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Moderate Aerobic Exercise Alters Migration Patterns of Antigen Specific T helper Cells within an Asthmatic Lung

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Abstract

Studies show that an escalation in both incidence and severity of allergic asthmatic symptoms can largely be due to increased sedentary lifestyles. In addition, moderate aerobic exercise has been shown to reduce the severity of asthma; albeit by an unknown mechanism. Studies do implicate the re-distribution of T helper (Th) cells as a means of moderate aerobic exercise altering an immune response. We have previously reported that exercise decreases T helper 2 (Th2) responses within the lungs of an ovalbumin (OVA)-sensitized murine allergic asthma model. Therefore, we hypothesized that exercise alters the migration of OVA-specific Th cells in an OVA-challenged lung. To test this hypothesis, wild type mice received OVA-specific Th cells expressing a luciferase-reporter construct and were OVA-sensitized and exercised. OVA-specific Th cell migration was decreased in OVA-challenged lungs of exercised mice when compared to their sedentary controls. Surface expression levels of lung-homing chemokine receptors, CCR4 and CCR8, on Th cells and their cognate lung-homing chemokine gradients revealed no difference between exercised and sedentary OVA-sensitized mice. However, transwell migration experiments demonstrated that lung-derived Th cells from exercised OVA-sensitized mice exhibited decreased migratory function versus controls. These data suggest that Th cells from exercised mice are less responsive to lung-homing chemokines. Together, these studies show that moderate aerobic exercise training can reduce the accumulation of antigen-specific Th cell migration into an asthmatic lung by decreasing chemokine receptor responsiveness.

Keywords

Bioluminescence; exercise; asthma; T cell migration; Th2 response

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Introduction

Asthma is a widespread disease currently affecting approximately 8% to 10% of the United States population. The rapid increase over the past twenty years has been attributed to many factors, such as indoor and outdoor pollution, childhood immunizations, and factors associated with a western lifestyle (Cohn et al., 2004). One factor further exacerbating this increase is the progressive decline in physical activity and the subsequent increase in sedentary lifestyle as reported by Lucas and Platts-Mills (Lucas and Platts-Mills, 2005; Lucas and Platts-Mills, 2006). In accordance with these and other studies, the American College of Sports Medicine and the American Thoracic Society recommends low to moderate aerobic exercise for all types of asthma patients (Cooper, 1992; Nieman, 1997; Nieman, 2003; Satta, 2000). Together, these studies indicate that moderate exercise can reduce the severity of asthmatic symptoms (Chowdhury et al., 2011; McMahon et al., 2011); yet, the mechanism responsible for the clinically observed exercise-mediated decrease in asthma severity is unclear.

The CD4⁺ Th differentiated cell subset type 2 (Th2) produces characteristic Th2 cytokines, including interleukin (IL)-4, IL-5 and IL-13, and play an important role in orchestrating the allergic asthma inflammatory processes. The Th2 response promotes eosinophilia, allergen-specific immunoglobulin (Ig)E production, mucus secretion, and goblet cell hyperplasia that facilitates the trademark allergic asthma characteristics: chronic airway inflammation and airway hyper-responsiveness (AHR) (Foster et al., 2000). Elevated Th cells have been consistently found in bronchio-alveolar lavage fluid (BAL) and bronchial biopsies of chronic asthma patients (Bentley et al., 1992a; Bentley et al., 1992b; Robinson et al., 1992). Furthermore, Th cells accumulate in the lungs following each subsequent allergen challenge generating a profound localized memory Th cell population that propagates the chronic airway inflammation (Bodey et al., 1999; Del Prete et al., 1993; Holgate et al., 1997; Montes-Vizuet et al., 2006). The later stages of an asthmatic response are characterized by an increased release of several different pro-inflammatory molecules, including chemoattractants such as lung-homing chemokines that promote the entry and accumulation of immune cells, specifically Th2 cells, into the lungs. Continual trafficking of Th cells into the lung perpetuates the chronic airway inflammation generating a positive feedback system that maintains the asthmatic microenvironment (Cohn et al., 2004; Islam and Luster, 2012). Notably, studies have demonstrated that Th2 cell recruitment to asthmatic lung tissue can be mediated through binding of the chemokine homing receptors, CCR4 and CCR8, with their respective ligands, TARC/CCL17 and I-309/CCL1 (Islam and Luster 2012). Unfortunately, the complete mechanism by which Th2 cells accumulate within asthmatic lungs is poorly understood.

Previous studies by others have shown that moderate intensity exercise causes a systemic T lymphocyte cell re-distribution into circulation from lymphoid organs and tissues in the absence of an inflammatory insult (Kruger and Mooren, 2007; Kruger et al., 2008). Specifically, during exercise there is an initial lymphocytosis that is followed by a lymphocytopenia in the recovery phase after exercise (Niederman et al., 1991; Nieman, 1997). The mechanism responsible for the decrease in circulating T lymphocytes remains unclear. Some investigators have suggested apoptosis as a means for the decrease in the post-exercise lymphocytopenia. However, most published reports have found very little evidence for programmed cell death as the predominant mechanism in this moderate aerobic exercise effect (Mooren et al., 2002; Mooren et al., 2004). Therefore, other processes, such as T lymphocyte trafficking and re-distribution, are more likely candidates to explain the changes in T lymphocyte numbers during and after exercise.

In this study, for the first time, we have shown in real-time the dynamic re-distribution of exercised Th cell trafficking in the presence of an inflammatory insult. Specifically, we visually and quantitatively show that moderate aerobic exercise significantly alters the migration patterns of luciferase-expressing OVA-specific Th cells within an OVA-challenged murine allergic asthma model. We also found that the exercise-mediated decrease in OVA-specific Th cells within the lungs accumulates with each repeated OVA aerosolization challenge suggesting a decrease in localized memory Th cell responses. Furthermore, the exercise-mediated attenuation of Th cell lung-homing does not appear to be driven by a change in chemokine gradients (TARC/CCL17 and I-309/CCL1) or chemokine receptor expression levels (CCR4 and CCR8) expressed on Th cells. Instead, moderate exercise had a significant impact on the functional capacity of these chemokine receptors on Th cells.

Materials and Methods

Mice

Six to eight week-old female wild type mice on a BALB/c background (Taconic) were used as recipients for all asthma-exercise experiments. Various donor cell populations were utilized as described in individual figures. First, for imaging experiments, DO11.10 T_{LUX} BALB/c mice were used as donors for Th cells (Azadniv et al., 2007; Chewning et al., 2009; Zinn et al., 2008). Briefly, DO11.10 T_{LUX} BALB/c mice express a luciferase reporter controlled by the T lymphocyte cell-specific-CD2 promoter. Luciferase is stably expressed beginning at double positive T cell development and remains expressed throughout the life cycle of the T cell regardless of activation status. When the substrate, D-luciferin, is provided, firefly luciferase yields a light wavelength that is greater than the wavelength absorbed by living tissues, thereby permitting the detection and quantification of the light emitted. The light emission is directly proportional to the number of CD4⁺ Th cells expressing luciferase *in vivo* (Chewning et al., 2009). DO11.10 T_{LUX} BALB/c mice generate CD4⁺ Th cells that express OVA-specific T cell receptors (TcR) that exclusively respond to ovalbumin (OVA). Thus, the DO11.10 T_{LUX} BALB/c mouse provides an OVA-specific Th cell population that can be tracked *in vivo* in real time by bioluminescent imaging without further invasive treatment to mice. Second, for all non-imaging experiments DO11.10 BALB/cJ mice (Jackson Labs) were used as donor Th cells. All mice were maintained in autoclaved Micro-isolator cages (Lab Products) and provided with food (Teklad) and water as needed at the University of South Alabama, College of Medicine, Department of Comparative Medicine, and Animal Care Facility.

Adoptive Transfer

DO11.10 T_{LUX} BALB/c (bred at University of Alabama at Birmingham) or DO 11.10 BALB/cJ (Jackson Labs) mice were sacrificed to harvest spleen and lymph nodes for OVA-specific T cell receptor (TcR) CD4⁺ Th cells. Organs were homogenized and red blood cells lysed to yield a single cell suspension. The concentrations of cells were adjusted to 4x10⁶ CD4⁺ Th cells in 300 μ L of no serum Dulbecco's Modified Eagle's Medium (DMEM). Each wild type BALB/c recipient mouse received cells via intravenous (i.v.) injection into the tail vein while in a mouse restrainer on Day -7 of the OVA-sensitization and challenge protocol (see Fig. 1).

OVA-sensitization and Challenge

Mice were randomly assigned to four experimental groups: (a) *Sedentary* OVA-sensitized (SO); (b) *Exercised* OVA-sensitized (EO); (c) *Sedentary* non-sensitized (S); and (d) *Exercised* non-sensitized (E). OVA-sensitized and challenged mice were challenged with ovalbumin (OVA) as previously described (Pastva et al., 2004). Briefly, OVA-sensitized

(SO, EO) mouse groups were injected intra-peritoneal (i.p.) with 200 μL of 0.25 $\mu\text{g } \mu\text{L}^{-1}$ (50 μg total) alum-precipitated, chicken egg ovalbumin (Imject Alum; Grade VII OVA 98% pure, Sigma Chemical) while non-sensitized controls received an i.p. injection of PBS on Days +0 and +14. Beginning on Day +21, OVA-sensitized mice (EO and SO) were exposed to aerosolized OVA at a concentration of 5 mg mL^{-1} in 0.9% w v^{-1} NaCl solution (saline) while non-sensitized mouse groups (S and E) received saline alone for 30 minutes per day for five consecutive days (Days +21-25). Aerosolized OVA [or saline] at the same concentration was administered for 10 minutes per day at 3 days per week for the remainder of the study (Days +28-49) (see Fig. 1). This method of OVA-sensitization and challenge has been demonstrated previously to induce an allergic response as indicated by increases in serum OVA-specific IgE levels (Pastva et al., 2004).

Exercise training

Exercise training was performed 3 days/week for a total time period of 4 weeks (see Fig. 1). The first week of the exercise regimen consisted of a training and acclimation period whereby mice were subject to running beginning at 10.0 m min^{-1} for 30 minutes and gradually increased to 13.5 m min^{-1} for 45 minutes. In the remainder of the protocol, mice exercised for 45 min at 13.5 m min^{-1} (0% grade). All exercise sessions included brief warm-up and cool-down periods so that the total treadmill time was approximately 60 min. To avoid novel effects, the control sedentary mice were placed on the treadmill for 10 min without exercise. No stimulus (i.e. electric shock or other mechanical stimulus) was used in this study. Sedentary mice were housed in the same room and were subjected to all the same conditions, without the exercise-training component. All exercise sessions commenced one hour post-OVA aerosolization challenge on Days +28-49 at 3 sessions per week frequency, as described above in the OVA challenge protocol (Pastva et al., 2004). Exercise training during those times was executed 3 days/week for a total of 10 sessions (see Fig. 1). Mice were sacrificed for analysis at either 10 or 18 hours after the final exercise session by i.p. injection of ketamine:xylazine (ketamine 160 mg kg^{-1} :xylazine 20 mg kg^{-1}).

Imaging of Live Mice (BLI)

Mice were anesthetized with isoflurane gas anesthesia and placed in a light-tight chamber. Ten minutes prior to image collection, mice received i.p. injection of the luciferase substrate, D-luciferin. The photographic (gray-scale) reference image was obtained at 10 minutes after D-luciferin i.p. injection; the bioluminescent image was collected immediately thereafter. Images obtained with a CCD cooled to -120°C , using the IVIS Imaging System (Xenogen Corp., Alameda, CA) with the field of view set at 10 cm height. The photographic images used a 0.2 second exposure, 8f/stop, 2 binning (resolution), and an open filter. The bioluminescent images used exposures of 600 seconds, 1f/stop, 8 binning and open filter. The bioluminescent and gray-scale images were overlaid using Living Image software (Xenogen Corp.) and Igor image analyses software (Wavemetrics, Lake Oswego, OR) was used to obtain a pseudo color image representing bioluminescence intensity (blue, least intense, and red, most intense). Regions of interest were drawn around organs and the total counts (photons) were summed into organ areas. The total counts in the region of interest were normalized to the total image time to obtain counts sec^{-1} (Zinn et al., 2008). The “whole body” region of interest (ROI) was quantified and represented by an oval gate designated GATE 1 as indicated in Fig.3. The “lung” region of interest was quantified and represented by a rectangle gate designated GATE 2 as indicated in Fig. 3. Quantification of ROI gates was assessed at each time point indicated.

Bronchio-alveolar Lavages

Mice were prepared for bronchio-alveolar lavage (BAL) as previously described (Pastva et al., 2004). Cell viability was determined via trypan blue exclusion and cell types were

differentiated on cytopsin preps using Wright-Giemsa stain. Cell differentials were determined from at least 500 leukocytes using standard hematological criteria.

Analysis of chemokine gradients

BAL fluid samples were analyzed for concentrations of mouse TARC/CCL17 and I-309/CCL1 using commercial ELISA kits according to the manufacturer's instructions (R&D Systems).

Isolation of CD4⁺ Th cells

Spleens, lymph nodes or lungs of DO11.10 or experimental recipient BALB/c mice were removed and single-cell suspensions prepared. The red blood cells were removed using lysis buffer, ACK buffer (KD Medical). Lung CD4⁺ Th cells were isolated from the single cell suspension using a cell sort staining protocol with CD4⁺-PE (ebioscience GK5.1) and cell sorted with the FACS Aria II (Bryant Hanks, University of South Alabama Flow Cytometry Core Facility). Spleen and lymph node CD4⁺ Th cells were magnetically separated using the negative CD4⁺ T cell selection II according to manufacturer's instructions (Miltenyi Biotec) (Cat #130-095-248).

Antibodies and Reagents

The following antibodies were purchased from eBioscience and Abcam and used for Flow Cytometry on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA): + CCR4 (Biolegend KH-4F5), CCR8 (Abcam ab1663), CD3 (ebioscience 145-2C11), and CD4 (ebioscience GK5.1).

Flow cytometry

Cells were stained for flow cytometry with FITC-, PE-, PerCP-Cy5, APC, or APC Cy7 mAbs (ebioscience and Biolegend) as indicated in the appropriate figure legends. For analytical flow cytometry, at least 50,000 gated live lymphocyte events with forward and side scatter properties of lymphocytes were collected on a BD Biosciences FACS Canto II and analyzed using FlowJo software (BD Biosciences, San Jose, CA). Compensation for each fluorochrome-tagged antibody was set with the auto-compensation (FITC, PE, APC, APC Cy7) feature on FACS Canto II by gating on single-labeled samples. FACS analyses were performed on single cell suspensions from mediastinal lymph node (mLN), spleen, other draining lymph nodes (cervical, axillary, brachial) and lung either 10 or 18 hours post-final OVA challenge / exercise training session. Analysis of chemokine receptor expression for figures was performed by initially gating on live lymphocytes, then gating on CD3⁺ expressing populations, and finally gating on CD4⁺ expressing cells for final chemokine receptor expression assessment (see Supp. Fig. 5). For final analysis, cell numbers were calculated by applying the FlowJO software percentages analysis to total cell counts collected at the time of organ homogenization.

Transmigration Assay

CD4⁺ Th cells were isolated from the lung-specific draining lymph node, mediastinal lymph nodes (mLN), and lungs by cell sorting as previously described (FACS Aria II). A 5 μ m Cyto-select 96-well Cell Migration Assay (Cell Biolabs) was used to assess extent of transmigration in response to chemokine. Briefly, 40,000 Th cells (4×10^5 Th cells mL⁻¹) were added to the top well of a transwell 96-well plate. The upper well was separated by a polycarbonate membrane (5 μ m pore size) from the lower well. The ligands for Th2 lung-homing chemokine receptors, CCR4 and CCR8, (CCL17 and CCL1, respectively) were individually assessed and added to the bottom well at 100ng/mL as a chemo-attractant. The assay was incubated for 5-6 hours (37°C). Cells in the bottom well and underside of the

membrane were lysed and stained with Lysis Buffer/CyQuant GR fluorescent dye solution (Cell Biolabs) that binds DNA and read by a fluorescence plate reader at 480/520nm. Cells were quantitated by a standard curve (cell number x fluorescence) plated simultaneously at the time of cell migration analysis. All samples were plated in triplicate.

Data Analysis and Statistics

Statistical significance between sedentary and exercised mice for bioluminescent imaging was calculated using repeated measures ANOVA. The effect of exercise on BAL cell counts, chemokine gradients, Th cell expression of chemokine receptors and Th cell trafficking function was analyzed comparing; exercised non-sensitized and sedentary non-sensitized or exercised-OVA sensitized and sedentary-OVA sensitized populations. These pair analyses used a paired student's t-test for all single variable comparisons. In order to distinguish significant differences between the four experimental groups: (1) exercised OVA-sensitized (EO); (2) sedentary OVA-sensitized (SO); (3) exercised non-sensitized (E); and (4) sedentary non-sensitized (S); data was analyzed using two-way ANOVA analysis between the two variables of exercise treatment and OVA treatment as indicated (GraphPad Prism 5). Results were then reported as group means $\pm$ standard error of the mean (SEM) with significance set at a level of $p = 0.05$ and interaction of variables.

Results

Moderate aerobic exercise decreases the number of lymphocytes within the lungs of OVA-sensitized mice as compared with sedentary controls

BALB/c mice received OVA-specific Th cells prior to undergoing OVA-sensitization and challenge / exercise training (Fig. 1) Eighteen hours post final exercise session, bronchio-alveolar lavage (BAL) was collected and assessed for changes in cellular content from each mouse group *Sedentary* OVA-sensitized (SO); *Exercised* OVA-sensitized (EO); *Sedentary* non-sensitized (S); and *Exercised* non-sensitized (E). Results in Figure 2 show that total cell numbers do not differ significantly between exercised OVA-sensitized (EO) mice and their OVA-sensitized sedentary (SO) counterparts. Additionally, no significant difference was observed in individual cell categories (eosinophils, macrophages, basophils, and neutrophils) when comparing exercised and sedentary OVA-sensitized mouse groups (Supp. Fig. 1) ($N = 7$ / group). However, an exercise-mediated significant decrease in cell number was observed in the lymphocyte population of OVA-sensitized mice as compared with sedentary mouse groups (Fig. 1) ($t(10) = 2.116$; $p = 0.0302$).

Moderate aerobic exercise decreases OVA-specific T lymphocytes as indicated by a bioluminescent reporter

The significant reduction in lymphocytes observed in BAL fluids of OVA-sensitized exercised mice (Fig. 2) led to the examination of the exercise –mediated re-distribution of allergen-specific T lymphocytes during an allergic airway inflammatory response. Mice received OVA-specific luciferase expressing Th cells prior to undergoing the OVA-sensitization and challenge / exercise training protocols (Fig.1) (Chewning et al., 2009; Zinn et al., 2008) Bioluminescence collected within regions of interest (ROI) over time indicated a significant reduction in OVA-specific Th cells within both the “whole body” ROI (GATE 1) and the “lung” ROI (GATE 2) of exercised OVA-sensitized mice as compared to sedentary OVA-sensitized controls (Fig. 3A and 3B). Kinetic analyses of bioluminescent measurements within the “whole body” ROI (Fig. 3C) and the lung ROI (Fig. 3D) indicated a significant decrease in OVA-specific Th cells in both gated compartments when OVA-sensitized mice underwent moderate aerobic exercise (two-way ANOVA repeated measures: body bioluminescence; exercise treatment $F(1,16)=8.10$, $p=0.012$, time $F(11,176)=3.83$, $p<0.0001$, interaction $F(11,176)=2.97$, $p=0.0012$; lung bioluminescence; exercise treatment

$F(1,16)=6.19$, $p=0.024$, time $F(11,176)=6.50$, $p<0.0001$, interaction $F(11,176)=2.55$, $p=0.0051$). Specifically, these data show that changes in OVA-specific Th cell trafficking commence on Day 28 at the initial application of exercise ($N=7/\text{group}$). Bioluminescent images and quantification for time points prior to Day +21 are included in Supplementary Fig. 2A-C.

Interestingly, exercised OVA-sensitized mice (Fig. 3E) demonstrated equivalent bioluminescence signals as compared with sedentary OVA-sensitized mice between hours 6 and 10 post-OVA challenge (Fig. 3A), but quickly decreased by 12 and 18 hours until additional administration of OVA-challenges (Fig. 3A and 3E). Therefore, for further daily kinetic analysis of trafficking patterns, mice were evaluated at 18 hours post-OVA challenge in order to assess the lymphocytopenic effects of exercise on Th cell re-distribution. As expected, studies investigating the bioluminescent signal of OVA-specific Th cells in non-sensitized mouse groups showed neither OVA-specific Th cell expansion nor any lung specific trafficking in response to saline aerosolization challenges (Supp. Fig. 2D).

Exercise alters Th2 cytokine production profiles

Because asthma is propagated by Th2 responses in the airways, lung Th2 cytokine levels were evaluated in the OVA-specific Th cell adoptive transfer of OVA-sensitized mice. These studies investigate the effects of exercise on the OVA-specific Th2 memory responses. At the end of the OVA-sensitization and challenge / exercise training protocols, mice from each treatment group were evaluated for Th2 cytokine, IL-4, IL-5 and IL-13, levels in collected BALs. In correlation with previous mouse models (Lowder et al.; Pastva et al., 2004), IL-4 was significantly decreased in OVA-sensitized exercised mice when compared to OVA-sensitized sedentary mice (Fig. 4) (Interaction, $F(1,15)=8.03$, $p=0.0126$; OVA treat, $F(1,15)=13.74$, $p=0.0021$; Exercise treat, $F(1,15)=4.58$, $p=0.0492$). In contrast, there was no significant change in IL-5 levels between treatment groups. There was, however, a significant increase in IL-13 levels when mice underwent a long term moderate aerobic exercise regimen, but only in the presence of an OVA-sensitized inflammatory micro-environment (Interaction, $F(1,20)=4.64$, $p=0.0436$; OVA treat, $F(1,20)=4.91$, $p=0.0384$; Exercise treat, $F(1,20)=2.25$, $p=0.1490$). As expected in the absence of an inflammatory insult, the non-sensitized controls exhibited significantly reduced Th2 cytokine levels as compared with their OVA-sensitized counterparts (Fig. 4).

Exercise does not differentially impact the protein expression of the lung specific Th cell homing chemokine, CCL1 or CCL17; nor their respective chemokine receptors, CCR4 or CCR8, in OVA-sensitized mice

Because the chemokines, CCL17 and CCL1, play a significant role in the migration of Th2 cells into an inflamed lung (Islam and Luster 2012), the OVA-specific Th cell adoptive transfer mouse model was evaluated for changes in CCL17 and CCL1 protein levels. As shown in Fig. 5, moderate aerobic exercise did not significantly alter CCL17 or CCL1 levels in OVA-sensitized mice BALs at 18 hours post-final OVA challenge / exercise session when compared to sedentary controls. At 10 hours post- final OVA challenge / exercise session, however, both CCL17 and CCL1 are reproducibly elevated in the exercised OVA-sensitized mice in comparison to their sedentary OVA-treated controls (Supp. Fig. 3).

The impact of exercise on the surface expression of CCR4 and CCR8, which bind to CCL17 and CCL1, respectively, on OVA-sensitized $CD4^+$ Th cells were evaluated by flow cytometry. $CD4^+$ Th cells were isolated from mediastinal draining lymph node (mLN), lung, spleen, and cervical/brachial/axial draining lymph nodes at 18 hours following the final OVA-challenge / exercise session and assessed. It should be noted that flow cytometric evaluation $CD4^+$ Th cells includes both the recipient mouse's endogenous $CD4^+$ Th cell

population as well as the donor OVA-specific Th cell population. Results in Figure 6 show no significant change in CCR4⁺ or CCR8⁺ CD4⁺ Th cell populations isolated from mLNs. Non-sensitized controls were not included for this analysis as mLNs are not detectable in the absence of OVA-sensitization. Together, these data show that exercise treatment has no significant effect on the expression of either CCR4 or CCR8 on CD4⁺ Th cells isolated from OVA-sensitized mice when compared with sedentary controls. Similarly, no difference in chemokine receptor expression was observed on CD4⁺ Th cells isolated from either lungs, other draining lymph nodes, or splenic tissues of exercised and sedentary OVA-sensitized mice (data not shown); nor was any difference in chemokine receptor expression found at 10 hours post-final OVA challenge / exercise training session (data not shown).

Exercise significantly decreases Th cell migration in vitro

In light of the differential effect of exercise on both lung-homing chemokine gradients and chemokine receptor expression in OVA-sensitized mice as described above, the function of CCR4 and CCR8 on lung-isolated CD4⁺ Th cells was evaluated. The isolated CD4⁺ Th cell population again included both the recipient's endogenous Th cell populations and the donor OVA-specific Th cell population. Flow cytometric analysis indicated the donor OVA-specific Th cell population (allotypic expression of Thy1.2⁺) within the lung isolated CD4⁺ Th cell population used for the transmigration studies was 2-3% for exercised OVA-sensitized mice and 5-6% for sedentary OVA-sensitized mice (data not shown). At the end of the OVA-sensitization and challenge / exercise training protocols, chemotactic function of lung-isolated CD4⁺ Th cells were evaluated *via* a transwell migration assay either in response to CCL17 (ligand for CCR4) or CCL1 (ligand for CCR8). The results demonstrated that moderate aerobic exercise significantly decreased the migratory capacity of Th cells in response to either chemokine (Fig. 7) (CCL1, $t(4)=3.619$, $p=0.0224$; CCL17, $t(4)=2.428$, $p=0.0361$). As expected, non-sensitized controls showed little to no migration in response to CCL1 or CCL17.

Discussion

Allergic asthma is a complex and inter-woven innate and adaptive immune response most commonly documented to be orchestrated by an allergen specific Th2 cell response. Other investigations have shown an overall reduction in Th2 presence within the lungs of moderately exercised OVA-sensitized mice and moderately exercised chronic allergic asthmatic patients translates to an increase in lung function (Cooper, 1992; Dugger et al.; Hewitt et al., 2009; Nieman, 1997; Nieman, 2003; Pastva et al., 2004; Satta, 2000). The mechanisms behind the exercise mediated decrease in Th2 cell presence in the lungs have been proposed to include both apoptosis and Th cell re-distribution (Brines et al., 1996; Cooper, 1992; Kruger and Mooren, 2007; Lucas and Platts-Mills, 2005; Mooren et al., 2004; Nieman, 1997; Nieman, 2003; Satta, 2000; Woods, 2005). Moderate aerobic exercise in the absence of an inflammatory insult is observed to induce an acute lymphocytosis followed by a more long-term lymphocytopenia when compared to pre-exercise lymphocyte levels where apoptosis was not the dominant factor in these lymphocyte changes (Mooren et al., 2004). Together, these studies and the data presented in this manuscript show moderate aerobic exercise induces a dynamic re-distribution of allergen-specific Th cells even in the presence of an allergen challenge (Fig. 3A-E). Additionally, these studies investigate one of the potential mechanism in which Th cell trafficking patterns are affected by exercise.

The significant reduction in lymphocytes documented from collected BALs of exercised OVA-sensitized mice using an OVA-specific Th cell adoptively transferred mouse model (Fig. 2) correlates with results previously published using wildtype OVA-sensitized / exercise trained mice (Lowder et al.; Pastva et al., 2004). These published studies indicate a reduction in total number and eosinophil cell populations of BALs in addition to decreased

lymphocytes. This report's findings are novel because the OVA-specific Th cell adoptive transfer mouse model investigated the isolated effect of exercise on long term allergen-specific memory Th responses rather than the overall alteration of a complex asthmatic immune response previously described. Additionally, we show, in part, that the lymphocyte reductions in the lung were due to hindered lung-homing capabilities. Specifically, lung homing chemokine receptors, CCR4 and CCR8, exhibit decreased sensitivity to their respective lung-homing chemokine gradients (Fig. 7).

The T-LUX bioluminescent technology allowed us, for the first time, to visualize allergen-specific Th cell trafficking patterns in real-time in a single mouse throughout the OVA-sensitization allergic asthma mouse protocol with and without a moderate intensity exercise regimen. The images showed that exercise allowed for significant re-distribution of OVA-specific Th cells from the lung tissue resulting in a reduced number of OVA-specific Th cells over time (Fig. 3A-D). These data further support Mooren *et al* and others that show moderate aerobic exercise can re-distribute Th cells systemically (Kruger and Mooren, 2007; Kruger et al., 2008). However, these data indicate that the proposed exercise effect on Th cell re-distribution does not exclude an allergen-specific Th cell population in the presence of an allergen insult. In addition, the hourly kinetic assessment of the bioluminescent images validated the exercise-induced acute lymphocytosis between 6-10 hours post-exercise / OVA-challenge followed by a long-term lymphocytopenia in the hours following (Fig. 3E). Further investigations are needed to illustrate how long the exercise-mediated effects on allergen-specific Th cell migration patterns are maintained.

The mechanisms behind the observed Th cell re-distribution from lung tissues in these studies could involve a large number of candidates based on the literature, including the chemokine receptors CCR3, CCR4, CCR8, and the PGD2 receptor, CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes). The levels of many of these chemokine receptor ligands are found to be raised in asthmatic airways, be it in chronic disease or following acute exposure to an allergen (Bochner and Hamid, 2003; Pilette et al., 2004; Vijayanand et al., 2010). However, these studies focused on CCR4 and CCR8 and their respective chemokine gradient (TARC/CCL17 and I-309/CCL1); since recent published reports find these chemokine receptors to play a dominant role in generating a Th2 lung-homing allergic asthma microenvironment (Andrew et al., 2001; Bonecchi, 2008; Bonecchi et al., 2009; Borroni et al., 2008; Borroni et al., 2009; Bumbacea et al., 2004; Campbell and Butcher, 2000; Galliera et al., 2008; Lappin and Campbell, 2000; Rivino et al., 2004; Rivino et al., 2010; Romagnani, 2001; Sallusto et al., 1998). In addition, CCR4 and CCR8 antagonists are at the forefront of potential asthma therapeutics in proposed clinical trials (Connolly et al., 2012; Karlsson et al., 2011; Mutalithas et al., 2010; Perros et al., 2009; Vijayanand et al., 2010). Th2 cell migration into the lungs can occur when one or both of these chemokine receptors are engaged by their respective chemokine ligands, CCL17 and CCL1.

Interestingly, data presented in this report indicates no significant change to either chemokine receptor levels or their cognate chemokine ligand gradients within the lungs at 18 hours post final OVA challenge / exercise session. The moderate CCL17 reduction seen in Figure 5 show BALs at 18 hours of exercised OVA-sensitized mice compared with sedentary controls. This CCL17 decrease observed in exercised OVA-sensitized mice could be an indirect result of the decrease in localized lung Th2 cytokines that control CCL17 gradients (Fig 4 and 5) (Islam and Luster, 2012). Whereas, given our adoptive transfer model investigates memory OVA-specific Th responses, the indicated unaltered CCL1 gradient at 18 hours of exercised OVA-sensitized mice compared to the sedentary controls is most likely due to the decrease in IgE induced mast cell degranulation (Supp. Fig. 1 and Fig. 5). It is this increase in IgE-mediated mast cell degranulation that stimulates CCL1

production (Islam and Luster, 2012). However, as previously published studies have shown moderate aerobic exercise does significantly decrease OVA-specific IgE production in wild type exercised OVA-sensitized mice, we expected to see a moderate decrease in CCL1 levels (Lowder et al.; Pastva et al., 2004). Notably, in those studies, CCL1 did show a moderate decrease as predicted in exercised OVA-sensitized mice (data not shown).

Next, chemokine receptor function (CCR4 and CCR8) was tested *via* a transmigration assay and was found to be insufficient for exercised OVA-sensitized Th cells to respond to either chemokine, CCL1 or CCL17 (Fig. 7). The transmigration assay findings provide a potential mechanism that correlates with both the bioluminescent and BAL findings that demonstrate a decrease in OVA-specific Th lymphocytes in the OVA-challenged lung (Fig. 2 and 3A-D). These data suggest exercised OVA-sensitized Th cells are unable to respond to their cognate chemokine to the same capacity as their sedentary counterparts, in part, due to the desensitization of both CCR4 and CCR8 (Fig. 3A-D and 7). Furthermore, the decrease in migratory function of exercised Th cells offers some explanation as to why equivalent chemokine gradients between treatment groups (Fig. 5) do not translate to the maintenance of equivalent OVA-specific Th cells within the lung (Fig. 3A).

Additionally, supplementary data presented in these studies also offer an additional mechanism in which the altered Th cell migration patterns may occur. When chemokine gradients were analyzed at 10 hours post-exercise / OVA challenge, the exercised mice showed a reproducible increase in both chemokine gradients, CCL17 and CCL1, when compared to their sedentary counterparts but was quickly lost by 18 hour analysis (Supp. Fig. 3). The 10-hour chemokine increase could provide one way in which the observed lymphocytosis in bioluminescent images collected between 6-10 hours post-OVA challenge of exercised mice may transpire (Fig. 3E). Specifically, the spike in chemokine levels in exercise mice may be necessary to overcome the reduced chemokine receptor sensitivity of exercised Th cells that will thereby allow for entry into the lung when presented with an allergen insult (Fig. 7). However, once the allergen challenge is no longer present and the chemokine gradient decreases, the exercise-induced decrease in chemokine receptor sensitivity no longer allows for sufficient activity that would maintain exercised Th cells within the lung. Therefore, exercised OVA-specific Th cells are allowed to exit the lung while sedentary OVA-specific Th cells remain trapped within the lung (Supp. Fig. 3 and Fig. 3E). Nonetheless, further studies are needed to identify all possible mechanisms responsible for the acute lymphocytosis followed by lymphocytopenia observed in exercised OVA-sensitized mice.

In addition, further studies are needed to investigate the mechanism in which chemokine receptor desensitization is occurring within exercised OVA-sensitized mice. One potential line of investigation is the cross activation of G-protein receptor kinases (GRKs) that in turn desensitize chronically activated G protein coupled receptors (GPCRs) in a promiscuous manner. One group of studies indicated in human Th cells that chronically activate CCR4 with CCL17 *in vitro*; can induce GRK2 and GRK3 activation. This GRK activation, in turn, desensitizes not only CCR4 but also β_2 -adrenergic receptors, both of which are GPCRs (Heijink et al., 2003; Heijink et al., 2004; Heijink et al., 2005; Iellem et al., 2001; Panina-Bordignon et al., 2001). Of interest to this manuscript, exercise is capable of communicating with Th cells by their expression of β_2 -adrenergic receptors. Therefore, a potential mechanism for CCR4 and/or CCR8 desensitization could involve chronic activation of β_2 -adrenergic receptors that results in cross-desensitization of additional GPCR chemokine receptors expressed, thereby altering Th cell migration capabilities. As Th cells differentiate into Th2 cells, the β_2 -adrenergic receptor expression has been shown to be decreased (Kin and Sanders, 2006). However, at various stages of activation prior to differentiation, Th cells

do express α_2 -adrenergic receptors at varying levels and could affect long-term chronic Th2 responses. Thus, this line of investigation requires further experimentation.

Another candidate that could explain the dynamic exit of Th cells post-exercise is CCR7, a chemokine receptor that facilitates Th cell exit from tissues towards lymphoid structures. CCR7 has been shown to be elevated in exercised human blood lymphocyte samples and has been hypothesized to contribute to the re-distribution of lymphocytes during exercise (Kruger and Mooren, 2007; Kruger et al., 2008). In supplemental data, CCR7 was elevated on exercised OVA-specific Th cells in our adoptive transfer OVA-sensitized model when analyzed at 18 hours but statistical significance was not acquired (N=10-12/group). However, exercised OVA-specific Th cells did show a significant decrease of CCR7 at 10 hours post-exercise / OVA challenge (10 hours, $t(6)=2.238$, $p=0.0333$; 24 hours, $t(19)=1.366$, $p=0.0939$; Supp. Fig. 4). The effect of exercise showed some degree of variability that could not be controlled. However, the published trend did remain the same in our OVA-specific Th cell adoptive transfer murine asthma model and could, in part, explain the image results indicating entry into the lung at 10 hours due to decreased CCR7 expression followed by exit from the lung at 18 hours due to increased CCR7 expression (Supp. Fig. 4 and Fig. 3E).

Finally, it is notable to discuss the exercise induced significant increase in interleukin [IL]-13 levels when mice are presented with an inflammatory insult, OVA-sensitization/challenge (Fig. 4). Allergic asthma is characterized by airway hyper-responsiveness (AHR), eosinophilic inflammation, mucus hypersecretion, subepithelial fibrosis, and elevated IgE levels. The Th2 cytokine profile, IL-4, IL-5, and IL-13, plays a pivotal role in orchestrating these indicators of allergic asthma pathogenesis. IL-4 is associated with increased IgE responses (Coyle et al., 1995) and was decreased in our exercised adoptive transfer mouse asthma model when compared to sedentary controls. IL-5 promotes local eosinophilia and mucus secretion (Foster et al., 2002; Hogan et al., 1998a) and was unaltered in our adoptive transfer mouse asthma model when compared to sedentary controls. These data further support previous reports by Schwiebert *et al* and others that show moderate exercise can reduce chronic asthma pathogenesis by decreasing Th2 cytokine levels (Lowder et al.; Pastva et al., 2004).

However, Interleukin-13 has a more ambiguous role in orchestrating the allergic asthma Th2 response, but is capable of inducing features of the allergic response; including airway hyperresponsiveness (AHR) and mucus production. (Grunstein et al., 2002; Hogan et al., 1998b; Laoukili et al., 2001; Mattes et al., 2002; Wills-Karp et al., 1998). Although we are unclear as to why this significant increase in IL-13 is observed in exercised OVA-sensitized mice, it is important to note that moderate exercise has been found to decrease AHR, mucus production and airway inflammation (Lowder et al.; Pastva et al., 2004). Therefore, it is reasonable to suggest that some additional unknown factor may be required to facilitate IL-13 in eliciting its pro-inflammatory properties. Without this additional factor in our exercised OVA-sensitized mice, this treatment group maintains the benefits of decreasing airway inflammation and AHR as previously published (Pastva et al., 2004). One such proposed mediator of IL-13 pro-inflammatory responses is IL-17 (Kinyanjui et al., 2013). These recent findings suggest IL-13 induced allergic responses of the airways are shaped by the overall amount of IL-17 present as well as by the cell types producing this cytokine. IL-17 was found to facilitate, enhance and block IL-13 mediated allergic airway responses depending on IL-17 quantity and/or source. Thus, further studies are needed to better illustrate what additional components could be missing in our exercised OVA-sensitized mice that prevent this increase in IL-13 from promoting further allergic asthma pathogenesis.

Taken together, these results suggest a regulatory role for moderate aerobic exercise in the dynamic Th cell trafficking patterns for allergen-specific Th cell responses in response to an allergen challenge. Specifically, these studies provide insight into how exercise supports the alterations in Th cell levels within tissues during an inflammatory insult through the desensitization of lung-homing Th2-specific chemokine receptors and potentially, indirect alterations of their respective chemokine gradients at earlier time-points (Fig. 8). These chemokine receptors are currently experimental therapeutic targets for chronic allergic asthmatics in clinical trials and have shown advantageous results in blocking their function on asthma pathogenesis. Moderate aerobic exercise may provide a feasible, cost-effective alternative to reducing allergen specific Th cell responses in the lung that will translate to increased lung function. However, the exercise induced increase in IL-13 levels within the inflammatory environment poses some concern for exercise- induced asthmatic events and should be thoroughly investigated in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Exercise decreases function of lung-homing chemokine receptors on Th cells.

Figure 1. OVA-sensitization and challenge / exercise training protocols timeline

Wildtype BALB/c mice received an ADOPTIVE TRANSFER (ADTX) (i.v.) of 4×10^6 DO11.10 BALB/c or DO11.10 T-LUX BALB/c CD4⁺ Th cells on Day -7. Recipient mice were randomly assigned to four experimental groups: (a) *Sedentary* OVA-sensitized (SO); (b) *Exercised* OVA-sensitized (EO); (c) *Sedentary* non-sensitized (S); (d) *Exercised* non-sensitized (E). On Days +0 and +14, OVA-sensitized mice were administered an i.p. injection of OVA-adjuvant (or PBS for non-sensitized) for antigen SENSITIZATION. On Days +21-25, OVA-sensitized mice were CHALLENGED with OVA at 5mg/mL (or saline for non-sensitized) via aerosolization for 30 minutes daily for 5 consecutive days in order to localize the OVA-specific Th cell response in the lungs. On Days +28-49, OVA-sensitized mice were further CHALLENGED with aerosolized OVA at 5mg/mL (or saline for non-sensitized) for 10 minutes at 3 doses/week. In addition, on Days +28-49, one hour after the 10-minute aerosol challenge, exercised mouse groups (E and EO) were placed on a treadmill for a moderate aerobic EXERCISE session (45 minutes). At the end of these protocols, exercised mouse groups have completed ten 10- minute OVA (or Saline) aerosol challenges followed by ten exercise training sessions.

Figure 2. Exercise decreased lymphocyte numbers but not total cell numbers in bronchio-alveolar lavage cell differential evaluations

OVA-specific Th cells were adoptively transferred (i.v.) to wild type recipient mice. Recipient mice underwent OVA-sensitization / exercise training regimen as indicated in Fig. 1. Post-protocol completions, bronchio-alveolar lavages (BAL) were performed. The cellular components of lavagates were analyzed for changes in total number of cells and lymphocytes. Lymphocytes were significantly reduced in exercised OVA-sensitized mice when compared to their sedentary counterparts. Data presented as mean $\pm$ SEM (N=7/group). For lymphocyte significance, *P<0.05 between groups where indicated.

Figure 3. Exercise decreased OVA-specific T lymphocytes in response to aerosolized OVA challenges as indicated by bioluminescent analysis

OVA-specific luciferase expressing Th cells were adoptively transferred to wildtype recipient mice. Recipient mice underwent OVA sensitization / exercise training protocols as indicated. The luciferase substrate, D-luciferin, was i.p. administered prior to each image collection. Five-minute images were collected at time-points indicated. A) Representative images of two sedentary OVA-sensitized (SO) and two exercised OVA-sensitized (EO) mice. Image collected 18-h post-protocol completion on Day 50. The number of OVA-specific Th cells was evaluated by the quantification of the bioluminescent signal. The color-density scale provided in the legend indicates amount of photon emissions. B) A representative mouse for both sedentary OVA-sensitized (SO) and exercised OVA-sensitized (EO) mouse groups was imaged at indicated time-points 18-h post OVA-challenge throughout the OVA-sensitization and challenge protocol. (Images for Days +0-21 are found in Supplementary Fig. 2A. Representative non-sensitized mouse control images are seen in Supplementary Fig. 2D.) Region of interest (ROI) quantification of bioluminescence signals over time throughout the OVA-sensitization and challenge protocol for C) “whole body” ROI (GATE 1); Exercise training significantly reduced bioluminescent signals after the initiation of exercise on Day +28. All data points presented as mean $\pm$ SEM (N=7/group). Significant effect of exercise, $*p<0.05$ using repeated measures ANOVA. Or D) “lung” ROI (GATE 2); Exercise treatment significantly reduced bioluminescent signals after the initiation of exercise on Day +28. All data points presented as mean $\pm$ SEM (N=7/group). Significant effect of exercise, $*p<0.05$ using repeated measures ANOVA. Lung ROI were drawn to exclude cervical lymph node bioluminescence signals as we have previously mapped in studies published by Chewning et al. (Chewning et al., 2009) (Both “whole body” and “lung” ROI bioluminescent signal quantification for Days +0-21 are found in Supplementary Fig. 2B and 2C.) E) Representative image of two exercised OVA-sensitized (EO) at 10-h post-OVA challenge and at 18-h post-OVA challenge after second 10-minute OVA challenge / exercise training session (Day 30 and 31).

Figure 4. Exercise altered Th2 cytokine levels in bronchio-alveolar lavagates
 OVA-specific Th cells were adoptively transferred (i.v.) to wild type recipient mice. Recipients underwent the OVA sensitization / exercise training protocols. 18-hour post-protocol completions, bronchio-alveolar lavages (BAL) were collected and analyzed for Th2 cytokine levels (Interleukin [IL] - 4, IL-5, IL-13). A significant decrease in IL-4 was detected in exercised OVA-sensitized samples when compared to their sedentary controls. However, a significant elevation was observed in IL-13 in exercised OVA-sensitized samples when compared to their sedentary controls. There was no significant change in IL-5 levels. Data presented as mean $\pm$ SEM (N=7 group). For both IL-4 and IL-13, *P<0.05 between groups where indicated.

Figure 5. Exercise did not alter production of CCL17 or CCL1 gradients produced in lungs OVA-specific Th cells were adoptively transferred (i.v.) to wild type recipient mice. Recipient mice underwent the OVA sensitization / exercise training protocols. At the end, bronchio-alveolar lavagates were measured for chemokine gradients that facilitate Th2 entry into the lungs: CCL17 (ligand for CCR4) and CCL1 (ligand for CCR8). No significant change for either CCL17 or CCL1 was detected when comparing exercised and sedentary OVA-sensitized mouse groups. Data presented as mean $\pm$ SEM (N=13/ group).

Figure 6. Exercise did not alter expression levels of CCR4 or CCR8 on CD4⁺ Th cells from mediastinal lymph nodes

OVA-specific Th cells were adoptively transferred (i.v.) to wild type recipient mice. Recipient mice underwent the OVA sensitization / exercise training protocols. A) At the end, mediastinal lymph nodes (mLN) were analyzed for expression of Th2 lung-homing chemokine receptors, CCR4 and CCR8. No significant change was observed for either CCR4⁺ or CCR8⁺ Th cells isolated from mLN. Data presented as mean $\pm$ SEM (N=6/group). Representative histograms/dot plots indicate method of analysis and flow cytometry gating (see Supp. Fig. 5).

Figure 7. Exercise decreased responsiveness of CCR4 and CCR8 on lung-isolated Th cells
 OVA-specific Th cells were adoptively transferred (i.v.) to wild type recipient mice. Recipient mice underwent the OVA sensitization / exercise training protocols. At the end of the protocols, 5×10^4 lung-isolated $CD4^+$ Th cells were cell sorted *via* FACS Aria II. Th cells were plated in the top well while 100ng/mL of chemokine (either CCL1 or CCL17) was plated in the bottom well of a 96-well transwell plate. At the end of incubation, cells were lysed and fluorometrically quantified in the bottom well. Exercised OVA-sensitized Th cells showed a decrease in migratory capabilities when compared to their sedentary counterparts. Data presented as mean $\pm$ SEM (N=3/group) from one experiment. The experiment was performed twice with the same results. For CCL17 and CCL1 transmigration functional significance, * $P < 0.05$ between groups where indicated.

Figure 8. A summary of the proposed regulatory role for moderate aerobic exercise in the dynamic allergen-specific Th cell trafficking patterns during an lung allergen challenge

Th2 cells expressing CCR4 and/or CCR8 interact with their respective chemokine gradient, CCL17 and/or CCL1, to enter the lungs in response to an inflammatory insult. Moderate aerobic exercise decreases CCR4 and CCR8 sensitivity to their chemokine ligand. At 10 hours post-allergen (OVA) challenge, exercised OVA-challenged lungs must generate increased CCL17 and/or CCL1 levels as compared to sedentary controls (Supp. Fig. 3) to overcome the exercise-mediated decrease in chemokine receptor sensitivity (Fig. 7). This allows for entry into the lung. However, at 18 hours post-allergen (OVA) challenge, exercised OVA-sensitized CCL17 and CCL1 levels are decreased comparable to sedentary control levels yielding a decrease in exercised Th cells in the lungs. The reduced function of lung-homing chemokine receptors on exercised Th cells no longer have sufficient levels of chemokine ligand to signal Th cell migration to the lung. Whereas, sedentary Th cell controls remain in the lungs at 18 hours due to full functioning lung-homing chemokine receptors engaged with equivalent levels of chemokine. In addition, at 10 hours, CCR7 levels on exercised OVA-sensitized Th cells are significantly decreased when compared to sedentary controls allowing for the lung-homing signal to play a dominant role. Furthermore, by 18 hours, CCR7 levels on exercised OVA-sensitized Th cells are reproducibly increased and may become the dominant migratory signal on Th cells allowing for exit from the lungs while lower levels of CCR7 on sedentary Th cells result in Th cells to be trapped in the lungs (Supp. Fig. 4 and Fig. 3A).