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Mobilizing Immune Cells with Exercise for Cancer Immunotherapy

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Abstract

Hematopoietic stem cell (HSC) transplantation and adoptive transfer immunotherapy are effective in treating blood cancers and post-transplant infections, but low circulating cell numbers in patients and donors is oftentimes a limiting factor. We postulate that a single exercise bout will increase the yield of patient and donor-derived HSC's and cytotoxic lymphocytes to improve this form of treatment for cancer patients.

SUMMARY for TOC:

Exercise increases the yield of donor-derived lymphocytes and hematopoietic stem cells (HSC) to improve adoptive transfer immunotherapy and HSC transplantation.

Keywords

Viral infections; adoptive transfer; exercise immunology; hematopoietic stem cell transplantation; tumor antigens; NK-cell; T-cell

INTRODUCTION

Every year around 60,000 people worldwide receive hematopoietic stem cell transplantations (HSCT) for the treatment of blood cancers and genetic disorders (16). While HSCT is a potentially curative treatment, viral infections and disease relapse are still common, accounting for ~78% of all deaths in the post transplant period. Adoptive transfer immunotherapy is the passive infusion of immune cells (usually lymphocytes) to a cancer patient recipient in order to prevent or treat potentially fatal viral infections and disease relapse after HSCT. Lymphocytes are typically taken from the blood of the patient

(autologous transfer) or healthy donor (allogeneic transfer) and either transferred directly without further manipulation or after a period of *in vitro* stimulation where antigens, growth cytokines and/or gene transfer are used to expand the cells so they recognize and kill malignant and virus-infected cells more effectively (7, 8, 21). Adoptive transfer immunotherapy is highly effective, but it can be limited by the very low frequency of specialized lymphocyte subsets [i.e. virus-specific T-cells (VSTs)] in the peripheral circulation, sometimes resulting in too few cells being transferred to the patient to reconstitute the immune system (7, 8, 21). Low cell numbers result in prolonged manufacturing times, thus delaying the delivery of the *ex vivo* expanded cell products that are vital to treat potentially fatal viral infections and refractory disease. It is therefore imperative to find efficient and economical ways to mobilize large numbers of lymphocyte subtypes from the tissues to the blood where they can be readily accessed and used therapeutically.

A single bout of dynamic exercise elicits a profound and almost instantaneous mobilization of all major leukocyte subtypes into the peripheral circulation (28, 29). This phenomenon, now known as ‘exercise-induced leukocytosis’, was first reported at the turn of the twentieth century. It has since been established that hemodynamic shear-stress, as a result of increases in cardiac output, blood pressure and blood flow, can cause leukocyte demargination from the vascular, pulmonary, hepatic and/or splenic reservoirs to markedly increase the number of leukocytes in the main axial blood flow of the peripheral circulation (28, 29). Moreover, catecholamines and glucocorticoids, which bind to adrenoreceptors and glucocorticoid receptors expressed by the exercise-responsive leukocytes evoke their mobilization and egress from the blood compartment both during and after a single exercise bout. This leukocytosis is not uniform, with those immune cell subtypes that have greater cytotoxicity (killing), antigen experience and tissue migration potential being preferentially mobilized into the blood with exercise (29). Within lymphocytes, NK-cells, CD8⁺ T-cells and γ T-cells are particularly exercise responsive, and the more differentiated subtypes [i.e. central memory (CM) and effector memory (EM) T-cells] within these parent cell populations are preferentially mobilized over their less differentiated counterparts (i.e. naïve T-cells) (28, 29). Moreover, T-cells mobilized with exercise are specific to multiple viral antigens, secrete a plethora of cytokines, and are more sensitive to activation and proliferation when stimulated with specific (i.e. viral peptides) and non-specific (i.e. mitogens, CD3/CD28 monoclonal antibodies) agents (28, 29), whereas NK-cells present in the blood during the recovery phase of exercise are more efficient killers of various cancer cell lines *in vitro* (4). Obtaining larger numbers of discrete lymphocyte subsets from both patients and healthy donors in this ‘primed’ state due to exercise might not only markedly increase cytotoxic lymphocyte recovery from blood, but also augment and hasten the *ex vivo* manufacture of cytotoxic lymphocyte cell lines for adoptive transfer immunotherapy. Furthermore, shifts in cell subpopulations and phenotypic changes with exercise might allow the exercise-mobilized lymphocytes to perform more effectively in the host after transfer, and because exercise also mobilizes CD34⁺ hematopoietic stem cells (HSCs), dynamic exercise may serve as a suitable adjuvant to current pharmacological methods that are used to mobilize HSCs from the bone marrow to the blood in healthy stem cell donors.

Here we present our integrated hypothesis that a single bout of exercise will enrich the blood compartment of ‘primed’ virus and tumor reactive T-cells and NK-cells in healthy donors that can be easily accessed and used to augment the manufacture of clinical-grade virus and tumor killing lymphocytes for adoptive transfer in the post transplant setting. We also present evidence that exercise-mobilized cells will be better suited for *ex vivo* expansion and might even perform better in the host after transfer. Finally, we discuss ways in which exercise can improve the recovery of HSCs from the bloodstream of healthy donors, which might help reduce donor burden and the reliance on additional pharmaceutical agents that have known toxicities and undesirable side-effects.

HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

HSCT is the preferred treatment for many patients with genetic disorders and blood (“liquid”) cancers. HSCs can be obtained from the transplant recipient (autologous HSCT) prior to treatment or from a suitable related (e.g. sibling) or unrelated donor to the patient (allogeneic HSCT). HSCs may be harvested from the bone marrow, peripheral blood or umbilical cord blood and delivered to the patient after they receive a conditioning regimen (chemotherapy and/or radiation) to eliminate the endogenous hematopoietic cell producing abilities of the patient prior to transplant. Conditioning helps clear the bone marrow of malignant cells and creates “immune space” for the development of a potent graft-versus-tumor (GvT) effect in the patient’s bone marrow after engraftment. The transfer of HSCs from an allogeneic source, however, is a double-edged sword. While the GvT effect of an allograft is potentially curative, the allograft has the potential to cause graft-versus-host disease (GvHD) when the donor effector cells target and destroy healthy cells and tissues in the host.

Despite the widespread success of HSCT, disease relapse remains a significant problem in a large portion of allo-HSCT patients as more than 85% of patients with high risk acute myeloid leukemia (AML) will relapse within 18-months of an initial remission, and the odds of surviving longer than 5-years following HSCT reduces markedly with advancing age (17). Moreover, viral infections are a major cause of morbidity and mortality after allo-HSCT and are fatal in 17–20% of cases (21). The elimination of the recipient’s immune system through a combination of immunosuppressive drugs and radiation exposure are associated with a significant risk of latent herpesvirus reactivation during the post-transplant period. In particular, common herpesviruses such as herpes simplex virus (HSV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), cytomegalovirus (CMV) and human herpesvirus-6/7 (HHV-6/7) pose a problem for immunocompromised patients after HSCT. Moreover, community viruses such adenovirus, parvovirus, respiratory syncytial virus (RSV) and influenza also cause significant morbidity and mortality after HSCT (21). Although the administration of potent antivirals such as ganciclovir and rituximab are sometimes successful in controlling CMV and EBV infections, respectively, these drugs can be ineffective, have significant toxicities, provide no long-term protection and might even generate drug-resistant variants (21). These agents also have no impact on restoring anti-viral immunity, which is the cornerstone of adequate viral protection during the post-transplant period.

ALLOGENEIC ADOPTIVE TRANSFER IMMUNOTHERAPY

Donor lymphocyte infusions (DLI) stemmed from the idea that blood lymphocytes from the HSC donor would contain T-cells capable of restoring antiviral and anti-tumor activity in the HSCT recipient. It was expected that the pre-existing VSTs in the donor would proliferate and persist in the recipient to provide adequate immune protection against a number of common viruses. Although met with some success, particularly for the treatment of adenovirus and EBV-associated lymphoproliferative disease, a major limitation of the DLI method was the substantially greater number of alloreactive cells that can cause GvHD relative to the very few numbers of VSTs in the donor lymphocyte pool. As such, DLI oftentimes caused GvHD without providing broad-spectrum antiviral immunity and is now considered a high-risk procedure to prevent and treat viral disease post-HSCT (21). While DLI is still considered standard treatment for refractory leukemias and lymphomas, it is generally considered to be more useful as a preemptive therapy to reduce relapse rates than for the effective treatment of leukemic relapse (7). This is largely due to the lack of lymphocytes in donor blood capable of reacting with tumor-specific antigens. Attention has now shifted toward obtaining, or manufacturing, antigen-specific T-cells from donor blood using direct selection and purification techniques or selective expansions *ex vivo* (7).

Direct Selection and Purification of Antigen-Specific T-cells

Many strategies have been developed with a view to increasing both the safety and efficacy of adoptive T-cell therapy (7, 8, 21). Inactivating or physically removing alloreactive T-cells with fusion proteins or monoclonal antibodies prior to transfer is a strategy used to improve DLI. Genetic modification methods involve incorporating a 'suicide gene' or 'safety switch' in donor T-cells, which are activated in the event of GvHD to kill or 'switch off' alloreactive T-cells. Other approaches involve the direct isolation of VSTs using multimers that are loaded with synthetic antigen-specific peptide/HLA molecules allowing them to bind to cognate receptors on the T-cells. This approach is limited, however, as it requires prior knowledge of immunodominant epitopes and is restricted by donor HLA type (21). Moreover, most commercially available multimers are made with HLA class I antigens restricting their selection to CD8+ T-cells which may limit the scope and duration of an immune response after transfer particularly if HLA class II restricted CD4+ T-cells are required (21). In this regard, T-cells are also selected by their ability to secrete effector cytokines such as IFN- γ in response to viral peptide stimulation (8, 21). This allows many T-cell subtypes (from both CD8+ and CD4+ subsets) to be selected and is not restricted to certain HLA types or specific peptides. However, a limitation of both the multimer and cytokine capture methods is the low number of antigen-specific cells found in the circulation of healthy donors (8, 21). This oftentimes results in insufficient numbers of antigen-specific T-cells being obtained from the donor to elicit adequate immune protection in the recipient after transfer.

Expansion of Antigen-Specific T-cells *ex vivo*

To overcome limitations associated with low circulating cell numbers, VSTs are typically expanded *ex vivo* prior to transfer (8, 21). The methods used to expand VSTs are broad, but the overall objective is to increase their number and specificity without expanding

alloreactive T-cells. This allows large numbers of VSTs to be generated from relatively low numbers of circulating memory T-cells, which can be a limiting factor for the direct isolation and transfer of donor-derived VSTs using multimer and cytokine capture methods.

Autologous antigen-presenting cells (APCs) [typically in the form of monocyte-derived dendritic cells (Mo-DCs)] that express HLA molecules containing antigen-derived peptides and co-stimulatory molecules that elicit robust T-cell activation and proliferation, are required to expand donor T-cells. Ex vivo expansion manufacturing processes, depending on the donor and the methodology used may be complex, may require infectious viral material, production of a clinical grade vector, and may require prolonged (10–12 weeks) periods of cell culture (8, 21). Recent advances have decreased manufacturing times when the donor has a population of pre-existing memory T-cells (i.e. CMV-specific memory T-cells from a CMV seropositive donor), whereas, the manufacture of VSTs from a non-infected (antigen naïve) donor is more prolonged but critical since recipients infected with a virus who receive T-cell products from a virus naïve donor are at the highest risk for viral reactivation/infection after HSCT (8).

Multi-VSTs can now be generated in as little as 10-days following a single exposure to clinically relevant peptides when the donor is virus experienced (15, 24). Donor peripheral blood mononuclear cells (PBMCs) are stimulated with overlapping peptide pools and growth cytokines that allow for the production of a single cell line with simultaneous specificity for multiple antigens. This method also eliminates the reliance on complex manufacturing processes that require infectious virus material, clinical grade vectors, Mo-DC manufacture, and prolonged cell culture. Whether obtained using direct selection or *ex vivo* expansion techniques, donor-derived VSTs have been shown to confer protection *in vivo* after transfer in 70–90% of recipients (8). However, these rapid manufacturing protocols still require donors to have high numbers of circulating VSTs to enhance the multi virus specificity of the final cell product and new strategies are sought to augment and simplify the manufacturing process so the method can be more broadly applied (8, 24).

The adoptive transfer of cytotoxic T-cells designed to detect and kill cancerous cells expressing tumor associated antigens (TAAs) has been shown to be effective in combating various cancer types such as metastatic melanoma, lymphoma, neuroblastoma, as well as lymphocytic and myeloid leukemias (14). This approach has been used to successfully manufacture functional TAA-specific T-cells from both healthy donors and cancer patients, thus making it a potentially effective treatment for both allogeneic and autologous adoptive transfer therapies (7). However, manufacturing TAA-specific T-cells from healthy donors has proved difficult (7). This is because non-viral TAA are often self-antigens, which are infrequent in healthy donors and have low antigen binding capabilities (avidity) (7). Moreover, T-cells specific to these self-antigens in healthy donors often fail to respond to their specific antigen (anergy) and the successful manufacture of TAA-specific T-cells from healthy donors is likely to involve the activation and differentiation of the naïve T-cell population. These limitations are overcome somewhat by prolonged manufacturing times and multiple stimulations with peptide-loaded Mo-DCs, although it can sometimes take several weeks and even months of cell culture to generate enough TAA-specific T-cells for adoptive transfer. Unfortunately, prolonged manufacturing time also increases the production of alloreactive T-cells that can cause GvHD. Clinical trials involving the adoptive transfer of

donor-derived TAA-specific T-cells are currently ongoing (7), but new methods are sought to hasten the manufacture of TAA-specific T-cells from healthy donors.

NK-cell Expansion and Adoptive Transfer

Allogeneic adoptive transfer of NK-cells has been used to curtail or reverse the spread of multiple hematologic [e.g. multiple myeloma (MM) and AML] and solid malignancies (e.g. non-small cell lung, ovarian, and breast cancers) (25). The procedure has a consistently high safety profile with low incidence of GVHD, but unfortunately the vast majority of poor prognosis cancer patients do not respond to NK-cell therapy (25). This could be due to low yields of alloreactive NK-cells from healthy donors, a loss of activating receptors and cytotoxic function following transfer, immunosuppressive tumor microenvironments, and/or the failure of the NK-cells to expand and persist in the host following transfer (27, 35). NK-cells are often expanded from donor PBMCs over 2–3 weeks using growth cytokines and transfected “feeder cells” that selectively expand large numbers of NK-cells with potent cytotoxic functions and increased activating receptor expression (30). However, limitations of NK-cell therapy include their short half-life *in vivo* (approximately 10 days) and their propensity to lose cytotoxic activity after transfer (25, 35). Mitogenic cytokines (i.e. IL-2 and IL-15) are delivered to the patient to promote *in vivo* expansion and activation of NK-cells, but this can also expand undesirable suppressor cells (i.e. regulatory T-cells) and results in a host of side-effects including hypotension, fever, and thrombocytopenia (10). While KIR-ligand mismatch has improved the efficacy of allogeneic NK-cell therapy, more complex strategies designed to overcome NK-cell tumor evasion are in development (i.e. genetic engineering of NK-cells and tumor-targeting antibodies, and antibody blocking of NK-cell inhibitory signaling) (25). Simpler manufacturing approaches to expand tumor-targeting NK-cells from healthy donors that are able to persist *in vivo* following adoptive transfer without introducing more side-effects and treatment-related pitfalls remains a critical need.

THE POTENTIAL FOR EXERCISE TO IMPROVE ALLOGENEIC ADOPTIVE TRANSFER IMMUNOTHERAPY

Immune cell collections from healthy donors are typically done under resting conditions via apheresis or standard phlebotomy. Exercise, through activation of the biological stress response, evokes a massive leukocytosis, increasing the number of circulating NK-cells and antigen-specific T-cells (28, 29). T-cells redeployed with exercise have increased cytokine production, longer telomeres, and a lower activation threshold to exogenous agents (28), indicating that exercise-mobilized cells would be excellent precursors for the isolation and manufacture of polyfunctional T-cell lines that will persist and expand *in vivo* following adoptive transfer. Moreover, exercise elevates the ability of NK-cells to kill HLA-E expressing lymphoma and multiple myeloma target cells during the recovery phase of exercise (4, 5). Here we discuss strategies by which exercise might improve allogeneic adoptive transfer immunotherapy, with a particular focus on DLI and the isolation/expansion of VSTs, TAA-specific T-cells and NK-cells.

Can Exercise Improve Donor Lymphocyte Infusions?

Exercise may provide better cell products for the preemptive prevention and treatment of disease relapse after HSCT through DLI. For instance, when a fixed number of lymphocytes are transferred to a patient, the proportion of NK-cells among the transferred lymphocytes will be 3–4-fold greater after exercise without affecting the proportion of VSTs (28). This would mean a similar number of VSTs being delivered to the patient with the added advantage of having more NK-cells that can suppress donor T-cell alloreactivity without compromising the GvT effect (23). In this regard, exercise may reduce or even eliminate the need for further cellular processing that is required to remove alloreactive T-cells prior to transfer. The lowered proportion of regulatory T-cells in PBMCs after exercise may also be advantageous as they are known to inhibit the expansion of antigen-specific T-cells both *ex vivo* and *in vivo* (33). One potential problem with exercise is the greater frequency of effector T-cells in the lymphocyte pool that could increase the risk of GvHD (28). As such, it will be important to show that exercise mobilized lymphocytes can augment the desired GvT effect without increasing the risk of GvHD before they can be used clinically.

Can Exercise Increase the Enrichment of multi-VSTs from Donor Blood?

Obtaining adequate numbers of VSTs from donor blood for direct transfer is highly desired. Not only does this expedite the delivery of the cell product to the patient, but also eliminates potential pitfalls associated with *ex vivo* expansion methods including epitope loss, functional exhaustion, prolonged manufacturing times and anergy (7, 8). Unfortunately, low numbers of circulating VSTs can inhibit the successful isolation and transfer of ‘non-manipulated’ cells from donor to patient. We have shown that exercise increases VSTs per unit of blood up to 5-fold depending on the target antigen (31). Exercise is a particularly potent stimulus for mobilizing VSTs reacting to herpesvirus antigens such as those derived from CMV and EBV (31, 32), but we have also found that VSTs specific to non-latent viruses (i.e. adenovirus) are also highly exercise responsive (unpublished). This is particularly important, as the frequency of VSTs to non-latent viruses in individuals without an active infection is extremely low, making the capture and rapid manufacture of these VSTs extremely difficult. This might pave the way for using exercise to augment the capture and *ex vivo* expansion of broad range multi-VSTs specific to a host of non-latent viruses such as adenovirus, BK virus, RSV, and influenza, which are known to contribute to morbidity and mortality following HSCT (8, 21).

New cytokine capture methods designed to enrich multi-VSTs from whole blood will be particularly useful for the direct capture of VSTs following exercise (Figure 1). Although VSTs are oftentimes enriched from a fixed number of isolated PBMCs, this would greatly dilute the mobilizing effects of exercise because of the substantially elevated proportions of NK-cells in post-exercise PBMC fractions (28). Stimulating whole blood with viral peptides removes the artificial ‘ceiling’ that is imposed when using fixed numbers of PBMCs, thus ensuring that all exercise-mobilized VSTs are accessible by cytokine capture.

Can Exercise Augment the *ex vivo* Expansion of multi-VSTs?

A population of pre-existing memory T-cells are required to rapidly expand VSTs from healthy donors. However, the frequency of VSTs can be <1% of the peripheral lymphocyte

population, which poses a significant obstacle to the successful *ex vivo* expansion of multi-VSTs for adoptive transfer. Noting that exercise evoked a greater CD8⁺ T-cell and EM T-cell redeployment in people with CMV (28), we also found marked increases in the number of VSTs reacting to the CMV antigens IE-1 and pp65 after a single exercise bout (31). Moreover, the exercise-mobilized VSTs reacted with a large number of IE-1 and pp65 epitopes indicating that they have broad antigen specificity (31). This led to our hypothesis that a single exercise bout would augment the *ex vivo* manufacture of multi-VSTs, which we tested by expanding multi-VSTs from healthy CMV and EBV seropositive participants before and after 30-minutes of steady state cycling exercise. Fixed numbers of PBMCs were isolated for the rapid manufacture of multi-VSTs over 10-days of cell culture (15). Exercise augmented the manufacture of VSTs reacting to CMVpp65 (average 2.6-fold), EBV LMP-2 (average 2.5-fold) and EBV BMLF-1 (average 4.4-fold) antigens compared to the resting condition (32). The VSTs expanded after exercise were phenotypically identical and equally capable of ‘per-cell’ antigen-specific and MHC-restricted killing of autologous target cells compared to the VSTs expanded at rest (32). Thus, although exercise did not appear to improve the functional properties of the expanded VSTs on a per-cell basis, it allowed us to generate a substantially greater number of VSTs with comparable ‘per-cell’ anti-viral properties to those expanded from resting PBMCs. The number of VSTs expanded after exercise was unrelated to the frequency of VSTs in the starting PBMC fractions (32), indicating that exercise-mobilized VSTs respond and proliferate to viral antigens on a per-cell basis more efficiently than their counterparts in resting blood. These findings provide evidence that a single exercise bout may serve as a simple, reliable and economical adjuvant to boost the production of multi-VSTs from healthy donors for allogeneic adoptive transfer immunotherapy (Figure 1). However, it will be important to show that the VSTs expanded after exercise do not have increased alloreactivity and that multi-VSTs reacting with a broad range of viral antigens (including those derived from non-latent viruses) can be expanded from a single exposure to clinically relevant peptide pools without introducing antigen dominance.

Can Exercise Augment the *ex vivo* Expansion of TAA-specific T-cells?

The adoptive transfer of TAA-specific T-cells evolved from an improved understanding of the antigens expressed by leukemia and lymphoma cells that could be targeted by cytotoxic T-cells (7). It has been shown that cytotoxic T-cell lines can be manufactured *ex vivo* to recognize a wide array of TAAs and minor HLA antigens, providing strong clinical application potential in both the allogeneic and autologous HSCT settings (7). However, the manufacture of TAA-specific T-cells, particularly from healthy allogeneic donors, is challenging due to difficulties priming and expanding sufficient cell numbers. Moreover, because non-viral TAAs are mostly self-antigens, T-cells specific to these antigens are not typically found in healthy donors and, if present, are of low avidity as self-reactive T-cells are typically anergic (7).

To determine if the biological stress response would facilitate the manufacture of TAA-specific T-cells, we asked healthy subjects to complete a single bout of maximal stair running exercise (duration: 73 to 200 seconds). Mo-DCs were also manufactured before and after exercise and pulsed with an array of TAA peptides including *melanoma-associated*

antigen-4 (MAGE-A4), *preferentially expressed antigen in melanoma* (PRAME), and *Wilms' tumor protein-1* (WT-1), and co-cultured with pre and post-exercise autologous lymphocytes for 14-days (19). A second stimulation with autologous peptide-pulsed Mo-DCs derived from resting blood was delivered after 7 days. We found that exercise improved the expansion of T-cells responding to at least one TAA in 84% of participants (19). Exercise increased the expansion of MAGE-A4 and PRAME-specific T-cells in 70% and 61% of participants, respectively (19). However, only 38% of participants displayed an increased expansion of WT-1 specific T-cells after exercise (19). Among the 'exercise responders', exercise increased the manufacture of MAGE-A4 and PRAME-specific T-cells 3.4 fold and 6.2 fold compared to the resting condition, respectively (19). Although we were unable to find demographic or physiological differences between the exercise responders and non-responders, it is important to note that exercise did not appear to inhibit the manufacture of TAA-specific T-cells in the non-responders. Moreover, the cells expanded post-exercise retained their ability to recognize and kill autologous target cells pulsed with TAA peptides and were phenotypically identical to the cells expanded under resting conditions (19). So while exercise might not increase the *ex vivo* manufacture of TAA-specific T-cells in all donors, it works more often than it does not and, because it did not ever inhibit the cell manufacturing process, we contend that it remains a worthwhile strategy to augment the manufacture of TAA-specific T-cells for adoptive transfer immunotherapy.

Despite increasing the *ex vivo* expansion of TAA-specific T-cells in the majority of cases (19), the exercise effects, in terms of both magnitude and consistency, were not as large as we have seen with the manufacture of VSTs (32). This is perhaps due to VSTs being manufactured from a population of precursor memory T-cells whereas TAA-specific T-cells are largely generated from the priming and expansion of naïve T-cells. It is also possible that the lack of consistent effects across all donors was due to our unsophisticated mode of exercise, which was relatively brief without controlling the intensity and duration of the bout (19). Taken together, these findings indicate that exercise might prime naïve T-cells to become more responsive to antigen stimulation and underscores the need to comprehensively assess the impact of different modalities of exercise (and their intensity and duration) on the priming and expansion of both naïve and memory precursor T-cells for immunotherapy.

Can Exercise Improve NK-cell Immunotherapy?

NK-cell therapy has great potential to improve the treatment of HLA-expressing malignancies, such as AML and MM. Unfortunately, current expansion methods tend to generate NK-cells with low alloreactivity and a higher expression ratio of the inhibitory receptor NKG2A relative to the activating receptor NKG2C (25), both of which ligate with the non-classical HLA molecule, HLA-E, that is overexpressed on primary AML and MM cells (22). While most metastatic tumors are characterized by HLA downregulation to evade detection by T-cells, disease progression in MM and AML is associated with reacquisition of HLA class I expression (including HLA-E) as a means of evading KIR-matched and NKG2A^{pos} NK-cells (22). In addition, the NKG2A^{pos} CD56^{bright} NK-cells that predominate during the early phase of immune reconstitution after HSCT secrete large amounts of IFN- γ which upregulates HLA-E expression by primary MM and AML cells, thus improving their

ability to evade reconstituted NK-cells (22). Conversely, this makes them more susceptible to being killed by NKG2C^{pos}/NKG2A^{neg} NK-cells (6), suggesting that new methods to selectively isolate or expand CD56^{bright}/NKG2C^{pos}/NKG2A^{neg} NK-cells from donor blood would be a useful approach to treat and prevent relapse in AML and MM.

Our work shows that exercise has great potential as an adjuvant for NK-cell therapy as it primes NK-cells to kill HLA-expressing tumor cells that are typically resistant to NK-cells (4, 5). Furthermore, the phenotypic and functional properties of NK-cells in the blood during the recovery phase of exercise (high cytotoxicity, low differentiation status, high activating receptor and low inhibitory receptor expression) appear better suited for the allogeneic adoptive transfer setting compared to NK-cells in blood at rest or immediately after exercise (4) (Figure 1). Indeed, 1h after completing a 30-minute cycling protocol, there are increased proportions of NKG2C^{pos}/NKG2A^{neg} NK-cells and their ability to kill target cells expressing both classic and non-classic HLA molecules is markedly elevated, particularly in HCMV seronegative donors (5). Thus, it might be better to expand NK-cells during exercise recovery as opposed to during or immediately after exercise, especially from HCMV seronegative donors, who tend to have lower numbers of NKG2C^{pos}/NKG2A^{neg} NK-cells and lower NKCA against HLA-E-target cells at rest (5, 6). To this end, we have shown that NK-cells derived from blood 1h post-exercise expand far more vigorously than those obtained before or immediately after exercise with no loss in activating receptor expression or cytotoxicity (3). We have also shown that NK-cells taken from blood 1h post-exercise are less-differentiated, which suggests that they will have greater replicative potential and possibly persistence *in vivo* (4, 5).

POTENTIAL MECHANISMS FOR THE AUGMENTING EFFECTS OF EXERCISE

Increased Enrichment of Cytotoxic Lymphocytes from Donor Blood

The biological stress response mobilizes cytotoxic lymphocytes to the periphery, potentially allowing a greater enrichment of VSTs and other cell types from whole blood using cytokine capture methods. Increases in cardiac output, blood pressure and blood flow, that accompanies activation of the sympathetic nervous system, increases hemodynamic shear-stress causing adherent leukocyte subtypes to detach from their endothelial ligands and enter the main axial flow of the peripheral circulation (29). The mobilization of certain lymphocyte subtypes is also dependent on the actions of catecholamines such as epinephrine and the neurotransmitter norepinephrine, which bind to β -adrenergic receptors (ARs) expressed by lymphocytes causing their mobilization into the peripheral circulation. Lymphocytes, particularly NK-cells, $\gamma\delta$ T-cells and memory CD8⁺ T-cells, preferentially express the β_2 -AR allowing them to be rapidly mobilized to the bloodstream in response to catecholamines (28). Viral specific T-cells predominantly exhibit a CM and EM phenotype, which, in turn, are known to express higher levels of the β_2 -AR in comparison to T-cells with a naïve or low differentiated phenotype (28). Moreover, in addition to increasing their mobilization, exposing T-cells to catecholamines elevates their production of IFN- γ in response to antigenic stimulation (27, 30), which may allow more antigen-specific cells to be enriched from the blood using peptide stimulation and cytokine capture methods.

Augmented expansion of cytotoxic lymphocytes *ex vivo*

Exercise augments *ex vivo* manufacture of VSTs even after adjusting for pre-expansion VST numbers (32). It is possible then that exercise is simply altering the proportion of discrete T-cell subtypes within the VST population that favor enhanced proliferative responses to peptide stimulation. For instance, a greater proportion of CM or EM cells among the peptide-responsive VSTs may allow for a more vigorous expansion of the memory T-cell pool *ex vivo*. Furthermore, we have found that CM and EM T-cells mobilized with exercise have a lowered expression of the T-cell exhaustion marker PD-1 (unpublished data). This, coupled with our previous findings that exercise mobilizes cytokine secreting CD8⁺ T-cells with long telomeres, suggests that exercise mobilized cells are more likely to proliferate, carry out effector functions, and persist in the host after transfer than comparable cells collected from resting blood (28, 29). The augmenting effects of exercise on *ex vivo* expansion might be due to shifts in the proportion of bystander cells within the stimulated PBMC fractions, such as lowered proportions of regulatory T-cells that can otherwise suppress T-cell proliferation to antigen stimulation (29, 33). Moreover, the ratio of antigen-presenting cells (i.e. monocytes and dendritic cells) to memory T-cells may shift favorably with exercise to promote enhanced T-cell activation and proliferation (20). Indeed, we found that the increased number of monocytes in the post-exercise PBMC fractions can increase the generation of Mo-DCs (20), which may have resulted in a greater level of antigen presentation in the post-exercise condition for the expansion of TAA-specific T-cells (19).

Within the NK-cell compartment, the proportion of CD56^{bright}/CD57^{neg} cells increases during exercise recovery (1h after exercise cessation) (4). These cells are considered to be a less mature population of NK-cells with greater proliferative potential, which might explain why NK-cell proliferation is enhanced during exercise recovery (3). Given the profound effects of exercise and exercise analogs (i.e. catecholamine infusion) in altering the composition of lymphocyte and monocyte subtypes within PBMCs (28, 29), it is likely that proportional shifts in cell subtypes are at least partially responsible for the augmenting effects of exercise on the *ex vivo* expansion of T-cells and NK-cells. It is also possible that exercise affects T-cells and NK-cells directly causing them to be more responsive to antigen/cytokine stimulation at the individual cell level. Alternatively, bystander cells involved in the process of antigen presentation and activation, such as monocytes and dendritic cells, could be directly impacted to enhance the activation and proliferation of T-cells and NK-cells to antigen stimulation. Soluble candidates likely involved are cytokines, catecholamines, growth hormone and thyroid hormones, which have all been shown to modulate memory T-cell function and expansion (29). Studies assessing the effects of exercise on *ex vivo* expanded cell products depleted of specific cell populations and/or in the presence of resting or post exercise serum would be illuminating.

Can β -AR agonist infusions act as an 'Exercise Surrogate'?

It is possible that a simple infusion with a β -AR agonist will elicit a robust mobilization of VSTs, analogous to exercise, as a means to increase the enrichment of VSTs from donor blood (28). Indeed, β_2 -AR agonist infusion might be preferred over exercise in certain clinical situations. For instance, high-intensity and/or prolonged exercise bouts might not be practical in cancer patients (autologous setting) or even healthy donors (allogeneic setting)

with low fitness levels (29). Moreover, exercise may have to be performed within the confinements of bed rest for some donor and patient populations. In this regard, β -AR agonist infusions for the purposes of mobilizing cytotoxic lymphocytes for direct isolation or *ex vivo* expansion would be preferred (27, 29). However, identifying the minimum intensity and duration of exercise required to enhance the mobilization and expansion of cytotoxic lymphocytes would certainly broaden its application as a clinical adjuvant.

Studies on the effects of *in vitro* catecholamine stimulation on T-cell cytokine production and proliferation have generated equivocal results. The use of synthetic β -AR agonists (predominantly targeting the β_2 -AR) to stimulate T-cells has been reported to both increase (30) and decrease (34) T-cell IFN- γ and IL-4 cytokine production, but impair T-cell proliferation (30, 34). Conversely, targeting the β_1 -AR has been shown to enhance T-cell proliferation and IL-4 secretion, but inhibit IFN- γ production (11). It has also been suggested that when β -AR stimulation precedes the antigenic challenge (as opposed to being simultaneously stimulated), T-cell activation, proliferation and cytokine secretion are more likely to increase (27), as would be the case when VSTs are expanded *ex vivo* following a single bout of exercise, or if synthetic catecholamines are used to mobilize VSTs to the circulation *in vivo* (32). Nevertheless, potential inhibitory effects of catecholamines on T-cell proliferation would be a non-issue if they are transient and catecholamines/ β -AR agonists are used as a mobilizing agent *in vivo* (11, 30). Waiting a short time for the effects of the catecholamines to dissipate might be all that is required before the mobilized cells are transferred to the patient or expanded *ex vivo* (11, 30). Conversely, if catecholamines acting on T-cell β -ARs are driving the augmenting effects of exercise on VST isolation and expansion, then more work will be required to determine the β -AR subtypes involved, the downstream signaling pathways (e.g. cAMP/PKA, p38/MAPK, β -arrestin, Gs), and the optimal stimulation kinetics prior to antigen challenge.

EXERCISE AS AN ADJUVANT FOR HEMATOPOIETIC STEM CELL MOBILIZATION

We propose that exercise will be a simple and cost-effective way to increase the yield of HSCs from healthy donors for transplant (Figure 2). Peripheral Blood Stem Cell (PBSC) collections typically occur after several days of granulocyte-colony stimulating factor (G-CSF) treatment, which is used to proliferate CD34+ hematopoietic stem cells (HSCs) in the bone marrow and mobilize them to the peripheral blood for collection via apheresis. Mobilizing PBSCs with G-CSF has several side effects including severe bone pain and nausea (9). There is also concern that G-CSF and other mobilizing agents, such as the chemokine receptor type-4 inhibitor, plerixaflor, can cause leukemia in healthy donors (2). The time commitment and discomfort associated with PBSC collections is an obstacle for many donors, with ~40% of donors requiring at least 2 apheresis sessions (each session takes 3–4h) in order to collect enough PBSCs for transfer (2). Subsequent apheresis sessions are often accompanied by further pharmacological interventions to facilitate PBSC collection.

New methods are sought to increase the percentage of donors reaching target HSC numbers in a single apheresis session, thus reducing reliance on pharmaceutical interventions that have known toxicities and debilitating side effects. In healthy people not treated with HSC mobilizing agents, circulating CD34+ HSCs may increase 2–4 fold after a single bout of even very brief exercise (12). Exercise appears to mobilize CD34+ HSCs independently of G-CSF and catecholamines (26), suggesting that hemodynamic sheer stress might be responsible for the demargination of previously mobilized CD34+ HSCs into the main axial blood flow of the peripheral circulation. The number of CD34+ HSCs mobilized with exercise is still considerably lower than the numbers found in the blood of healthy donors following G-CSF therapy so exercise is unlikely to completely replace G-CSF as an HSC mobilizer (12). However, it is probable that a large proportion of the HSCs mobilized by G-CSF will also enter the marginal pools making them inaccessible by standard apheresis procedures, which would pave the way for exercise to be used as a simple and economical adjuvant to increase the yield of CD34+ HSCs during apheresis through its effects on hemodynamic shear-stress (Figure 2). Future studies are required to determine if exercise, or pharmaceutical interventions that evoke an analogous biological stress response, can be used as an adjuvant to G-CSF therapy for mobilizing and collecting HSCs from the blood of healthy donors. This might reduce donor burden by preventing the need for additional apheresis sessions and pharmaceutical mobilization agents that are expensive and beset with debilitating side effects.

CONCLUSIONS

Leukocytosis in response to exercise was first reported at the beginning of the twentieth century and has long been considered a fascinating phenomenon, albeit one that was not believed to harbor any real clinical significance or application (29). More than a century later, HSCT and adoptive transfer immunotherapy has come of age and we propose that exercise-induced leukocytosis can serve as a potent adjuvant for cellular immunotherapy by increasing the number of cells that can be isolated from donor blood, accelerating the manufacturing process, and improving the quality of cell product being delivered to the patient (3, 4, 19, 20, 32). Moving forward, we should determine the extent by which exercise has broad application across multiple different cell types and methods used to isolate and expand cell products for immunotherapy. For instance, $\gamma\delta$ T-cells are preferentially redeployed with exercise (1) and are expanded *ex vivo* using phosphoantigens (i.e. zoledronic acid) to treat a range of hematologic malignancies (follicular lymphoma, multiple myeloma, acute and chronic myeloid leukemia) and solid tumors (renal cell, breast and prostate carcinomas) (13) (Table), but it remains to be seen if exercise can enhance the expansion or function of $\gamma\delta$ T-cells. We have also shown that exercise augments the *ex vivo* manufacture of Mo-DCs (20), which could be useful to augment dendritic cell vaccination therapy for the treatment of diseases such as chronic B-cell lymphocytic leukemia (18). Identifying the mechanism(s) by which exercise augments the isolation and expansion of cytotoxic lymphocytes from donor blood would broaden the clinical application of this work, particularly if the exercise effects can be replicated by a simple pharmaceutical intervention (i.e. infusion with an β -AR specific agonist) (11, 30). It will also be important to assess the safety profile of the expanded cell products to ensure that the use of exercise-

mobilized/expanded cells will not increase the risk of GvHD. Finally, the exercise protocols and timing of blood draws will have to be optimized for the clinic in order to maximize the isolation and expansion of specific cell products during and after exercise.

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KEY POINTS

- Viral infections and disease relapse are major causes of morbidity and mortality after hematopoietic stem cell transplantation (HSCT) for the treatment of blood cancers
- Allogeneic (donor to patient) lymphocyte infusions can effectively treat and prevent post-transplant viral infections and disease relapse after HSCT; however, low numbers of antigen-specific cytotoxic lymphocytes in donor blood can be a limiting factor.
- A single exercise bout mobilizes large numbers cytotoxic lymphocytes to the blood and augments the *ex vivo* manufacture of antigen-specific T-cells in donors previously exposed to viruses that cause morbidity and mortality after HSCT. Infusing more of these donor-derived cells to cancer patients after HSCT could greatly improve post-transplant outcomes.
- Exercise may serve as an effective and economical adjuvant to boost cytotoxic lymphocyte numbers in donor blood to improve the efficacy of adoptive cell transfer after HSCT.
- Potential mechanisms and the translation of this work to the clinic are addressed.

Figure 1.

Exercise as an adjuvant for viral specific T-cell (VST) isolation and *ex vivo* expansion. Successful adoptive transfer of donor-derived VSTs is oftentimes limited by low numbers of circulating VSTs in donor blood. A single bout of exercise mobilizes VSTs into the peripheral circulation, increasing their concentration per unit volume of whole blood. Stimulating whole blood samples with viral peptide pools allows 2–3 times more VSTs to be isolated from the donor during exercise using IFN- γ capture antibodies and magnetic cell sorting for direct transfer to the hematopoietic stem cell transplant (HSCT) patient (Direct Isolation Method). Similarly, isolating and expanding a fixed number of peripheral blood mononuclear cells (PBMCs) from donors after exercise allows 2–5 times more VSTs to be manufactured when co-cultured with viral peptides and growth cytokines over 8–10 days prior to adoptive transfer (*ex vivo* Expansion Method).

Figure 2.

Exercise as an adjuvant for CD34+ stem cell mobilization following growth-colony stimulating factor (G-CSF) treatment. A single bout of exercise mobilizes CD34+ stem cells adhered to the vascular endothelium, causing a 2-fold increase in the number of CD34+ stem cells in the main axial flow of the peripheral blood compartment. Peripheral blood stem cell (PBSC) collections by apheresis typically begin when the cell concentration exceeds 10 CD34+ cells/µl following G-CSF therapy. Although exercise alone is unlikely to generate or maintain these cell concentrations for an entire PBSC collection procedure lasting 3–4h, the hemodynamic effects of exercise may act as an adjuvant to increase CD34+ stem cell yields following G-CSF therapy. Exercising healthy donors during apheresis following G-CSF therapy may reduce PBSC collection time and the number of PBSC collection sessions required to achieve target CD34+ stem cell counts, thus reducing donor burden and the need for further pharmaceutical interventions that have known toxicities and potentially debilitating side-effects.

Table 1.

The potential of a single exercise bout to enhance the isolation and manufacture of specific cell types obtained from healthy donors for the purpose of allogeneic stem cell transplantation and adoptive transfer immunotherapy. The methods likely to be improved or be unaffected by exercise are indicated by + and –, respectively.

Cell Type/Product	Direct Selection Method	<i>Ex Vivo</i> Expansion Method	Clinical Application	Reference
Multi-VSTs	+	+	Adoptive T-cell Therapy, prophylaxis and treatment of viral infections post-HSCT	(8, 15, 21, 24, 31, 32)
TAA-specific T-cells	–	+	Adoptive T-cell Therapy for bloodborne malignancies, relapse prophylaxis and treatment of refractory disease	(7, 14, 19)
$\gamma\delta$ T-cells	+	+	Adoptive T-cell Therapy for bloodborne malignancies and solid tumors	(1, 13)
NK-cells	+	+	Adoptive transfer for the treatment of HLA-expressing malignancies, including AML and MM	(1, 3, 5, 6, 22, 25)
Dendritic Cells and Monocyte-Derived Dendritic Cells	–	+	Dendritic cell vaccination therapy	(18, 20)
CD34+ HSCs	^a +	N/A	Hematopoietic Stem Cell Transplantation	(12)

^a Cell numbers sufficient for clinical use are only likely to be obtained if exercise is used as an adjuvant to mobilize HSCs in donors following prior G-CSF treatment