Moderate Exercise Early After Influenza Virus Infection Reduces the Th1 Inflammatory Response in Lungs of Mice

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Running Head: Exercise and immune response to influenza virus infection in mice

Abstract

We have previously shown that moderate exercise significantly increased survival after influenza virus (A/PR/8/34) infection in mice. We hypothesized that this brief duration of exercise would either increase innate immune defences and/or shift the immune response from a Th1 inflammatory to a Th2 anti-inflammatory response resulting in decreased lung pathology. Adult male BALB/cByJ mice (5-6 months old) were infected with 50 μL of A/PR/8/34 virus (40HAU) intranasally and randomized to either an exercise (EX) or sedentary (SED) group. EX mice performed 20-30 min of moderate exercise (8-12 m/min) on a motorized treadmill 4 hr post-infection and then exercised similarly for 4 consecutive days. SED mice were exposed to similar environmental conditions but did not exercise. Mice from both EX and SED groups were sacrificed 1, 3, or 5 days post-infection (p.i.) and lungs, mediastinal lymph nodes (MLNs) and spleens were harvested. EX significantly reduced total cellular infiltration and IFN-γ gene expression in lungs at Days 3 and 5 p.i. and there was a qualitative shift in the expression of cytokines in the lung from a Th1 to a Th2 response. There was also a tendency toward a reduction in influenza M1 protein mRNA expression. There was no difference in IFN-β protein levels between groups. These data suggest that moderate exercise when applied early after infection shifts the immune response away from a Th1 profile in mice infected with influenza virus. This exercise-induced shift in immune response may be responsible for improved survival after influenza virus infection. (Exerc. Immunol. Rev. 12, 2006: 97-111)

Keywords: influenza, exercise, inflammation, immunity, Th1, interferon-γ

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Introduction

Many laboratories, including ours, have conducted research examining the influence of exercise on immune responses. While far from certainty, the prevailing data suggest that moderate exercise improves immune function (43), whereas strenuous, prolonged exercise decreases many aspects of immune functioning (18, 19, 29). Aside from a few recent studies with herpes simplex-1 virus and lymphocytic choriomeningitis virus (LCMV) (6, 19, 43), the majority of research evidence comes from studies examining in vitro immune responses using pathogen non-specific immune challenges. For instance, several studies have examined the response of isolated peripheral blood mononuclear cells to polyclonal mitogens or examined natural killer cell activity from cells obtained before and after exercise (14, 24, 33). Examination of the effects of exercise on the immune response to clinically relevant challenges is lacking.

Each year, diseases associated with the influenza virus are responsible for an estimated 36,000 deaths and up to 150,000 hospitalizations in the United States making it a significant public health problem (4). Both the innate and the adaptive immune systems are activated following primary infection with influenza virus. Early innate detection of influenza virus in the respiratory tract occurs through plasmacytoid dendritic cell (pDC) toll-like receptor (TLR)-7 leading to the induction of type 1 interferons (IFNs), NF-κB activation, and pro-inflammatory cytokine expression (26). TLR-7-triggered production of IFN-α and IFN-β induce an anti-viral state important to limit the initial spread of the virus, and these Type I interferons also begin to drive the innate immune response to influenza (5, 32). Perhaps more importantly, the initial triggering of TLR-7 makes the pDCs key players driving the early events that ultimately lead to the development of Th1-dominated cell-mediated immune responses. The pDCs secrete multiple pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-18) and chemokines (e.g. RANTES, MCP-1) that directly and indirectly lead to increased antigen processing and presentation by macrophages and increased cytotoxicity by natural killer (NK) cells. Ultimately, TLR-7 signaling can be linked to activation of antigen-specific immune responses by the production of IFN-γ, which is the principal mediator of Th1 responses. That link is further manifested in the subsequent proliferation and recruitment of antigen-specific $CD8^+$ T lymphocytes (the expansion of which depends on $CD4^+$ Th1 cells) in the draining mediastinal lymph nodes (MLNs). These antigen-specific $CD8^+$ T cells play a significant role in viral clearance and recovery from influenza infection (42). Interestingly, while a strong Th1 response is important in influenza viral clearance, such an inflammatory response may also lead to tissue damage and death (38). It has been suggested that Th2 cytokines, such as IL-4 and IL-10, counteract potentially harmful high levels of Th1 cytokines (and their induced responses) in influenza infection (1).

We have previously determined that 4 days of moderate exercise applied early after infection with influenza virus significantly increases survival rate in BALB/c mice (25). In contrast, prolonged exercise led to higher mortality rates. In this study, we explored moderate exercise-induced changes in immune responses of mice infected with influenza virus in an attempt to begin to understand why it is that exercise protected mice from death due to influenza virus challenge. We hypothesized that this brief duration of exercise would result in

decreased lung pathology and shift the immune response from a Th1 inflammatory response to a Th2 anti-inflammatory response.

Materials and Methods

Animals. Specific pathogen-free inbred male BALB/c mice aged 5-6 months (n=110) were bred in our facility and used in all experiments. Mice were housed individually in micro-isolated shoe box cages in facilities maintained at a temperature of 23 ºC. All mice were kept on a 12:12-h light-dark cycle (0700-1900 dark) and given autoclaved food (8640 Harlan Teklad 22-5 Harlan, Madison, WI) and water *ad libitum*. All animal treatments were approved by the Institutional Animal Care and Use Committee at the University of Illinois @ Urbana/Champaign and within the guidelines set by the NIH for the care and use of laboratory animals.

Influenza Infection. We challenged BALB/c mice intranasally (i.n.) with a dose of virus (40 hemmaglutinating units [HAU]) that resulted in ~50% lethality of sedentary control mice as in our previous study (25). In this study, 50 μL of influenza virus (40 HAU of A/Puerto Rico/8/34) was delivered i.n. into mice lightly anesthetized with Isoflurane.

Exercise Paradigm. Mice were infected 2-3 hr into their dark cycle on Day 0. Four hr post-infection (p.i.) (1300-1400, Day 0) mice were exercised using a motorized treadmill at a speed of 8-12 $m.min^{-1}$ and 5 % grade for 30 min. This treadmill speed was approximately 55-65 % of their maximal oxygen uptake (VO_2max) (23). Our own studies measuring VO_2max in BALB/c mice of various ages have confirmed these relative exercise intensities (data not shown). Mice then exercised similarly for 3 more consecutive days (Days 1-3), with each exercise session separated by 22-24 hr. We expected the effects of exercise to be greatest when exercise was performed in the early stages of infection. Mice were not exercised past Day 3 because they exhibited illness symptoms. Mice ran without electric shock or prodding. Control mice were exposed to the treadmill environment for a similar time but did not exercise. All mice were denied access to food and water during the exercise period.

Tissue Collection and Processing. Lungs isolated for mRNA or protein analysis were excised and immediately flash-frozen in liquid nitrogen in 2.5 mL micro-centrifuge tubes and then stored at -80 °C until analysis by real-time reverse transcriptase (RT)-PCR. Lungs isolated for cellular analysis were placed in 5 mL of media (RPMI 1640) and homogenized. The homogenate was added to 25 mL of media for a total volume of 30 mL. This was followed by the addition of 1,500 units of Type I collagenase per sample (Worthington Biochemical Corporation, Lakewood, New Jersey). This mixture was placed in a beaker and stirred using a magnetic stir plate for 45 min at 4°C. Following this digestion, the mixture was centrifuged at 200 x *g* for 5 min. The supernatant was removed, residual red blood cells were lysed with ammonium chloride solution, the cells were resuspended, counted, and single-cell suspensions were

adjusted for antibody staining and flow cytometric analysis. In addition to isolating lungs, we also examined cell content and phenotype of the draining MLNs and spleens. Single cell suspensions from MLNs and spleens were obtained by passing these tissues through a wire mesh screen (Sigma-Aldrich, St. Louis, MO). Residual red blood cells were lysed with ammonium chloride solution, cells were resuspended, counted, and single-cell suspensions were concentration adjusted for antibody staining and flow cytometric analysis.

Cytospin Preparation. Following sacrifice, lungs were lavaged with 3-5 mL of PBS. Cells were centrifuged at 250 x *g* for 5 min, resuspended in PBS, counted and adjusted to 0.5 x 10^6 cells per mL. Cells were kept on ice, and 100-200 µL of this solution was used for Wright's staining on microscope slides. Cell identification was performed at 40X in triplicate in 3 previously determined fields in a blinded fashion, with the three cell count (minimum of 200 cells counted per field) identifications averaged for each mouse. Neutrophils, lymphocytes, and macrophages are presented as a percentage of the total number of cells counted.

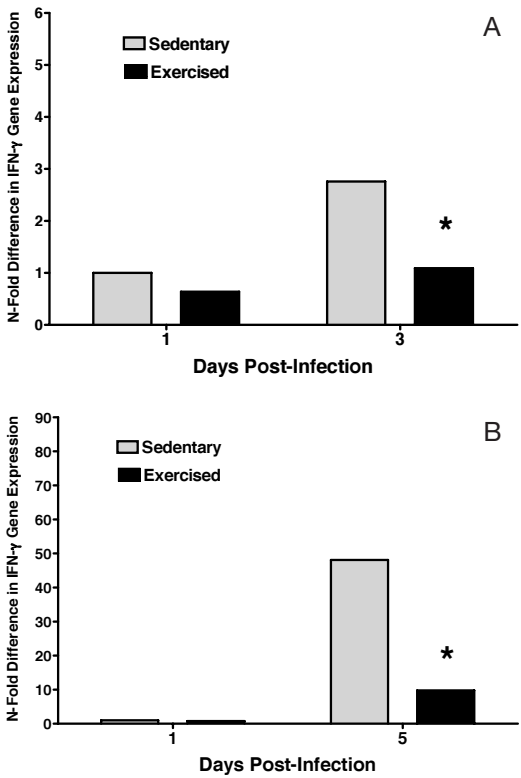

Figure 1. IFN-γ gene expression in lungs of mice on Day 3 (Figure 1a; n = 8 and 7 per group at Days 1 and 3, respectively) and Day 5 (Figure 1b; n = 8 and 10 per group at Days 1 and 5, respectively) following infection with A/PR/8/34. Note the differences in the y-axis scale between Figure 1a and 1b. *Significantly ($p < 0.05$) lower than SED group on same day.

Flow Cytometry. Cells (0.5 x 10^6) were added to 1 mL of PBS/2 % FBS/0.1% sodium azide and centrifuged at 250 x *g*. Cells were agitated following removal of supernatant and 100uL of PBS/2 % FBS/0.1 % sodium azide was added along with appropriate labeled antibodies (0.5 µg phycoerythrin-conjugated anti-CD8, Pharmingen, San Diego, CA; or 0.5 µg Rat Anti-Mouse CD49b/pan–RPE, Southern Biotechnology, Birmingham, AL) for 30 min at room temperature. Cells were then washed and fixed in PBS/1 % buffered Formalin (Sigma) and stored at 4°C until analysis using a Becton Dickinson LSR Benchtop Flow Cytometer with a 488 nm argon-ion laser. A minimum of 10,000

events were analyzed in each sample. The autofluorescence gate was set using an unstained sample. All autofluorescent samples fell within the first log, with stained samples falling between the first and third log.

Gene Expression. Lung tissue samples were homogenized using a standard single-step TRIzol RNA isolation procedure (GIBCO BRL, Gaithersburg, MD). After isolation, total RNA was quantified spectrophotometrically at 260 nm and 280 nm. RNA was reverse transcribed into cDNA according to manufacturer's protocol (Promega, Madison, WI). The reverse transcription system consisted of 25 mM $MgCl_2$, 10X reverse transcription buffer, 10 mM dNTP mixture, rRNase in ribonuclease inhibitor, avian myeloblastosis virus (AMV) reverse transcriptase, and random primers. Samples were incubated at 42˚C for 60 min, 100˚C for 5 min, and then cooled to 4˚C. All samples underwent real-time polymerase chain reaction (PCR), in order to quantify the nucleotide sequences of interest. 18S RNA was used as an internal control in all samples. Primers were purchased from Invitrogen Life Technologies (Carlsbad, CA), and probes were purchased from Applied Biosystems (Foster City, CA). Primer sequence information can be found in Table 1. The master mix for real time PCR consisted of 2X Universal Master Mix (PE Biosystems, Roche Molecular Systems, Branchburg, New Jersey), 0.9 μM of cDNA 5'primer, 0.9μM of cDNA 3'primer, and 0.25μM of cDNA probe. Samples were subjected to 40 cycles of amplification each consisting of 2 min at 50˚C, 10 min at 95˚C, 15 s at 95˚C, and 1 min at 60˚C. Real-time PCR was performed using a Prism 7000 Sequence Detection System Software (Applied Biosystems, Foster City, CA). Samples obtained from sedentary animals 24 hr post-infection were used as the reference control.

M1 Protein	**Sequence**
Forward	5'-GGACTGCAGCGTAGACGCTT-3'
Reverse	5'-CATCCTGTTGTATATGAGGCCCAT-3'
Probe	5'-CTCAGTTATTCTGCTGGTGCACTTGCCA-3'
IFN-γ	**Sequence**
Forward	5'-AGCAACAGCAAGGCGAAAA-3'
Reverse	5'-CTGGACCTGTGGGTTGTTGA-3'
Probe	5'-CCTCAAACTTGGCAATACTCATGAATGCATCC-3'
IL-10	**Sequence**
Forward	5'-TTTGAATTCCCTGGGTGAGAA-3'
Reverse	5'-ACAGGGGAGAAATCGATGACA-3'
Probe	5'-TGAAGACCCTCAGGATGCGGCTG-3'

Table 1. Primer sequences used for real-time RT-PCR.

Qualitative Protein Analysis. We utilized an inflammatory cytokine antibody array (RayBiotech, Lakewood, New Jersey) to probe for qualitative differences in protein expression in lung homogenates pooled from the two different groups. As this was a costly assay, only mice from Day 3 were used (n=6 SED and n=8 EX). Ten mg of lung was removed and added to 250 μL of lysis buffer, 250 μL PBS, and 50 μL of protease inhibitor cocktail (Sigma, St. Louis, MO). This mixture was homogenized and centrifuged at 2500 x *g* for 5 min. The resulting super-

natant was frozen at -80 °C. Samples from individual mice were then pooled (equal amounts of protein from each mouse) and analyzed according to manufacturer's specifications (RayBiotech, Lakewood, New Jersey).

Lung IFN-β ELISA. Analysis of lung IFN-β protein expression was performed using a commercially available (PBL Biomedical Laboratories, Piscataway, NJ) ELISA kit.

Data Analysis. Cell counts and percentages and lung IFN-β concentration are reported as mean ± standard error of the mean (SEM). For these variables, significant treatment effects (e.g. EX vs. SED) were determined using General Linear Model univariate analysis of variance (SPSS 14.0, Chicago, IL). Significant effects were followed up by a series of Bonferroni-adjusted post hoc tests. To assess the influence of exercise on gene expression during influenza infection, the quantity of specific RNA (e.g. IFN-γ, IL-10, or influenza M1 protein) obtained from a lung sample was compared to the quantity of the same RNA species present in sedentary lungs Day 1 post-infection. EX treatment did not affect baseline gene expression in uninfected lungs (data not shown). Briefly, real-time PCR generates a C_t value, which is the PCR cycle where amplification of the cDNA of interest begins exponential expansion. For analysis, the C_t value for the internal standard (i.e., 18S RNA) was first subtracted from the C_t value for the cDNA of interest. This subtraction controls for differences in reverse transcription and sample loading, and the value is denoted as the ΔC_t. Next, the ΔC_t value generated from referent tissue samples were then subtracted from the ΔC_t for each experimental sample. This equation sets the control sample to a reference value of 0 and generates a $\Delta\Delta C_t$for each unknown. And finally, these values were averaged for each treatment group; these mean values were used to generate the N-fold difference in RNA expression relative to the control using the equation: $2^{(-\Delta\Delta C_t)}$; using this equation the control = 1. Although this data transformation accurately illustrates the logarithmic amplification following each PCR cycle and is used in each of the figures, statistical evaluation was performed using the $\Delta\Delta C_t$ value. Ranked sum tests with a Student's *t-test* were performed using Sigma Stat statistical software version 2.0. p values of less than 0.05 were considered significant. Due to the logarithmic transformation of the

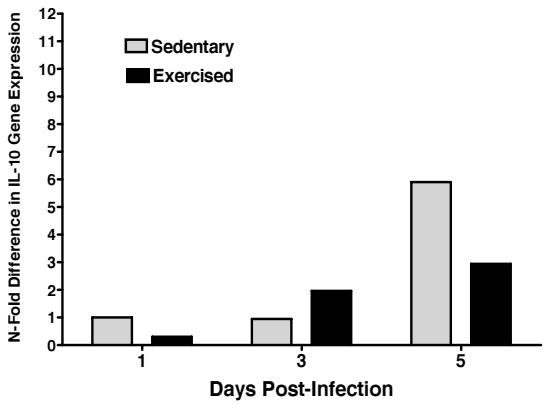

Figure 2. IL-10 gene expression in lungs of mice on Days 3 and 5 following infection with A/PR/8/34. (n = 12 and 10 in each group for Days 1 and 5 post-infection, respectively).

data, it is visually misleading to depict a standard deviation or standard error of the mean; thus the figures that illustrate real-time PCR data do not include such error bars.

Results

Exercise reduces cell influx into the lungs and secondary lymphoid organs of influenza infected mice. Infection resulted in significant cellular infiltration into the lung when compared to non-infected SED and EX mice (data not shown). Importantly, there was a significant reduction of total cells infiltrating the lung on Day 5, but not Day 3 (2.5 ± 0.6 vs. 2.5 ± 0.4x 10^6 for SED and EX, respectively; p = 0.98), p.i. in the EX when compared to the SED group (Table 2). We prepared cytospin slides of cells obtained from the lungs to determine whether exercise preferentially reduced specific phenotypes of cells including neutrophils, lymphocytes or macrophages. Despite the reduction in total cell number, there were no significant differences in the percentage of neutrophils (34 ± 4 % vs. 36 ± 5 %, for SED and EX, respectively, p = 0.78), lymphocytes (42 ± 4.7 vs. 42.5 ± 4.2 %, for SED and EX, respectively, p = 0.93) or macrophages (24 ± 3.7 vs. 21.6 ± 3.5 %, for SED and EX, respectively, p = 0.64) between EX and SED mice at Day 5 post-infection indicating that the anti-inflammatory effect of exercise was cell non-specific, at least as grossly defined by cell morphology. Furthermore, we found no significant differences in the percentage of cells positive for the cell specific surface markers CD8 (cytotoxic T lymphocyte) (Table 2) or CD49 (12.8 ± 3.6 and 15.9 ± 2.1, for SED and EX, respectively). EX mice exhibited significantly reduced numbers of $CD8^+$ cells in the spleen and a tendency for reduced $CD8^+$ cells in the MLNs and lung (Table 2).

Tissue	Total Cells ($x10^6$)		Percent $CD8^+$		$CD8^+$ ($x10^6$)	
	SED	EX	SED	EX	SED	EX
Lung	5.4 ± 0.44	4.1 ± 0.34*	12.6 ± 0.8	14.5 ± 2.7	0.68 ± 0.06	0.59 ± 0.1
MLN	4.8 ± 0.64	2.8 ± 0.66+	12.7 ± 2.9	11.7 ± 2.2	0.65 ± 0.19	0.33 ± 0.08+
Spleen	20.1 ± 5.7	12.9 ± 2.3	16.8 ± 3.7	15.9 ± 2.4	2.8 ± 0.4	1.7 ± 0.17*

Table 2. Effects of exercise on cellularity and CD8 phenotype in lung, MLN, and spleens of influenza infected mice 5d post-infection (N=5-8 per group). *p<0.05; +p<0.08

Exercise reduces IFN-γ gene expression in lungs of influenza infected mice. We measured lung cytokine gene expression to determine whether exercise modulated the Th1 (IFN-γ) or Th2 (IL-10) immune response to influenza infection. EX mice exhibited a significantly reduced expression of IFN-γ mRNA on both Day 3 (Figure 1a) and Day 5 (Figure 1b) post-infection. Irrespective of group, IFN-γ mRNA levels were significantly increased on Day 3 and even more so on Day 5 when compared to Day 1. Note the large y-axis scale difference between Figures 1a and 1b. While influenza infection increased IL-10 gene expression significant-

Cytokine	EX	SED
BLC	2,988.29 △	1,366.63
CD30L	1,282.62 △	0.00
Eotaxin	1,897.55 △	0.00
Eotaxin-2	1,503.41 △	0.00
FAS ligand	305.27 △	0.00
Fracktaline	4.08 △	0.00
G-CSF	0.00	0.00
GM-CSF	4,023.74	4,153.74
IFN-γ	43.34 ▽	2,165.44
IL-1α	4,312.05	2,578.38
IL-1β	2,554.57 △	243.31
IL-2	591.69	0.00
IL-3	0.00	0.00
IL-4	255.96 △	0.00
IL-6	0.00	0.00
IL-9	0.00	0.00
IL-10	0.00	0.00
IL-12p40/p70	338.24 △	0.00
IL-12p40	0.00	0.00
IL-13	1,679.28	2,988.31
IL-17	0.00 ▽	765.12
I-TAC	0.00 ▽	1,379.57
KC	0.00 ▽	834.00
Leptin	0.00 ▽	93.23
LIX	1,841.02 ▽	3,732.08
Lymphotactin	0.00 ▽	1,076.71
MCP-1	2,661.98	3,004.55
M-CSF	404.20 ▽	894.15
MIG	0.00	0.00
MIP-1-α	0.00	0.00
MIP-1-γ	10,887.22	6,527.00
RANTES	7,405.86	9,855.47
SDF-1	0.00 ▽	2,017.47
TCA-3	4,489.18	3,879.45
TECK	637.86	1,037.31i
TIMP-1	5,689.52	4,475.25
TIMP-2	1,271.95	1,801.83
TNF-α	1,776.01	1,728.44
sTNFrI	2,522.22	1,542.28
sTNFrII	1,968.53 △	743.47

Table 3. Qualitative protein expression (expressed as arbitrary units) in lungs 3 days post-infection. Numbers in green or △ indicate a > 2-fold increase in expression compared to SED. Numbers in red or ▽ indicate a > 2-fold decrease in expression compared to SED.

ly by Day 5 p.i., we found no significant differences between EX and SED in IL-10 gene expression on Day 3 or 5 p.i. (Figure 2); although there was a tendency ($p = 0.10$) for IL-10 gene expression to be lower in EX mice on Day 5 p.i. We also compared IFN-γ and IL-10 gene expression in the lungs of exercised (for either 1 or 4 days and sacrificed 24 hr after the last exercise bout), but non-infected, mice with that of sedentary non-infected mice. We found that exercise alone did not appreciably affect IFN-γ or IL-10 gene expression in the lung when compared to sedentary mice. Moreover, the level of IFN-γ and IL10 gene expression in lungs of non-infected sedentary or exercised mice was very low compared to infected mice.

Exercise causes a qualitative reduction in Th1 and an increase in Th2 proteins in the lungs of influenza infected mice. We used an antibody array to qualitatively assess inflammatory cytokine protein expression in pooled (equal amounts of protein per mouse) lung samples from SED and EX mice at Day 3 p.i.. As can be seen in Table 3, the levels of many inflammatory proteins were altered by exercise. Most notably, when compared with SED mice, exercise resulted in a 2-fold reduction in IFN-γ, RANTES, IL-13, IL-17, I-TAC, SDF-1, KC, LIX, M-CSF and lymphotactin. Two-fold increases in eotaxin, eotaxin-2, IL-2, IL-4, BLC, sTNFrI and II and CD30L were seen in exercised mice, as well as IL-1α and IL-1β. However, the pro-inflammatory cytokine TNF-α was similar in both groups and IL-6 was undetectable in either.

Exercise does not affect lung IFN-β expression. IFN-β aids in the early antiviral response through transcriptional activation of many genes, including the double-stranded RNA activated protein kinase (PKR) and Mx proteins, which in turn disrupt the replication of influenza virus (32). While IFN-β expression in infected lungs tended ($p = 0.11$) to be higher Day 1 post-infection in the EX mice there were no statistical differences between groups (Figure 3).

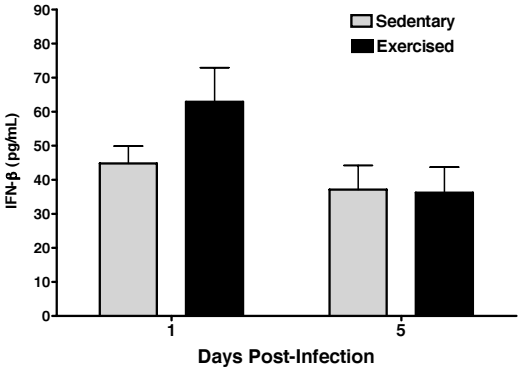

Figure 3. Effects of exercise on IFN-β protein expression in lungs of mice following infection with A/PR/8/34. (n = 8 per group).

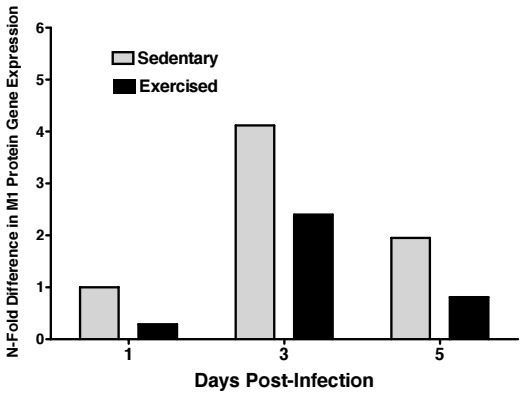

Figure 4. Effects of exercise on influenza virus M1 gene expression. Sedentary 24 hr post-infection sample was used as referent. While not statistically significant, there was a tendency towards lower viral replication in exercise when compared to sedentary mice. M1 gene expression in lungs of mice following infection with A/PR/8/34 (n = 11, 8, and 10 in each group at Days 1, 3, and 5 post-infection, respectively).

Exercise tends to reduce early viral replication in lungs of influenza infected mice. Influenza A/PR/8/34 infection in lung was measured using real-time PCR with a primer/probe set that amplified the virus-encoded matrix (M1) gene. Using the expression of this late viral gene product as a surrogate marker for virus replication (11), the data demonstrated a tendency for exercise to reduce viral M1 gene expression in the lung (Figure 4). While there were no significant treatment effects between EX and SED groups, at Days 1, 3 and 5 M1 protein gene expression was 40-60 % of that of SED mice. Not surprisingly, there was a significant increase in viral mRNA on Day 3 and Day 5 p.i. when compared to Day 1.

Discussion

After intranasal inoculation with A/Puerto Rico/8/34 (H1N1) virus, mice develop a lower respiratory tract infection with lung consolidation resulting in death starting on Day 6 after infection (38). In a previous study (25), we found

that moderate exercise, when performed during the initial stages of a mounting primary immune response to influenza infection, improved survival in mice. In this follow-up study, we hypothesized that exercise would shift the immune response from a Th1 inflammatory phenotype to a Th2 anti-inflammatory response. We hypothesized that such a shift would result in a decrease in immune-mediated lung pathology and would thus explain why exercised mice had higher survival rates as seen in our earlier study. To address this hypothesis, we examined the lungs and secondary lymphoid organs (MLNs and spleen) of exercised and sedentary mice at Days 1, 3, or 5 post-infection with influenza A virus.

In support of the hypothesis, the data illustrated that moderate exercise, performed in the early stages of a mounting primary immune response to influenza virus infection, resulted in a significant reduction in the total number of cells infiltrating the lung. This suppressive effect of exercise was without regard to cell type. In addition, the data showed that exercise significantly reduced the expression of the potent Th1 inflammatory cytokine IFN-γ, while not altering gene expression of the Th2 cytokine IL-10. While it is well known that Th1 mediated immune responses are responsible for influenza virus clearance and recovery from infection (42), there is some evidence to suggest that an exaggerated Th1 inflammatory response to influenza virus can cause lung pathology and increase mortality (38). Indeed, injection of neutralizing anti-TNF-α antibodies at the time of influenza infection reduced lung lesion severity and prolonged survival by 24 hr (31). Moreover, treatment with IL-1 receptor antagonist produced a small but statistically significant increase in food intake and survival rates after influenza infection in mice (37). We also found that exercise had no effect on lung expression of the early anti-viral protein IFN-β. This, coupled with a failure to detect a reduction in viral replication (although there was a trend), suggests that moderate exercise did not affect early innate immune defences in this influenza model that could contribute to reduced mortality.

In agreement with our gene expression data, qualitative antibody array protein analysis of lung homogenate obtained on Day 3 post-infection revealed an exercise-induced shift from a Th1 towards a Th2-type immune response. Exercise resulted in a greater than two-fold reduction in protein levels of IFN-γ, lymphotactin, IL-17, IL-13, I-TAC, KC, leptin, SDF-1, and LIX and a greater than two-fold increase in eotaxin, eotaxin-2, IL-2, IL-4, BLC, FasL, IL-12p40/p70, and CD30L. While not entirely consistent with our hypothesis due to elevated IL-12 (although expression of this protein was low), it is noteworthy that exercise led to an increase in many cytokines that are characterized as Th2-like and a decrease in those that are indicative of a Th1 response. For example, in addition to the reduction in IFN-γ, exercise also reduced the LPS-induced CXC neutrophil chemoattractant LIX which has been shown to be involved in tissue pathogenesis in the dextran sodium salt-induced model of colitis (22). Moreover, exercise reduced interferon-inducible T cell α chemoattractant (ITAC) which plays a pivotal role in attracting effector T cells into the sites of Th1-type inflammation and is critically involved in the development of multiple Th1-type inflammatory diseases (9, 27) and allograft rejection (17).

Conversely, exercise increased expression of IL-4 (the prototypical Th2 cytokine) and the eotaxins; which act as chemoattractants for eosinophils and are induced by the Th2 cytokines IL-5 (34). While the role of eosinophils in

defence against viral infection is controversial, eosinophil granules contain abundant ribonucleases that degrade single-stranded RNA containing viruses (34). Interestingly, exercise also resulted in an increased expression of IL-1α and IL-1β in lungs of infected mice which appears at odds with an anti-inflammatory or Th1 reducing effect of exercise. However, while IL-1 is responsible for acute lung immunopathology it also leads to increased survival in response to A/PR/8/34 (35). Lastly, lungs from the exercised group displayed higher levels of soluble TNF receptors with no change in TNF-α indicating induction of anti-inflammation. The results of this qualitative antibody array analysis should be interpreted with caution (although the analysis verified that IFN-γ protein was lower, in agreement with our gene expression data) and future studies will need to statistically verify changes in protein expression using data from individual animals. We predicted an increase in lung histopathology, although in the few animals that we examined it did not appear that exercise had a marked effect on lung tissue damage, these studies were far from conclusive because histopathology was only examined on Days 1, 3, and 5 post-infection, likely too early to distinguish differences between groups.

There is precedent that exercise and other stressors can cause shifts away from Th1 toward Th2 immune responses (2, 36) and it has long been known that glucocorticoids and prostaglandins of the E series (PGE_2 in particular) can decrease Th1 and increase Th2-mediated immune responses (8, 15). While catecholamines may also play a role, Kohut et al. (2004) found that β blockade exacerbated exercise training-induced increases in HSV-induced *in vitro* IFN-γ production in young mice, but decreased this same response in old mice (21). Neuroendocrine hormones are clearly involved in acute and chronic stress-induced alterations in leukocyte trafficking and increases, decreases, or changes in the composition of the cellular influx could have beneficial or detrimental consequences dependent on the immunologic context (39). Unfortunately, a definitive role for specific neuroendocrine molecules as being responsible for exercise or stress-induced shifts in the Th1/Th2 balance awaits further study. It is interesting to note that while complete shifts away from Th1-mediated immune responses would be detrimental in virally infected animals a more subtle counter-regulation of Th1 responses may be beneficial.

Exercise dosage and the timing of exercise relative to infection appear critical in determining whether exercise reduces or increases morbidity and/or mortality to infectious diseases. The data suggest a hormetic effect where single bouts of prolonged exercise or intense exercise during symptoms exacerbates disease (6, 10, 12, 13, 16, 18, 29), whereas prior moderate exercise training or moderate exercise during early times after infection (but before overt symptoms) lessens disease severity (3, 7, 16, 25, 30) or is without effect (40, 41).

Only a few studies have examined exercise-induced alterations in the immune response that could potentially explain changes in morbidity or mortality. Kohut et al. (2001) examined the *ex vivo* cytokine responses to i.n. HSV-1 infection after a single bout of prolonged exercise (19), which they had found previously increases mortality to such a challenge (6). This ~2.5 hr run resulted in reduced ex vivo splenocyte production of both Th1 (IL-2, IFN-γ and IL-12) and Th2 (IL-10) cytokines on Day 2, but not Day 7 (except for IL-12) post-infection (19). Moreover, Kapasi et al (2004) found that either single or multi-

ple bouts of exhaustive exercise decreased LCMV-specific $CD8^+$ T cell numbers and their production of IFN-γ in response to LCMV infection in young, but not old mice (18). The clinical consequences of this, however, were not examined. These data are consistent with our data examining *in vivo* responses to influenza in the lung except that we did not document a decrease in IL-10. It is interesting to note the different dose-response effects of moderate exercise (increased survival) vs. prolonged exercise (decreased survival) on mortality despite similar changes in cytokine production.

Employing a different exercise paradigm, Davis et al. (2004) found that 1 hr of moderate exercise performed daily for 6 days prior to HSV-1 infection resulted in a decrease of 45 % in morbidity and 38 % in mortality when compared to sedentary mice (7). A mechanistic role for exercise-induced changes in alveolar macrophage function was recently confirmed when this group demonstrated that depletion of alveolar macrophages with clodronate encapsulated liposomes could abrogate the moderate exercise-induced increase in morbidity and mortality (28). Seemingly at odds with the exercise-induced reduction in IFN-γ seen in our study, Kohut et al. (2004) found that 8 weeks of moderate exercise training prior to HSV-1 infection increased IFN-γ and IL-2 production in splenocyte cultures stimulated with HSV-1 one week post-infection in aged, but not in young mice (20). This effect was likely mediated by exercise-induced increases in catecholamines acting through β-adrenergic receptors, at least in old mice (21). The different results in their study when compared to ours can likely be attributed to different aged mice, exercise protocols (training vs. acute) and virus challenge.

While the mechanistic underpinnings of the differential dose response to exercise are difficult to reconcile, it may be that moderate exercise and prior training reduce immune-mediated damage without severely affecting immune effector functions. Strenuous exercise, on the other hand, may be of sufficient intensity to reduce effector functions allowing infectious disease progression. In support of this, we have found that moderate exercise, when applied during the initial stages of a mounting immune response to influenza, causes a shift in the immune response away from a Th1 and toward a Th2 response without altering early anti-viral defences. This may be responsible for the increased survival rates in exercised mice because a strong Th1 response may lead to immune mediated pathology and death.

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