18.0-28.3)	23.3 ±2.9 (19.9-25.1)	21.5 ±3.1 (18.0-25.5)	24.6 ±2.6 (19.9-28.3)
PA rating (1-7)		6.3 ±0.7 (5-7)	5.3 ±0.6 (5-6)	6.5 ±0.6 (5-7)	5.7 ±0.8 (5-7)
VO_2max (ml/kg/min)		50.5 ±6.3 (38-59)	50.0 ±2.6 (47-52)	50.7 ±3.6 (48-56)	50.2 ±6.5 (38-59)
CMV serostatus (% seropositive)		25%	33%	50%	14%
CMV IgG titer of seropositive (IU/ml)		16.15 ±6.7 (5.1-18.4)	24.07	16.15 ±6.7 (5.1-18.4)	24.07
EBV serostatus (% seropositive)		75%	66%	100%	57%
EBV IgG titer of seropositive (IU/ml)		140.4 ±73.4 (22-199)	154.19±64.8 (108-200)	178.37±70.9 (22-199)	168.27±40.9 (108-200)
Time to completion (sec)		105±19 (73-133)	111±16 (96-128)	106±17 (93-133)	107±20 (73-131)
Maximum heart rate obtained (bpm)		168±14 (142-185)	182±5 (177-187)	174±10 (164-185)	171±15.5 (142-187)
Blood lactate (mmol)	Resting	1.3±0.5 (0.75-2.1)	1.4±0.4 (1-1.9)	1.5±0.5 (1-2.1)	1.2±0.4 (0.75-1.9)
	Post exercise	8.5±2.3 (5-11.7)*	9.5±2.5 (7-12)*	8.8±2.3 (6.9-11.7)*	8.8±2.5 (5-12)*
Serum Neopterin (nmol/L)	Resting	6.15±3.9 (1.7-11.8)	9.3±3.9 (5.7-13.5)	4.9 ±3.9 (1.7-10.4)	8.2±3.8 (2.4-13.5)
	Post exercise	7.4±4.2 (1.8-12.4)*	11.5±5.8 (6.8-18.0)*	6.6±4.1 (1.8-10.9)	9.7±5.1 (2.0-18.0)
Serum Cortisol (ng/ml)	Resting	193±163 (52-506)	105±16 (87-118)	76 ±20.9 (52-90)	205±153 (87-506)
	Post exercise	202±190 (54-582)	89.6±9.4 (79-97)	77±26 (54-106)	208±185 (79-582)
Serum epinephrine (pg/ml)	Resting	61.8±19.3 (40.5-90.3)	46.9±3.26 (44.8-50.7)	55.2±14.4 (40.6-74.9)	59.2 ±20.2 (40.7-74.9)
	Post exercise	320±323 (55.3-1022)*	115±59.6 (80.9-184)*	190±152 (55.3-383)*	307±347 (80.9-1022)*
Serum norepinephrine (pg/ml)	Resting	511±261 (208-1036)	1192±372 (908-1613)	641±280 (384-1036)	728±502 (208-1613)
	Post exercise	1960±1858 (482-5553)*	3396±1248 (2189-4680)*	2018±2374 (482-5553)*	2543±1536 (749-4680)*
Lymphocytes (x10^3 cells/µl)	Resting	1.54±0.21 (1.2-1.9)	1.53±0.51 (1.1-2.1)	1.45±0.21 (1.2-1.7)	1.59±0.33 (1.1-2.1)
	Post exercise	3.49±0.42 (2.3-4.9)*	3.77±1.39 (2.6-5.3)*	3.52±0.58 (2.9-4.3)*	3.58±0.84 (2.6-5.3)*
Monocytes (x10^3 cells/µl)	Resting	0.36±0.98 (0.25-0.50)	0.47±0.15 (0.30-0.60)	0.34±0.11 (0.25-0.50)	0.41±0.12 (0.30-0.60)
	Post exercise	0.98±0.26 (0.5-1.3)*	1.23±0.42 (0.9-1.7)*	0.94±0.15 (0.75-1.1)*	1.11±0.37 (0.50-1.7)*

there were more females in the exercise-responder groups than in the non-responder groups, this did not reach statistical significance ($p>0.05$). Compared to exercise-non-responders, exercise-responders tended to be slightly older (MAGE-A4 exercise-responders: 27.4 ±2.4 yrs, MAGE-A4 exercise-non-responders: 24.3 ±2.5 yrs; $F=1.73$,$t(10)=-2.479$, $p=0.033$) **(Table 5)**. No other differences in participant demographics, such as fitness or viral serostatus, or in physiologic responses to exercise, including maximum heart rate and circulating levels of stress hormones, were found between the groups; all participants demonstrated a significant increase in blood lactate, hormone levels, and cell counts following exercise **(Table 5)**.

In the unstimulated cells (pre-expansion), PRAME exercise responders had a smaller proportion of $CD4^+$T-cells and a greater proportion of $CD8^+$T-cells compared to PRAME exercise non-responders,as well as a greater number of late-differentiated cells ($CD45RA^-CD62L^-$ $CD4^+$ T-cells and $CD45RA^+CD62L^-$ $CD8^+$ T-cells) **(Fig. 5A and 5B)**. The two groups did not differ in the exercise-induced mobilization of these cell subsets. Following two weeks of stimulation, the PRAME exercise responders had a greater proportion of late differentiated cells($CD45RA^-CD62L^-$ $CD4^+$ and $CD8^+$ T-cells) and a smaller proportion of central memory cells compared to PRAME exercise non-responders **(Fig. 5C)**. T-cell subsets did not differ between the MAGE-A4 exercise-responders and non-responders either before or after CTL expansion.

DISCUSSION

While the *ex vivo* expansion of virus specific T-cells from healthy seropositive donors is becoming increasingly routine (28), expansion of TAA-specific T-cells has proven more difficult, partially due to low numbers of naturally occurring TAA-specific T-cells among healthy individuals. As acute dynamic exercise elicits a profound and almost instantaneous leukocytosis (41), we hypothesized that a single bout of exercise could serve as a non-invasive and economical approach to increase the number of activated mononuclear cells in the blood. This strategy could thus augment the manufacture of TAA-specific T-cells. In agreement with this hypothesis, brief maximal exercise increased the expansion of CTLs specific to at least one of three TAAs in 84% of the healthy adults sampled, with exercise increasing the number of CTLs specific for MAGE-A4 and PRAME in 70% and 61% of all participants respectively. Both resting and post exercise expanded CTL lines were functional, as evidenced by their ability to secrete IFN-y in response to peptide stimulation and to kill autologous peptide-pulsed target cells in an antigen-specific manner.The CTLs expanded post exercise included both CD4+ and CD8+ T-cells with central and effector memory phenotypes, which is associated with persistence after infusion of antigen-specific CTLs (20).

Compared to CTLs expanded from resting blood, the exercise-responders demonstrated a 3.4- and 6.2- fold increase post exercise in the numbers of MAGE-A4- and PRAME-specific CTLs, respectively. Although only 38% of participants exhibited an exercise-enhancement for the expansion of WT-1-sepcific CTLs, the WT-1 exercise-responders showed a marked (6.1-fold) increase in the number of WT-1-specific CTLs post exercise. As very large numbers of TAA-specific CTLs are required for adoptive transfer (patient doses range from 4×10^7 to 3.3×10^9CTL/m^2 per transfer, 2-8 transfers) (6, 43), any increase in the number of TAA-specific CTLs is desirable. Exercise used in conjunction with apheresis, a clinical technique used to increase the number of leukocytes collected from a donor, could lessen the volume of blood

processed, thereby decreasing the potential risks and discomforts associated with this procedure.Because exercise increases the number of TAA-specific CTLs, it is possible that a sufficient number of these cells could be manufactured and transferred to the patients more quickly than with traditional protocols using resting blood. Current protocols typically require 4 to 12 weeks to stimulate and expand TAA-specific CTLs, limiting the applicability of this therapy to many patients (4, 40). Although we did not perform a time course experiment in the present study, it will be important to determine if exercise can reduce the time needed to manufacture clinically sufficient numbers of TAA-specific CTLs. The rapid generation of TAA-specific CTLs from healthy exercising donors could improve the efficacy of TAA-specific CTL transfer as both a prophylactic and early stage relapse treatment for a range of hematologic malignancies.

Although exercise did not increase TAA-specific CTL expansion from all participants in this study, it is important to note that exercise did not appear to substantially impair the expansion of TAA-specific CTLs either. That is, in the exercise-non-responders, the numbers of TAA-specific CTLs expanded post exercise were often similar to the numbers generated from resting blood. It is not known why exercise was less effective at expanding WT-1-specific CTLs compared to MAGE-A4- and PRAME-specific CTLs. Differences in antigen size seem unlikely, as the number of amino acids in the WT-1 peptide pool is greater than PRAME but less than-MAGE-A4. It would be interesting for future studies to broaden the tumor antigen repertoire examined post exercise, including tumor antigens involved in a variety of both solid organ and hematological cancers such as Aurora kinase (27), BMI-1 (33), and survivin (2).

It would be advantageous to identify individuals in whom exercise is most likely to benefit TAA-specific CTL expansions. We therefore attempted to identify demographic and physiological predictors of the exercise-responders in the current study. Although persistent herpes viruses such as CMV are believed to impair T-cell responses to novel antigens (34), CMV and EBV serostatus did not differ between the exercise-responders and non-responders. Moreover, although the physiological responses to exercise were not controlled, we did not find any differences in peak heart rate, exercise duration, blood lactate concentration, leukocyte mobilization, or serum hormones between the groups. It is therefore unlikely that differences between the groups were due to variations in demographic characteristics or the physiological responses to exercise. We also considered if exercise-induced changes in the composition of lymphocyte subsets differed between groups. Although PRAME-exercise-responders had a greater proportion of $CD8^+$ T-cells in initial cell cultures, and thus a greater proportion of T-cells with potential cytotoxic effector function, the groups no longer differed in the number or proportions of these cells following two weeks of stimulation. And while PRAME-exercise-responders had a greater proportion of EM $CD8^+$ cells among the expanded cells than non-responders, there was no exercise effect on the proportions of these cells in either group. The fact that exercise did not impact cell redeployment differently in the two groups suggests that shifts in cell proportions due to exercise can not fully explain differences between exercise-responders and non-responders. However, we acknowledge that this analysis may be limited by the small sample size and it remains possible that certain demographic characteristics and/or physiological responses to exercise may serve as good predictors of those donors likely to have an increased TAA-specific CTL response after exercise. Moreover, because the exercise protocol used in this study was rather rudimentary, future studies should make better attempts to optimize the intensity and/or duration of exercise for augmenting the manufacture of TAA-specific CTL and also include a non-exercise control condition to account for potential variability in CTL generation between blood draws.

The mechanisms that underpin the effects of exercise on TAA-specific CTL expansion are unknown. Shear stress, due to increases in cardiac output and blood pressure, and the actions of catecholamines binding to adrenergic receptors on leukocytes are thought to underlie many of the exercise-induced changes in immune cells (11, 14, 41). However, in the present study, epinephrine and norepinephrine levels increased to a similar extent in both the exercise-responders and non-responders, and the groups did not differ in maximum heart rate. This indicates that these physiological responses to exercise cannot alone explain the effect of exercise on TAA-specific CTL expansion. As has been documented elsewhere (10, 18, 19, 36), we observed a preferential mobilization of NK-cells and late differentiated subsets of T-cells, which in turn decreased the proportion of naïve cells within the PBMCs post exercise.We did not account for this increase in cells which are unlikely to respond to TAA (such as T-cells specific to alternate antigens), as a fixed ratio of mo-DC: PBMCs was used in all cultures. Despite beginning with a smaller proportion of naïve T-cells post-exercise, the two-week expansion using mo-DC stimulation yielded similar numbers of cells. This could suggest that the naïve T-cells mobilized by exercise were more susceptible to the stimulatory signals provided by the mo-DCs. Although not measured in these experiments, other studies have shown that T-cells mobilized with exercise express activation markers such as HLA-DR (15). Future work should account for exercise-induced changes in the composition of T-cell subsets and culture equal numbers of naïve cells from rest and post exercise.

The idea of using exercise as a simple adjuvant to improve immune-based treatments is quite new (35). Millard et al recently found that exercise increases peripheral blood NK-cells without causing substantial changes in their function, thus rendering them useful for some *in vitro* experiments requiring large numbers of NK-cells (24). We have also shown that many of the T-cells mobilized into the blood by exercise are specific to viruses such as CMV (37), indicating that a single bout of exercise could augment the manufacture of virus-specific CTLs as a means to prevent or treat viral infections after HSCT. The current study adds to this literature, demonstrating that exercise can also increase the expansion of TAA-specific CTLs. Although the current results are limited to donors capable of completing a maximal exercise bout, future studies able to identify the mechanism(s) by which exercise improves the expansion of TAA-specific CTLs may highlight pathways that could be manipulated by pharmacological

means to mobilize desirable cell populations to the peripheral blood compartment. This would expand the results of the present study to allogeneic donors lacking the fitness level necessary to complete an exercise bout, or to cancer patients requiring an autologous transfer but too ill to exercise.

In conclusion, we have shown for the first time that a short bout of maximal exercise in healthy adults yields greater numbers of functional CTLs specific for the tumor-associated-antigens MAGE-A4 and PRAME, and in some cases WT-1. Therefore, due to its simplicity, cost-effectiveness, likelihood of success and low risk of impairing the response, a single bout of exercise immediately prior to blood donation should be considered a worthwhile means to augment the manufacture of TAA-specific CTLs in healthy allogeneic donors. Future research should aim to optimize the intensity and duration of the exercise protocol that will best elicit this response, and determine if exercise could be used to minimize the time required to manufacture clinically sufficient numbers of TAA-specific CTLs for adoptive transfer immunotherapy.

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