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Dendritic Cells Are Involved in the Effects of Exercise in a Model of Asthma

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Running title: Exercise modulates immune response in asthma.

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Conflict of interest statement
All authors declare no conflict of interest related to this study.

Abstract

Introduction: Investigate the effects of aerobic exercise (AE) on both the maturation of dendritic cells (DCs) and the activation of lymphocytes in a mouse model of chronic allergic airway inflammation. **Methods**: C57Bl/6 mice distributed into Control, Exercise, OVA and OVA+Exercise groups were submitted to OVA sensitization and challenge. Treadmill training was performed for 4 weeks, and mice were assessed for classical features of chronic allergic airway inflammation, as well as dendritic cell activation and T lymphocyte response. **Results**: AE reduced OVA-induced eosinophilic inflammation as observed in bronchoalveolar lavage fluid (BALF) ($p<0.001$), airway walls ($p<0001$) and also reduced collagen deposition ($p<0.001$). AE also reduced BALF cytokines (IL-4; $p<0.001$), (IL-5; $p<0.01$), (IL-6; $p<0.001$), (IL13; $p<0.01$) and (TNF-α; $p<0.01$). Cells derived from mediastinal lymphnodes (MLN) of AE animals that were re-stimulated with ovalbumin, produced less IL-4; ($p<0.01$), IL-5; ($p<0.01$) and IL-13; ($p<0.001$). In addition, AE reduced both DC activation, as demonstrated by reduced release of IL-6 ($p<0.001$), CXCL1/KC ($p<0.01$), IL-12p70 ($p<0.01$) and TNF-α ($p<0.05$) and DC maturation, as demonstrated by lower MCH-II expression ($p<0.001$). **Conclusion**: AE attenuated dendritic cell and lymphocyte activation and maturation, which contributed to reduced airway inflammation and remodeling in the OVA model of chronic allergic airway inflammation.

Key words: asthma, airway inflammation, exercise immunology, cytokines, dendritic cells, lymphocytes.

Introduction

Asthma is a chronic inflammatory, heterogeneous airway disease affecting millions of people around the world (7, 19). Asthma is characterized by partially reversible airflow limitation, hyperresponsiveness (AHR), wheezing, cough, and airway remodeling of variable severity (7, 13, 19). Using ovalbumin (OVA) to sensitive mice, this study models allergic asthma, which is characterized primarily by a T-helper type 2 lymphocyte (Th2) mediated-pathology. The pathophysiology of the murine ovalbumin (OVA) model is more similar to allergic asthma in humans, which reproduces several allergic asthma phenotypes, such as eosinophilic airway inflammation, remodeling and hyperresponsiveness, as well as increased Th2 cytokines and IgE levels, and is distinct from exercise-induced asthma (EIA) or exercise-induced bronchoconstriction (EIB) both of which are not primarily Th2-mediated.

The mechanisms underlying the beneficial effects of AE presented in clinical studies, such as improved exercise capacity and quality of life, reduced bronchospasm, reduced anxiety and depression, attenuated airway AHR, decreased use of corticosteroids, and reduced eosinophilic inflammation (4, 14, 15, 21) remain unclear. Murine models of allergic asthma have suggested some mechanisms by which moderate AE may be beneficial to allergic asthma patients. For example, in mouse models, low and moderate intensity AE was found to inhibit redox imbalance, attenuate NF-kB activation, and increase pulmonary T regulatory cells and corticosteroid receptors (1, 8, 12, 16–18, 20, 22–24, 26, 27).

In humans, myeloid dendritic cells (mDCs) have been implicated in allergic asthma as they trigger and maintain asthmatic airway inflammation (6, 9). mDCs are considered pro-inflammatory cells as they prime naïve T cells in the lung and also in the lymph nodes, which results in Th2 differentiation and subsequent Th2 cytokine production, a key feature of asthma

(6, 9). In order to prime T cells, immature DCs (iDCs) must develop into mature myeloid DCs (mDCs), which are characterized by the expression of surface markers such as CD40, CD80, CD83, CD86 and the major histocompatibility class II (MHC-II) (6, 9). Thus mDCs are essential in the very early phase of asthma, and also play a key role in sustaining unresolved Th2-mediated inflammation (6, 9).

Whether AE affects the maturation and activation of DCs and activation of lymphocytes, has neither been investigated in humans with allergic asthma nor in murine models. Given the importance of DCs in mediating the allergic asthma phenotype in humans (6, 9) and in mouse models of allergic asthma (9), this study aimed to evaluate the effects of moderate AE on DC maturation and activation and subsequent T-cell activation in the murine ovalbumin model of allergic asthma.

Materials and Methods

All experiments were approved by the local ethical committee of Freiburg and also by the ethical committee of Nove de Julho University (AN004.2014) and were carried out according to the Helsinki convention for the use and care of animals published in 2013.

Animals and experimental groups

Male C57Bl/6 mice (6-8 weeks old) were bred at the animal facility of the University Hospital Freiburg and also in the animal facility of Nove de Julho University under specific pathogen free conditions.

Animals were divided into four experimental groups as follows: Naïve (Control; non-exercised and non-sensitized; n=20), Exercise (Exe; exercised and nonsensitized; n=20),

ovalbumin (OVA) (OVA; non-exercised and ovalbumin sensitized; n=20) and OVA+Exercise (OVA+Exe; ovalbumin sensitized + exercised; n=20).

Induction of chronic allergic airway inflammation

In total, the experiment lasted 58 days, with the exercise protocol beginning on day 28 and ovalbumin sensitization on day 0. Briefly, animals were sensitized with single intra-peritoneal injections (20ug/mouse) of ovalbumin on days 0, 14, 28 and 42. In addition, mice were challenged with aerosolized ovalbumin solution (1%) 3x/week, 30 minutes/session, beginning on day 21 and ending on day 55 (5 weeks), as previously described (22).

Aerobic treadmill test and training

All mice were adapted to treadmill training for 3 days (days 24-26), followed by a physical test on the fourth day (days 27) as previously described (22). Beginning on day 28, Exe and OVA+Exe groups were trained on a treadmill for mice at low intensity (60% of maximal velocity reached in the initial physical test) for one hour, 5x/week for four weeks (days 28-56). (22). A physical test was repeated on day 57, testing for maximal velocity, and animals were euthanized on day 58.

Analysis of airway inflammation

Airway inflammation was assessed through bronchoalveolar lavage fluid (BALF) and also by quantitative histological analysis. Under anesthesia (10mg/kg ketamine and 100mg/kg xylazine), animals were tracheotomised, and a cannula was inserted into the lungs. Lungs were washed with 3.0mL of PBS. Total number of cells in BALF were counted using a hemocytometer and differential evaluations were performed by FACS (Accuri C6, BD Biosciences) as previously described (25). Briefly, after counting and washing, BALF cells were

stained for 30 minutes with anti-I-Ad/I-Ed FITC (macrophages/DC), anti-CCR3 (eosinophils), anti-CD3 and anti-CD19 cy-chrome/PE-Cy5 (lymphocytes) and anti-CD11c APC (macrophages/DCs) in PBS (25). In addition, airway inflammation was also assessed through quantitative histomorphometric analysis as previously described (24). Briefly, 5µm thickness lung slices were stained with hematoxylin and eosin for analysis of lung structure and eosinophils count in the peribronchial space (5 complete airways per mouse) (24). The peribronchial space corresponded to the area between the epithelial basement membrane (outer boundary of the bronchial epithelium) and the airway adventitia (corresponding to the beginning of the lung parenchyma). Thus, after measuring the peribronchial area using the Image ProPlus version 4.5 (Media Cybernetics, MD, USA), the number of eosinophils into this area were counted using hematological criteria (24). The results were expressed as number of eosinophils per square millimeter of peribronchial area (24).

Analysis of airway remodeling

Five µm lung slides were stained with picrosirius red staining kit (Abcam ab150681) for evaluation of collagen deposition in the airway walls, and a quantitative histomorphometric method was applied as previously described (24). Briefly, the peribronchial space corresponded to the area between the epithelial basement membrane (outer boundary of the bronchial epithelium) and the airway adventitia (corresponding to the beginning of the lung parenchyma). Thus, after measuring the peribronchial area using the Image ProPlus version 4.5 (Media Cybernetics, MD, USA), the red stained area, which correspond to collagen fibers, were measured using the same software. The results were expressed as percentage of collagen fibers related to the total peribronchial area (24).

Generation and culture of bone marrow-derived dendritic cells (BMDCs)

Bone marrow cells were collected from bone marrow lavage of naïve and exercised animals, as previously described [4]. Bone marrow lavages were centrifuged for 5 minutes, 4°C at 1500rpm and cell pellets were re-suspended and cultured in RPMI 1640 supplemented with 10% FCS, 1% gentamicin and

recombinant murine GM-CSF (200UI/mL) for 8 days. On the 9^{th} day of culture, cells were pulsed with ovalbumin (100µg/mL) and the supernatant was collected for cytokine measurement (as an indication of DC activation) and the cells used for analysis of BMDCs maturation by FACS analysis [4].

Activation of Lymphocytes from Mediastinal Lymph Nodes (MLN)

MLNs were collected from all animals from all experimental groups and passed through a 70µm cell strainer followed by 40µm in RPMI 1640 medium supplemented with 10% FCS and 1% gentamicin in a sterile environment. Cells were counted and resuspended in the medium described above and then placed in 96 well plates.

Th1, Th2 and Anti-inflammatory Cytokines Analysis

Cytokines were analyzed by ELISA R&D Systems DuoSet ELISA kits (IFN-γ DY458; TNF-α kit DY410; IL-6 kit DY406; IL-12p70 kit DY419; IL-4 kit code DY404; IL-5 kit code DY405; IL-13 kit code DY413 and IL-10 kit code DY417) according to the manufacturer' recommendations in the supernatant of BALF, DC supernatant, and also in the supernatant of re-stimulated mediastinal lymphnodes (MLN) as previously described (24).

Statistical Analysis

The Sigma Stat 3.5 software was used for statistical analysis. The Graph Pad Prism 5.0 software was used to build the graphs. Normality analysis revealed parametric data that were expressed as means ± SD. Comparisons between all groups were carried out by two-way analysis of variance (ANOVA), followed by

Student-Newman-Keuls post-hoc test. Values were considered significant at $p<0.05$.

Results

Aerobic exercise reduces inflammatory cells in BALF

In order to verify robust asthmatic response due to OVA injury, and to assess the extent by which moderate AE attenuated OVA injury, a differential analysis of cells present in the bronchial alveolar lavage fluid (BALF) was performed. Total cell number (Figure 1A), eosinophils (Figure 1B), neutrophils (Figure 1C), lymphocytes (Figure 1D), and macrophages (Figure 1E), were increased significantly in BALF fluid of OVA-treated mice suggesting considerably increased inflammation in OVA-treated mouse lungs. Exercise alone did not increase the number of inflammatory cells. The presence of inflammatory cells in the BALF of OVA-treated mice that performed moderate intensity exercise (AE) was significantly decreased compared to OVAtreated sedentary mice.

Aerobic exercise positively modulates the pulmonary cytokines levels

To further assess inflammatory levels, inflammatory cytokines were measured (Figure 2). In concordance with inflammatory cell counts (Figure 1), an increase in pro-inflammatory cytokines IL-4 (Figure 2A), IL-5 (Figure 2B), IL-6 (Figure 2C), IL-13
(Figure 2F) and TNF-α (Figure 2G) was observed in OVA-treated mice. Pro-inflammatory cytokines were attenuated in OVA mice performing AE, and slightly reduced in exercise only groups. Anti-inflammatory cytokine IL-10 (Figure 2C) was increased in exercise only and OVA+Exe groups suggesting that moderate AE significantly increased IL-10 in the presence or absence of OVA treatment. AE slightly increased Th1 cytokine IL-12p70. IL-12p70 was decreased in the OVA-only group, indicating a primarily Th2 response in this model, and remained at the same level as the non-treated group in the OVA+Exe group, indicating that exercise may result in a more balanced Th1/Th2 response (Figure 2E).

Aerobic exercise reduces chronic allergic airway inflammation and remodeling

In order to assess the number of eosinophils present in the conducting airways, quantitative histological analyses were performed on hematoxylin and eosin stained sections, and eosinophils were counted (Figure 3A-D). The number of eosinophils was unchanged in the exercise only group and increased to approximately 130/mm^2 in the sedentary OVA group. Moderate intensity AE reduced eosinophil counts to approximately 50/mm^2 (Figure 3A), matching observations of eosinophils in the BALF fluid (Figure 1B).

As in asthma, the OVA model also leads to structural remodeling of the airways. Picrosirius staining indicated no change in exercise only mice, and an increase in collagen fibers in OVA mice compared to control (Figure 3F). Exercised OVA mouse airways underwent significantly less remodeling (Figure 3F). Results were quantified as the percent of airway

collagen (Figure 3F). In summary, these data demonstrate that moderate intensity AE decreases airway remodeling due to

Th2 inflammation in a model of OVA.

Aerobic exercise inhibits T lymphocytes activation

Immature dendritic cells that sense and collect antigen, like OVA as used in the present study, in the lung, will then continue on to the mediastinal lymphnodes (MLN) where they will present the OVA antigen to naïve T-cells. Naïve T-cells that have become Th2 cells are robust producers of IL-4, IL-5 and IL-13. Therefore, measuring these cytokines in the MLN also serves as an measurement of the Th2 cell population activation (24). In exercise only mice, the cytokine levels were similar to those of the control, while in OVA treated mice, Th2 cytokines were increased. As expected, reduced levels of Th2 cytokines were observed in exercised OVA mice (Figure 4A, B, C). IFN-γ is produced predominantly by natural killer (NK), natural killer T (NKT) cells, and also by cytotoxic Th1 cells. IFN-γ was not significantly regulated between groups in this model (Figure 4D).

Aerobic exercise inhibits dendritic cells activation and maturation

Next, a model of dendritic cell maturation using OVA stimulation of dendritic cells isolated from bone marrow lavages (BMDCs) derived from either sedentary or exercised animals was performed (24). On the ninth day of culture, cells were pulsed with ovalbumin (100µg/mL) and the supernatant was collected for cytokine measurement using ELISA (Figure 5) and also for FACs analyses for surface markers of dendritic cell maturation (Figure 5). Results are expressed as the level of cytokines detected in cell free medium versus the level of cytokines detected in the cell free medium of BMDCs of sedentary animals (Naïve/Medium) or exercised-only animals

(Exe/Medium). In addition, BMDCs derived from non-stimulated sedentary mice were compared to OVA-stimulated BMDCs isolated from sedentary mice (Naïve/OVA). In addition, cytokine levels of non-stimulated BMDCs derived from exercised mice were compared to those of BMDCs from exercised mice stimulated with OVA (Exe/OVA). In summary, BMDCs derived from exercised only mice were more resistant to OVA-induced cytokines IL-6, CXCL1/KC, IL-12p70, and TNF-α (Figures 5A-D).

FACs analyses revealed higher expression of mature dendritic cell marker MHC-II in OVA-stimulated BMDCs derived from sedentary animals than in OVAstimulated BDMCs derived from exercised animals (Figure 6A). Other markers for mature dendritic cells: CD80 (Figure 6B), CD83 (Figure 6C), and CD86 (Figure 6D) were only slightly reduced in OVA-stimulated BDMCs derived from exercised animals. These results suggested that BMDCs derived from exercised animals might be less mature than BMDCs derived from sedentary animals. Thus the attenuated Th2 response observed in the OVA+Exe groups may be partly due to exercise's stifling effect on dendritic cell maturation. Figure 6E and 6F are representative FACS

histogram from CD80 and CD83 expression, respectively.

Discussion

Allergic asthma is a disease characterized by a prodigious Th2 response, which is instigated by mDCs. Although low and moderate AE has been shown to attenuate the Th2 response in mouse models of allergic asthma (1, 8, 12, 16–18, 20, 22–24, 26, 27), whether moderate AE affects the maturation and activation of DCs and the subsequent lymphocyte response has not been studied. In this study, OVA-sensitized mice ran on a treadmill at 60% of

their maximal capacity (an intensity similar to jogging or light running) for 5 days a week, 60min a day, for 5 weeks, and showed a decreased Th2 response. While these results suggest that moderate AE may attenuate the Th2 activation in allergic asthmatics, ovalbumin model of chronic allergic airway inflammation is not part of the true pathology of allergic asthma, therefore these results require cautious interpretation.

DCs derive from hematopoietic stem cells in the bone marrow and hone to sites of pathogen invasion. Antigen acquisition and processing by DCs stimulates their maturation, which is partly characterized by MHCII expression. Next, mDCs migrate to mediastinal lymph nodes (MLNs) to educate naïve T-cells. Thus mDCs influence whether the lymphocyte response to a pathogen is primarily Th1 or Th2 mediated. In this study, OVA-treatment of both sedentary and exercised mice resulted in an overall increase in lymphocyte infiltration in the lung (Figure 1). However, their respective cytokine profiles were significantly different (Figure 2). Reduced Th2 cytokines (IL-4, IL-5, and IL-13) in the OVA-Exe animals suggested incomplete maturation of dendritic cells in exercised mice, which likely contributed to the attenuated lymphocyte accumulation in OVA+Exe animals. Likewise, increased IL-6 production in OVA mice indicated that in the absence of exercise, dendritic cells illicit a primarily Th2 response (2). Furthermore, the reduction of the Th1 cytokine IL-12p70 in OVA sedentary mice also indicated a major recruitment of Th2 cells in this model. In contrast, the maintenance of IL-12p70 production in OVA+Exe mice suggested that moderate AE may result in a more balanced Th1/Th2 response to OVA. Since allergic asthma in humans is characterized primarily by a robust Th2 response, these results along with other allergic asthma model animal study results (1, 8, 12, 16–18, 20, 22–24, 26, 27), suggest that moderate AE may attenuate an allergic asthmatic's

Th2 response to allergens, however clinical studies exposing allergic asthmatics to a regimen of moderate intensity AE are needed.

In both exercise and OVA+Exe mice, anti-inflammatory cytokine IL-10 was increased. *In vitro*, IL-10 inhibits both cytokine production and antigen-presenting function by inhibiting IL-12 production as well as the expression of MHCII, the co-stimulatory molecules CD80, CD86, and the DC marker CD83 (11). Interestingly, IL-10 reduced MHCII expression following activation of monocytes with LPS (28). Exposure to increased levels of IL-10 following exercise may explain why MHCII expression was strongly reduced in activated dendritic cells derived from exercised mice and significantly but not strongly for the other markers of mDCs maturation such as CD80, CD83, and CD86 (Figure 6).

AE has also been shown to increase intracellular cyclic adenosine monophosphate (cAMP) (3). cAMP signals not only via protein kinase A (PKA), but also activates exchange protein activated by cAMP (Epac), a second major cAMP effector. In the context of dendritic cell maturation, one study showed that cAMP- activated Epac blocked MHCII expression by dendritic cells (5). Moreover, cAMP and cAMP-elevating agents have been demonstrated to repress antigen presentation by LPS-stimulated BMDCs (10). Taken together, increased cAMP due to AE may be another potential mechanism by which the expression of MHCII was reduced by BMDCs harvested from exercised mice and stimulated in vitro with OVA. However, it remains unclear why co-stimulatory molecules CD80, CD86, and the DC marker CD83 expression remain elevated in exercised animals. In the future, co-cultures of dendritic cells and lymphocytes harvested from this model may provide further insight into the mechanisms involved.

In summary, moderate AE led to decreased maturation of DCs indicated by a reduction of MHCII, CD80, CD83 and CD86 expression in this model (Figure 6) which likely contributed to decreased Th2 differentiation and activation, resulting in the blunted pro-inflammatory cytokine response observed in OVA+Exe mice (Figure 2). These results further support the anti-inflammatory effects of moderate intensity AE in ovalbumin model of chronic allergic airway inflammation, and provide evidence that AE may stifle dendritic cell maturation and activation and T lymphocyte activation thereby promoting a more balanced immune response.

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Figure Legends

Figure 1: Inflammation in BALF.

BALF was isolated from non-stimulated (Control); moderate exercised (Exe) sedentary, ovalbumin-treated (OVA); and animals that performed moderate exercise and received ovalbumin treatment (OVA+Exe) and cytospin was performed. At least 300 cells were counted per cytospin. Total cell number (A) total eosinophils (B), neutrophils (C), lymphocytes (D), and macrophages (E). Inflammatory cells in the BALF of OVA-treated mice that performed moderate intensity exercise versus OVA-treated sedentary mice. ***$p<0.001$.

Figure 2: Inflammatory cytokine production.

ELISAs were performed on BAL fluid isolated from non-stimulated (Control); moderate exercise (Exe) sedentary; ovalbumin treated (OVA); and moderate exercised plus ovalbumin treated (OVA+Exe). IL-4 (A), IL-5 (B), IL-6 (C), IL-10 (D), IL12p70 (E), IL-13 (F) TNF-α (G). ***$p<0.001$; **$p<0.01$, *$p<0.05$.

Figure 3: Airway inflammation and remodeling

Non-stimulated (Control); moderate exercise (Exe) sedentary; ovalbumin-treated (OVA); and moderate exercised plus ovalbumin-treated (OVA+Exe). Hematoxylin and eosin staining of conducting airways was performed and eosinophils counted in the peribronchial space (5 complete airways per mouse per group). Control (A), Exe (B), OVA (C), OVA+Exe (D). Results are expressed as the number of eosinophils per mm^2 of conducting airways (E).

Picrosirius staining for collagen fiber quantification in Control, Exe, OVA and OVA+Exe groups were quantified and expressed as percentage of collagen fibers related to the total peribronchial area (F). ***p<0.001.

Figure 4: Th2 cytokines in the mediastinal lymph nodes

Non-stimulated (Control); moderate exercise (Exe) sedentary; ovalbumin-treated (OVA); and moderate exercised plus ovalbumin treated (OVA+Exe). Elisa was performed on MLN for IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D). ***p<0.001; **p<0.01, *p<0.05.

Figure 5: Elisa measurements of supernatant collected from bone marrow derived dendritic cells (BMDCs) cultures stimulated with ovalbumin from sedentary or exercised mice

On day ninth day of culture, cells isolated from lavages were pulsed with ovalbumin (100µg/mL) and the supernatant was collected for cytokine measurement using ELISA for cytokines. Results are expressed as the level of cytokines detected in cell free medium versus BMDCs collected in sedentary animals (Naïve/Medium) or cell free medium versus BMDCs derived from exercised-only (Exe/Medium) animals. BMDCs derived from sedentary mice stimulated with OVA were compared to non-stimulated BMDC isolated from sedentary mice (Naïve/OVA) as well as non-stimulated BMDCs derived from exercised mice versus BMDCs from exercised mice stimulated with OVA as (Exe/OVA). BMDCs derived from exercised only mice were more resistant to OVA induced cytokines IL-6, (A) CXCL1/KC (B), IL-12p70 (C), and TNF-α (D). ***p<0.001; **p<0.01, *p<0.05.

Figure 6: FACS analyses of bone marrow derived dendritic cells (BMDCs) cultures stimulated with ovalbumin from sedentary or exercised mice

Results are expressed as the level of cytokines detected in cell free medium versus BMDCs collected in sedentary animals (Naïve/Medium) or cell free medium versus BMDCs derived from exercised-only (Exe/Medium) animals. BMDCs derived from sedentary mice stimulated with OVA were compared to non-stimulated BMDC isolated from sedentary mice (Naïve/OVA) as well as non-stimulated BMDCs derived from exercised mice versus BMDCs from exercised mice stimulated with OVA as (Exe/OVA). MHC-II, (A) CD-80 (B), CD-83 (C), and CD-86 (D). Figure 6F and 6G are representative FACS histogram from CD80 and CD83 expression, respectively.

***$p<0.001$; **$p<0.01$, *$p<0.05$.

FIGURE 1

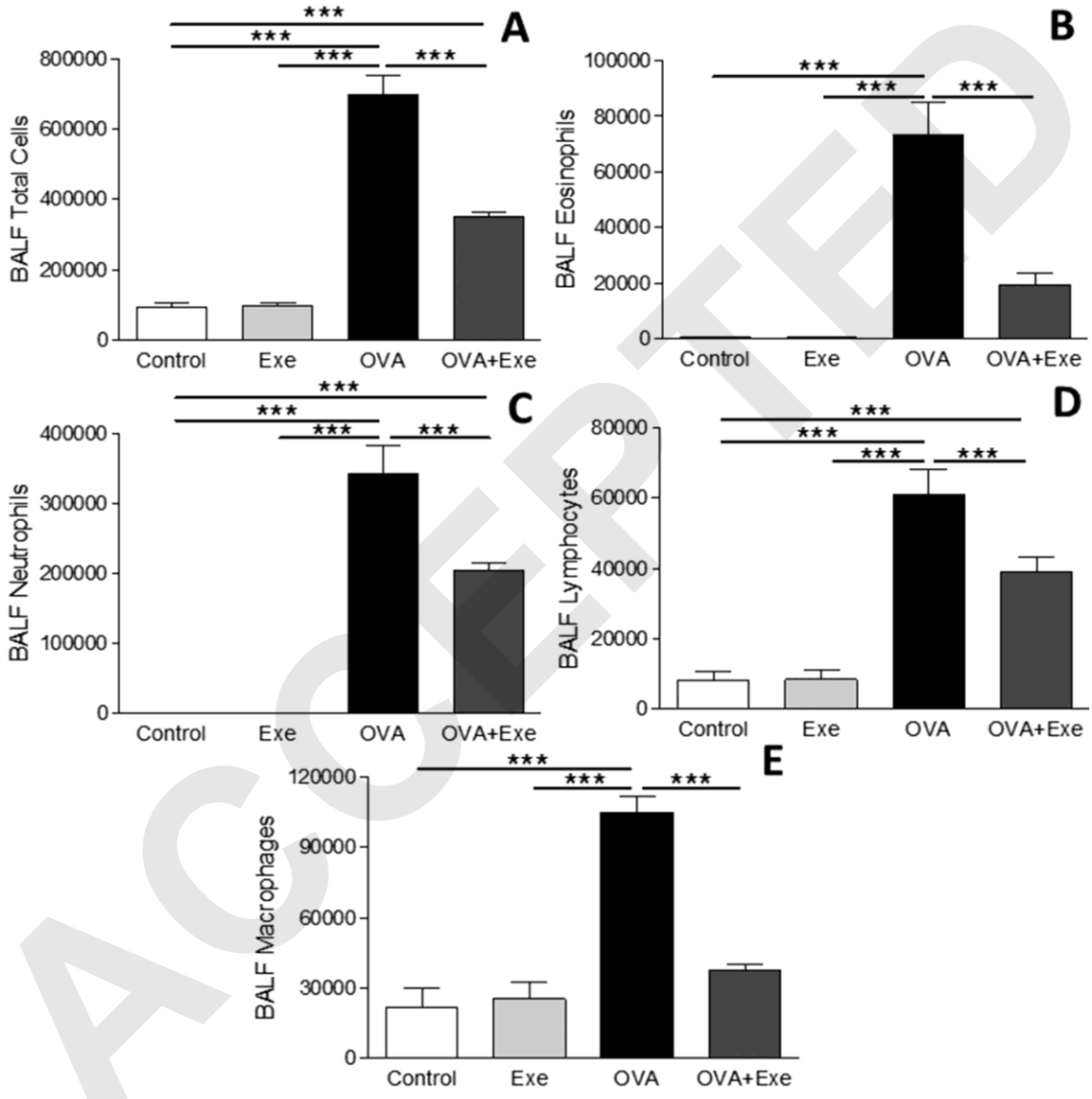


FIGURE 2

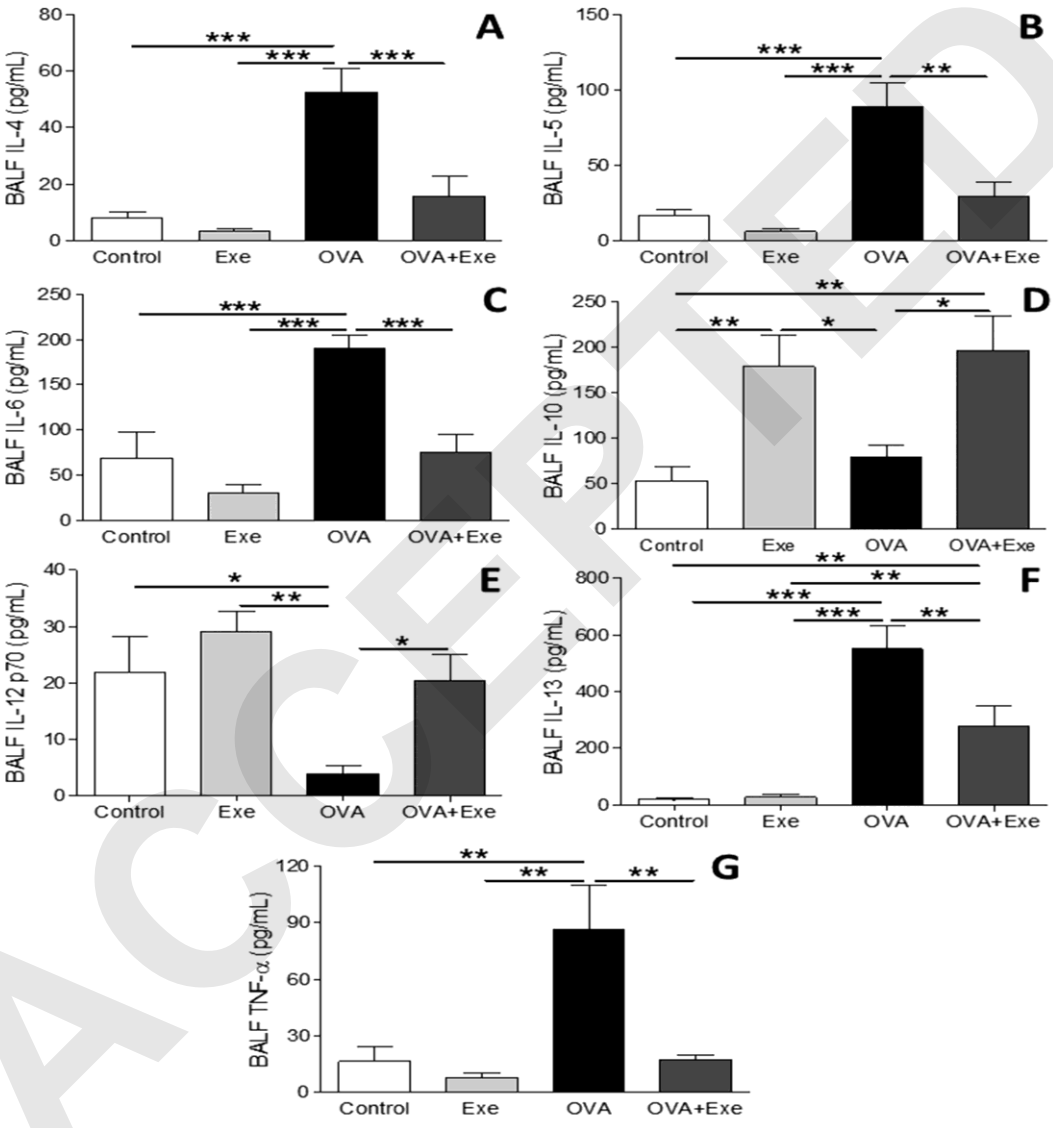


FIGURE 3

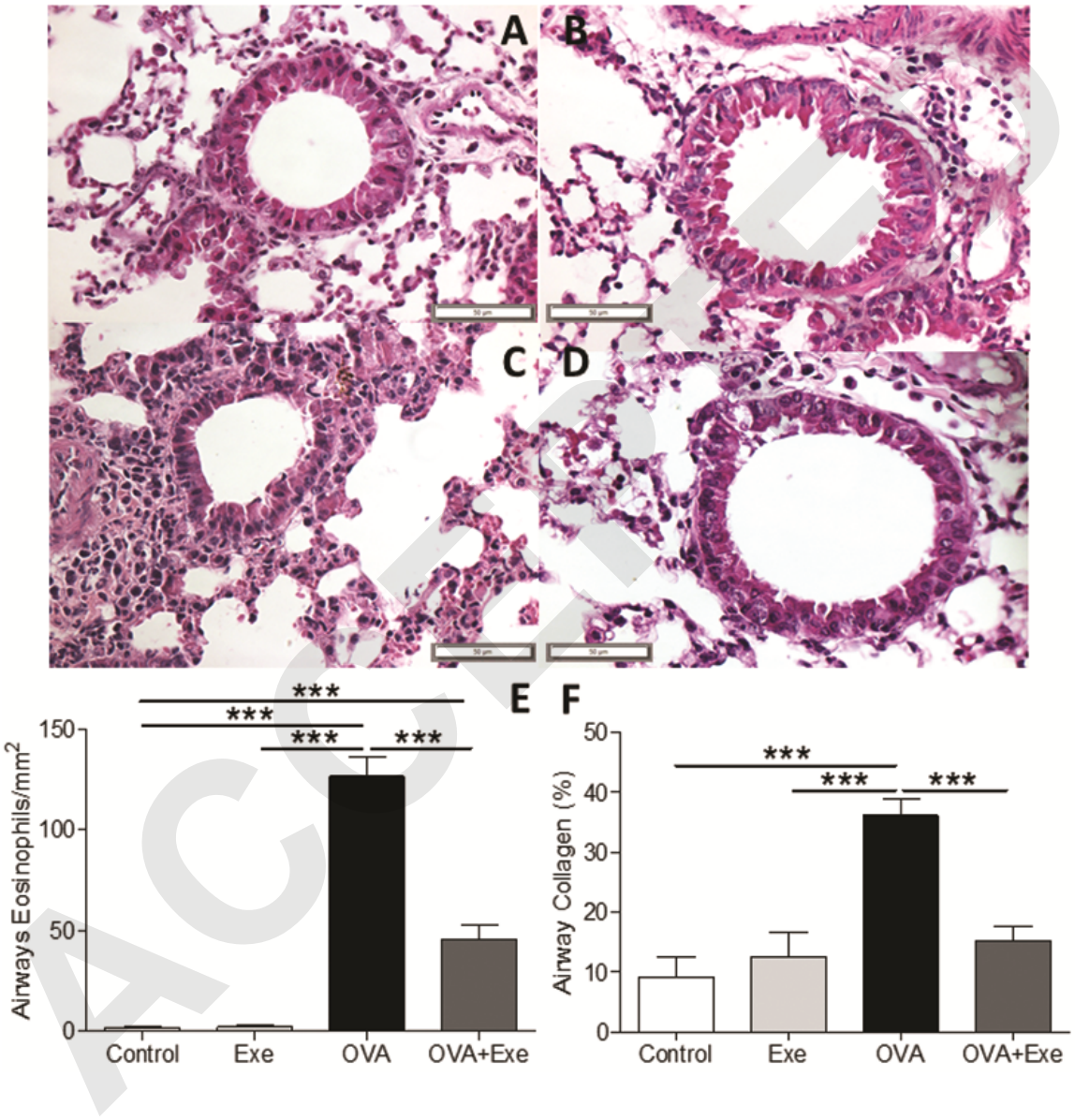


FIGURE 4

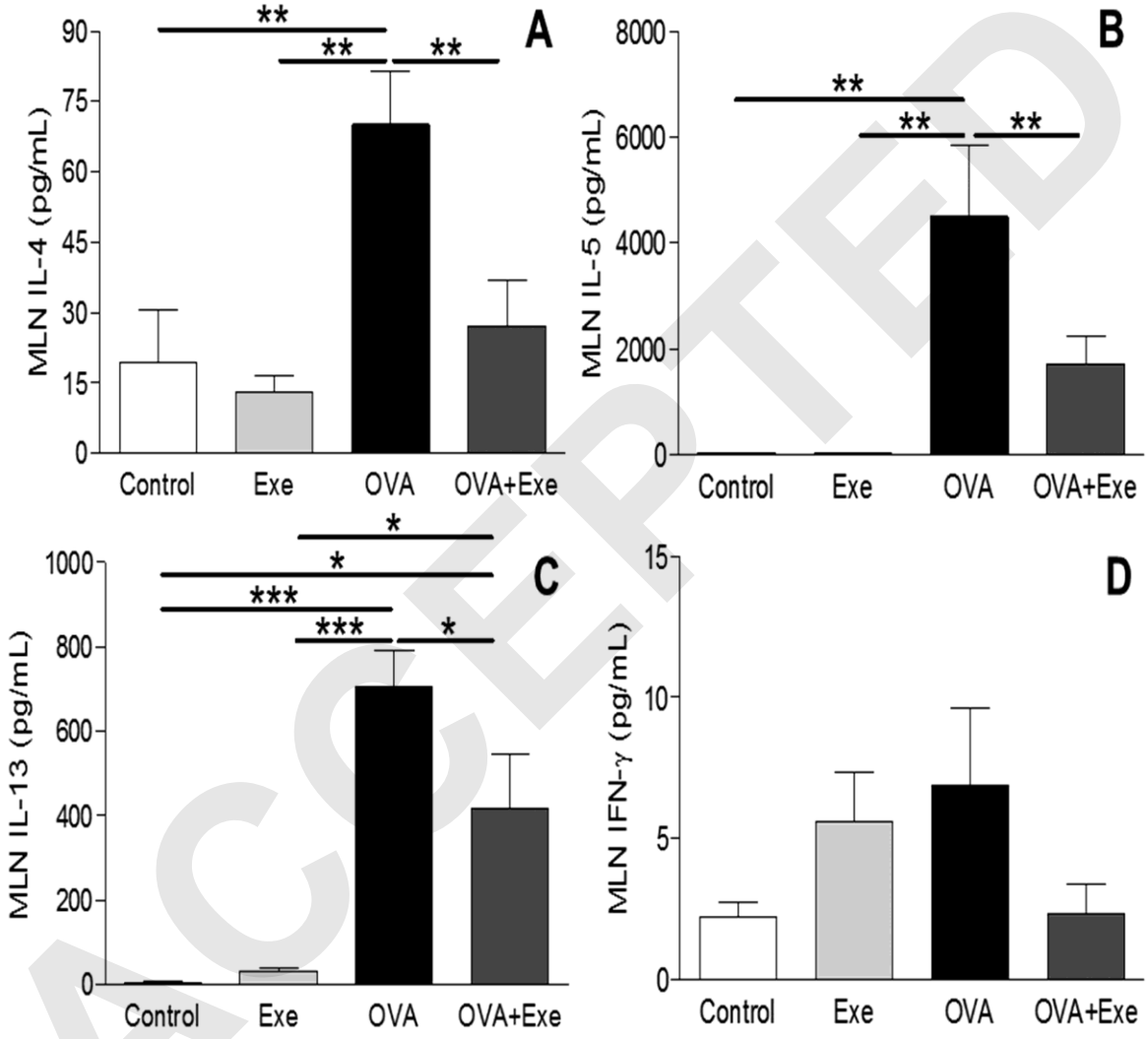


FIGURE 5

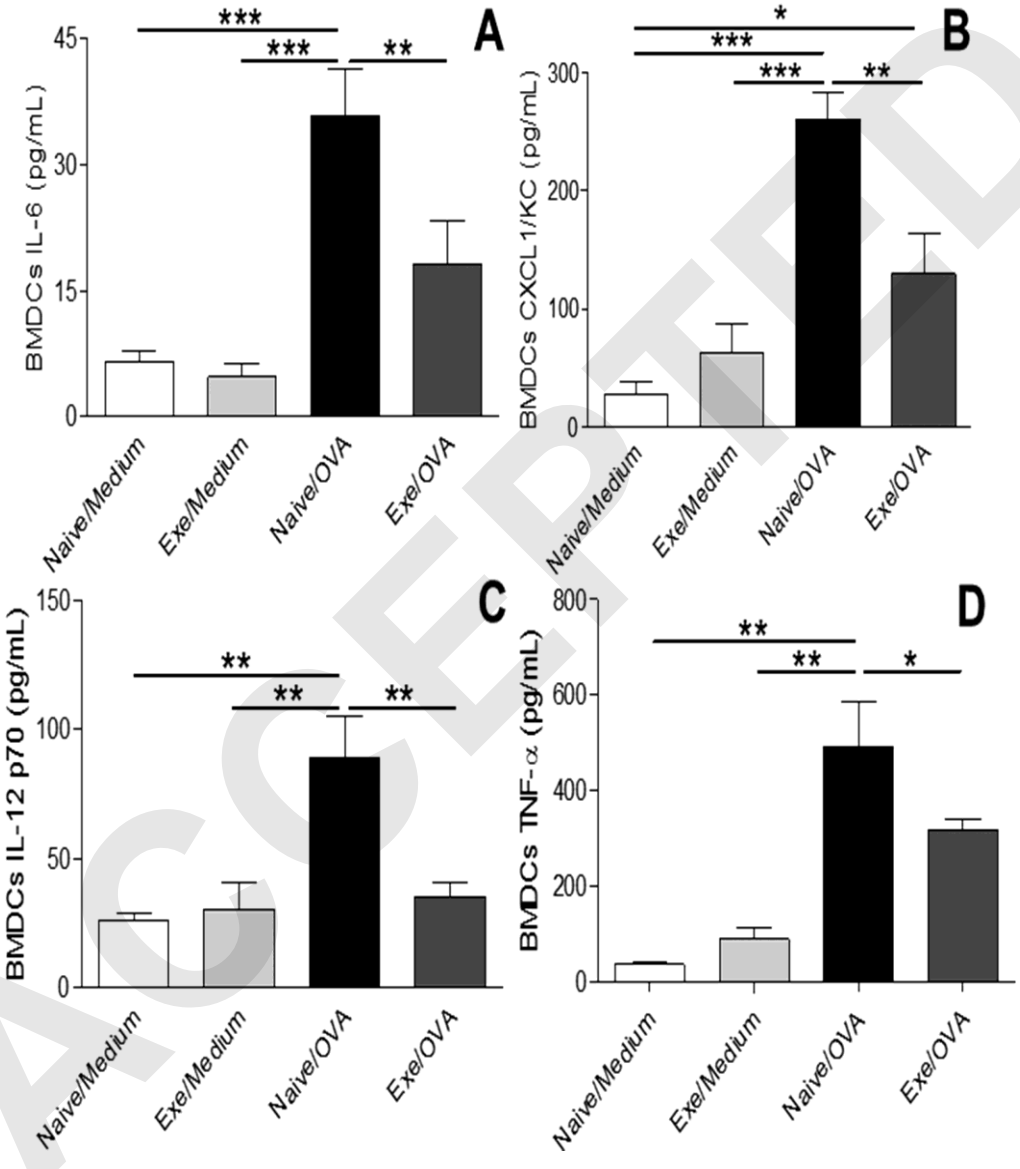
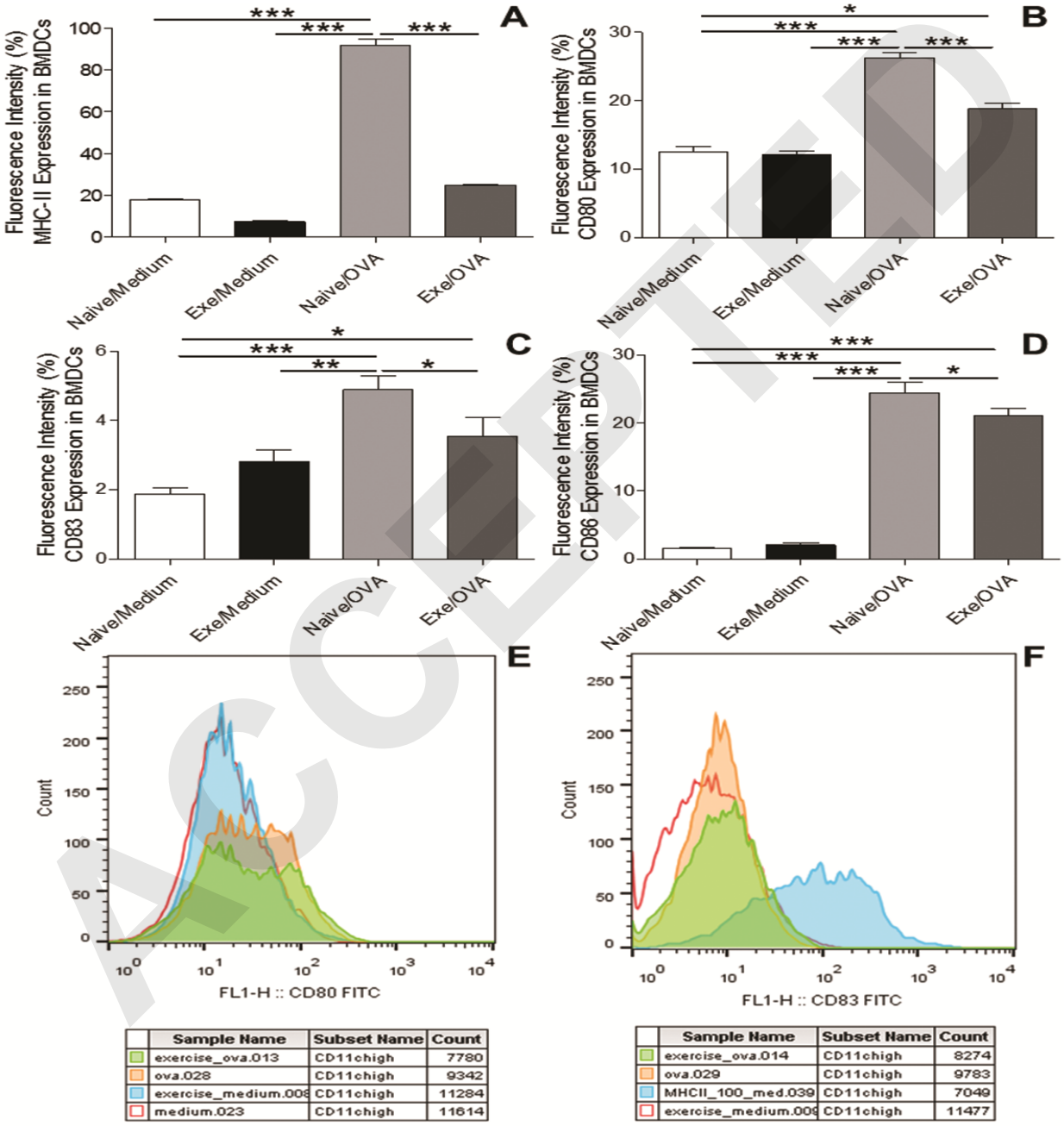


FIGURE 6



Sample Name	Subset Name	Count
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ova.028	CD11chigh	9342
exercise_medium.008	CD11chigh	11284
medium.023	CD11chigh	11614

Sample Name	Subset Name	Count
exercise_ova.014	CD11chigh	8274
ova.029	CD11chigh	9783
MHCII_100_med.039	CD11chigh	7049
exercise_medium.009	CD11chigh	11477