Characterizing Lung Inflammation in Agricultural Respiratory Exposures Between the Sexes

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the College of Medicine

University of Saskatchewan

Saskatoon

By

Kaitlin Sarah Jane Merkowsky

Permission to use

In presenting this thesis/dissertation in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/ dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation

Disclaimer

Reference in this thesis/dissertation to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan and shall not be used for advertising or product endorsement purposes. Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head of the Department of Medicine
3544 Royal University Hospital
103 Hospital Drive
University of Saskatchewan Saskatoon, Saskatchewan S7N 0X8 Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

Abstract

Agriculture workers, in Saskatchewan and worldwide, are exposed to numerous potential pollutants, including grain dust and pesticides. Although female workers make up approximately 25% of the agricultural working population, the majority of current research on the respiratory and inflammatory effects in agriculture has been conducted on male animal models and workers; very little has been studied of the female response. Females are able to mount a more robust and efficient early immune response leading to improved prognosis in surviving acute infections arising from a variety of pathogens (bacteria, viruses, trauma) compared to males. However, increased efficiency mean females are more predisposed to developing autoimmune diseases. Agriculture workers are commonly exposed to more than one pollutant at a time; how the interaction between glyphosate and lipopolysaccharide (a component of grain dust) will differentially affect the sexes is not known. Currently, there has been minimal work done to evaluate how a respiratory glyphosate exposure may differentially impact the sexes.

The following study evaluates the differences in the inflammatory respiratory response between the sexes following a short-term agriculture respiratory exposure and is the first study to do so. It uses a mouse model. C57BL/6 mice were intranasally treated with glyphosate (1µg), lipopolysaccharide (LPS) (0.5µg), combined LPS + glyphosate (LPS: 0.5 µg + glyphosate: 1µg), or Hank's Balanced Salt solution (HBSS) for 5 days. These studies were performed to characterize the inflammatory effects in mice following a short-term intranasal exposure to LPS plus glyphosate including 1) evaluating inflammatory effects of the combined exposure to glyphosate and LPS in female mice compared to exposure to each individual agent; 2) comparing the female response to the combined LPS plus glyphosate exposure vs. the male response; and 3) observing the structural lung changes of the combined exposure to glyphosate and LPS in female mice as measured using multiple image radiography.

Female mice, exposed to LPS and glyphosate for 5 days showed higher levels of inflammatory mediators compared to control animals, or those treated with only LPS or glyphosate. Inflammatory mediators, such as proinflammatory cytokines, were elevated in the LPS plus glyphosate treated animals, indicating that after 5 days, the addition of the glyphosate

impacts the ability of female mice to ameliorate the effects of LPS, compared to the animals treated only with LPS or glyphosate.

Further, this study revealed that female mice display a different inflammatory respiratory response compared to male mice. Female mice demonstrated: less lung architecture damage across treatment groups; significantly lower levels of inflammatory markers; and lower levels of proinflammatory cytokine expression as compared to male mice. This is the first study to validate that a significant difference exists between the male and female immune response following a short-term agriculture respiratory exposure to LPS and glyphosate.

Finally, comparisons of lung effects using multiple techniques (multiple image radiography, and histology) were utilized to evaluate a short-term common agriculture respiratory exposure in female mice. Histology revealed greater recruitment of cells into alveolar regions in the lungs of the mice and disruption to the bronchial epithelium from the combined LPS and glyphosate treated group as compared to other treatment groups. MIR images revealed mice exposed to LPS and both LPS plus glyphosate showed compromised lung tissue compared to other treatment groups. Taken together, these results reveal that female mice exposed to the combination of LPS and glyphosate displayed physiological and structural effects that were different from mice exposed to LPS or glyphosate alone. However, the inflammatory effects of the combined exposure were not as pronounced in the female mice compared to the male mice, highlighting the importance of using a structural evaluation technique such as multiple image radiography to reveal the impact to the lungs of such exposures.

Overall, we observed that female mice, exposed to an agriculturally relevant concentration combining LPS plus glyphosate for 5 days, exhibited respiratory inflammatory effects significantly different compared to each single exposure. Additionally, we demonstrated that there is a significantly different respiratory inflammatory response between the females and males at 5 days of LPS plus glyphosate exposure. While the precise mechanisms remain to be elucidated, the differences may be due to the protective effects of estrogen.

This study is the first research to characterize a short term respiratory inflammatory exposure to LPS plus glyphosate in female mice, to compare these results to those obtained from male mice, and to utilize multiple image radiography technology to do so. We were able to detect the differences between exposure groups using MIR and refined this technique during our study. The results suggest that MIR may become a paramount tool in future lung imaging experiments.

Acknowledgements

Thank you to my supervisor, Dr. Shelley Kirychuk, for her wisdom, patience, and guidance throughout my PhD journey. Dr. Kirychuk is truly a role model for me and I am in complete awe of what she has accomplished as a researcher, as a woman, and as a mother. I hope I can be half as successful as Dr. Kirychuk when I grow up!

Thank you to my advisory committee members Dr. Baljit Singh, Dr. Dean Chapman, and Dr. Caroline Duchaine for their expertise. I'd like to extend a special thanks to Dr. Gurpreet Aulakh who went above and beyond her duties as an RAC member by lending her time and experience during our experiments at the synchrotron, assisting me with the analysis, and always being quick to answer my questions. Also, thanks to my graduate chair, Dr. Niels Koehncke.

Thank you to the other members of the Kirychuk lab, Dr. Upkar Pandher, Dr. David Schneberger, and Dr. Amanda Nascimento, for assisting in the mice experiments and sample collection. Special thanks to our laboratory manager Brooke Thompson for her help with my assays, statistical analysis, and being a great sounding board for my struggles and frustrations throughout my program. Thank you to Dr. Aditya Manek for his help at the histology core facility, and Larhonda Sobchishin for her microscope assistance at the veterinary pathology department.

I am incredibly grateful for the research support I have received throughout my studies: College of Medicine Graduate Student Award (CoMGRAD), Health Sciences Graduate Scholarship (HSGS), and the NSERC CREATE Integrated Training Program in Infectious Diseases, Food Safety and Public Policy (ITraP) which provided me a once in a lifetime opportunities to travel to Brazil and The Netherlands.

Thank you so much to my parents Jennifer and Alan who nurtured a love for science in me from an early age and supported and encouraged me throughout my academic career. Thank you to my incredible friends who were always available for a coffee or wine whenever I needed to vent even when they didn't have a clue about what I was talking about! Special shout out to Dr. Kathlyn Woolfson who went through this harrowing process before me and was always just a text away whenever I complained or needed extra encouragement.

Finally thank you to my fiancée Matt for being my support system and cheerleader throughout. This journey would have been much tougher and less enjoyable without him by my side. Thank you for feeding me, cheering me up, believing in me 100%, and all the other little things. I love you, and now you get to marry a doctor!

Also, thank you to my amazing dog Gary who literally has no idea how much he has helped me through this journey!

Table of Contents

List of Figures

List of Abbreviations

A20	Tumor necrosis factor alpha-induced protein 3 (TNFAIP3)
AHR	Airway Hyperresponsiveness
ALI	Acute lung injury
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
B	Bronchus
B cells	B lymphocytes
BALf	Bronchoalveolar lavage fluid
BMIT-BM	Biomedical imaging and therapy-Bending Magnet
BV	Blood vessels
CCHSA	Canadian Centre for Health and Safety in Agriculture
cDNA	Complementary DNA
CLS	Canadian Light Source
COPD	Chronic obstructive lung disease
Ct	Threshold cycle
DAMPs	Damage-associated molecular patterns
DEI	Diffraction enhanced imaging
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
EPO	Eosinophil peroxidase
EU	Endotoxin Unit
FEF	Forced Expiratory Flow
FEV1	Forced Expiratory Volume in 1 second
g	Gram
GLY	Glyphosate

H&E	Hematoxylin and eosin
H2O2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution/saline
Hsp72	Heat shock protein 72
ICAM-1	Intracellular adhesion molecule-1
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
KC	Keratinocyte chemoattractant
kDa	kiloDalton
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
MIR	Multiple Image X-Radiography
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
PA	Pulmonary Artery
PBS	Phosphate buffered saline
PEF	Peak expiratory flow
PFRA	Paraformaldehyde
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Real time PCR
SD	Standard Deviation
T cells	T lymphocytes
TH	T-helper type cell
TLR	Toll-like receptor

TNF	Tumor necrosis factor
USAXS	Ultra-small angle scatter
VCAM-1	Vascular adhesion molecule-1
vWF	Von Willebrand factor
WBP	Whole-body plethysmography

Chapter 1: Introduction and Literature Review

1.1 Introduction

Agriculture is an important economic driver, producing commodities and resources, not only in the province of Saskatchewan, but in Canada and worldwide. However, evidence indicates that workers in the agriculture industry are at risk for developing deleterious respiratory effects from workplace exposures to airborne contaminants. Airborne contaminants include grain dust and pesticides, which have been linked to negative effects in the lung. It is unclear if exposure to glyphosate – an herbicide prevalently used in the agricultural industry – may affect the lungs. Moreover, agriculture workers are commonly exposed to more than one airborne contaminant at a time, commonly, grain dust and glyphosate. How the interaction between glyphosate and lipopolysaccharide (a component of grain dust) contributes to negative respiratory effects has yet to be determined. In Saskatchewan, 25% of farm operators are women (1), yet the majority of current research on the respiratory inflammatory effects of agriculture exposures has been done on male subjects. Furthermore, no molecular research has yet to compare the sex differential response following exposure to airborne contaminants, specifically the combination of LPS and glyphosate.

This is the first research to study the respiratory inflammatory effects of LPS and glyphosate exposure on lungs of female mice, and to compare differences in lung inflammatory responses between the sexes following exposure to a combination of glyphosate and LPS. We have further developed research techniques for studying airborne contamination by using imaging techniques to evaluate the structural effects in the lungs of mice exposed to glyphosate and LPS.

In this dissertation Chapter 1 is comprised of the literature review that covers: a) lung inflammation and key mediators involved in the process; b) LPS exposure, glyphosate exposure and their associated effects; and c) sex differences in pulmonary inflammation. Chapter 1 concludes by highlighting the gaps in the current literature. Chapter 2 outlines the hypotheses and research objectives. Chapters 3 to 5 cover related topics that look at LPS plus glyphosate co-exposure in female mice; the respiratory inflammatory differences in this exposure between

males and females; and utilizing imaging technology to study the co-exposure of LPS plus glyphosate. Chapter 6 covers the discussion arising from these three research topics and Chapter 7 is the works cited throughout.

1.2 Lungs

The lungs are an organ that reside in the thoracic cavity. In humans, the left lung is divided into upper and lower lobes while the right lung possesses an additional middle lobe (2) In a mouse, the left lung is comprised of one large lobe and the right of four lobes. The trachea is divided into left and right bronchi which are further subdivided into bronchioles. At the end of the bronchioles are the alveoli, where gas exchange occurs. The alveoli are made up of type 1 and type 2 epithelial cells. Supporting the alveoli is the interstitium which contains small blood vessels called capillaries, and inflammatory cells. The recruitment of inflammatory cells plays an important role during acute and chronic lung inflammation. The recruitment of activated inflammatory cells such as neutrophils often leads to acute lung injury. Alveolar macrophages are another important inflammatory cell type present in the lungs. Macrophages are important innate immune cells, involved in phagocytosis, antigen presentation and produce important inflammatory mediators (3).

1.3 Inflammation

Inflammation is a major response of the immune system to tissue damage and infection (4). It is the body's normal response to injury or infection and acts to remove the causative agent or substance in order to repair and heal the surrounding tissue (5). This response may be provoked by mechanical injury, toxins, microorganisms or hypersensitivities (5) and can arise in a matter of minutes following damage (6). Three steps occur during the inflammatory response: increased blood flow to the infected area; increased capillary permeability; and white blood cell migration from the capillary vessels to the interstitial spaces of the injured site (5). This inflammatory response is modulated by immune cells and biological mediators that act as cell-to-cell communicators (5). Inflammation is classically defined by the following five symptoms:

redness, swelling, heat, (which occur from the dilation and increased permeability of blood vessels) as well as pain and loss of function (5,7).

The inflammatory response is nonspecific: no matter the exposure or cause of damage, the response is similar (5) and is an essential mechanism of the innate immune response (8). The innate immune response is activated by pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) which are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) (9). Cells such as neutrophils and macrophages become activated when PAMPs and DAMPs bind to PRRs which in turn secrete molecules to trigger the inflammatory process (6). While inflammation occurs throughout several different areas of the body, in this thesis the main focus will be on inflammation occurring in the lungs.

1.3.1 Neutrophils

A major component of the immune response are neutrophils: granulocytes (white blood cells with a cytoplasm composed of granules) that are characterized by their multilobulated nucleus (10). Neutrophils are the predominant leukocyte in the blood and around two thirds of bone marrow stem cell activity is dedicated to production of neutrophils (6). Neutrophils are highly mobile, resulting in a quick arrival at damaged tissues and are typically the first of the leukocytes to arrive at the site of inflammation (6,10). Most neutrophils are stored within capillaries in the liver, spleen, lungs and bone marrow and then begin circulating during times of infection (6). Notably, the lungs seem to have a much larger neutrophil population during normal conditions in comparison to other organs, as indicated by intravital microscopy (11). During inflammation, cells release molecules that signal neutrophils to slow down, bind to endothelial cells lining blood vessel walls, and migrate into tissues (6,10).

The following steps are involved in the leukocyte recruitment cascade: tethering, rolling, adhesion, crawling, and transmigration (10). PRRs and DAMPs activate the capillary endothelium to upregulate P-selectin (CD62P) and E-selectin (10). P-selectin migrates from cytoplasmic granules to the cell surface within minutes (6) and E-selectin is upregulated within 90 minutes following endothelial cell stimulation (10). P-selectin binds a protein on the surface

of neutrophils called L-selectin (CD62L) which slows (tethers) the free-flowing neutrophils causing them to roll along the surface of the endothelial cells (6,10). During the rolling phase, the endothelial cells trigger the neutrophils to express leukocyte (or lymphocyte) function-associated antigen-1 (LFA-1) (6,10) which binds to intercellular adhesion molecule-1 (ICAM-1/CD54) expressed on the endothelial cells (6,10). Binding between LFA-1 and ICAMs slow down the neutrophil until it eventually stops (12). Rolling neutrophils have increased contact with chemokine covered endothelium (10). Activated endothelium cells produce CXCL8 (IL-8 in humans) which attracts, activates and promotes neutrophil adhesion to endothelium (6,10). An important proinflammatory cytokine, Tumour necrosis factor alpha (TNF-α), acts on the endothelium cells to produce IL-1, another proinflammatory cytokine. TNF-α also increases expression of additional adhesion proteins (6). To facilitate their emigration out of blood vessels, neutrophils increase vascular permeability and disrupt intercellular junction to create gaps between endothelial cells (6). Integrins, CAMs vascular cell adhesion protein-1 (VCAM-1) and other junctional proteins all work to facilitate neutrophil transmigration (10). Platelets are also present in inflamed tissues (10) and contribute to neutrophil recruitment during LPS induced inflammation (13).

The phagocytotic process begins once neutrophils reach inflammatory sites (6). Neutrophils are directed towards the invading organisms/damaged tissues by the chemotactic molecules being released (6,10). Neutrophils phagocytose microbes through various mechanisms including opsonization and PRRs (6). Neutrophils surround the invading organism via extending a projection called a lamellipodia that engulfs the bacterium into a vacuole called a phagosome (6). The bacterium may be destroyed through respiratory burst (6,10) which result from an increased uptake of oxygen by the neutrophils via activation of cell surface enzyme complex, NADPH oxidase (NOX) (6). Reactive oxygen species generated via this NOX complex work to resolve inflammation, though this process is unclear (10). The pathogen may also be destroyed by enzymes and peptides released from intracellular granules (10).

Neutrophils are short-lived cells that are active immediately following their release and are limited to performing a small number of phagocytic events (6). Following resolution of inflammation, neutrophils die in the tissue (10) and are then phagocytosed by macrophages

which produce IL-23 (6). IL-23 leads to the production of IL-17 by lymphocytes and IL-17 promotes Granulocyte colony-stimulating factor (G-CSF) (6,10). G-CSF is also the cytokine that regulates the production of neutrophils (6). Thus the rate of neutrophil production equals their rate of removal via apoptosis (6).

The quick response of neutrophils enables them to be the first line of defense against invading organisms. Following their effect, neutrophils degranulate and release DAMPs that activate the next line of defense: recruitment and activation of macrophage and dendritic cells (6).

1.3.2 Macrophages

The mononuclear phagocyte cellular system plays various important roles in the innate immune response to injury or pathogens (14). Macrophages are important cells involved in inflammation and are derived from monocytes (5). Monocytes are immature macrophages that circulate in the bloodstream and then migrate from the bloodstream to the tissue where they become macrophages (5,6). Tissue macrophages are involved in phagocytosis, antigen presentation, interact with proinflammatory cytokines, and nitric oxide production (15). These various functions mean many different subpopulations of macrophages exist (6). Examples of macrophage subpopulations include Kupffer cells in the liver, microglia in the brain, alveolar macrophages in the lung alveoli, and pulmonary intravascular macrophages in the lung capillaries (6,16). Compared to neutrophils, macrophages take longer to respond but are more robust in their antimicrobial abilities and are able to initiate the adaptive immune response (6).

Mononuclear phagocytes develop in the bone marrow from myeloid stem cells (6,16). From there they enter the blood where they circulate for approximately 3 days before migrating into various tissues and differentiating into the aforementioned subpopulations of macrophages (6). Macrophages express numerous surface, vacuolar and cytosolic molecules that aid in the detection of invading organisms (16). Macrophages also express numerous receptors such as Toll-like receptors and opsonin receptors such as CD64 which binds the FC region of antibodies (6). Macrophages produce numerous secretory molecules such as pro and anti-inflammatory cytokines like IL-1, IL-6, IL-12, IL-18 and TNF-α (6,16). Macrophages play a

role in neutrophil recruitment in response to tissue damage as well as neutrophil emigration from blood vessels (6,14). As inflammation progresses, monocytes within the blood respond by binding to vascular endothelial cells through adherence, rolling and eventually stopping in a similar fashion as neutrophils (6,14). Intracellular adhesion molecule 1 (ICAM-1) expressed in endothelial cells bind monocytes so they emigrate into tissues where they become macrophages (6).

Macrophages reach inflammatory sites hours after neutrophil arrival (6,14). Macrophages arrive via molecular signals such as DAMPs and ones sent out by neutrophils themselves (6). Neutrophil granules contain macrophage chemoattractants; dying neutrophils attract macrophages to inflammatory sites (6). Macrophages phagocytose and destroy bacteria via both oxidative and nonoxidative mechanisms and are able to execute repeated phagocytic activity (6). Pathogen degradation is performed by lysosomal proteases while matrix metalloproteinases (MMPs) work to remodel the extracellular matrix (14). Macrophages are also capable of phagocytising apoptotic neutrophils (6).

Macrophages may become activated through more than one pathway (6). During classical activation PAMPs or PRRs activate macrophages that produce proinflammatory cytokines such as TNF-α and IL-12 (16). These proinflammatory cytokines activate Natural Killer cells or TH1 lymphocytes which produce interferon-γ (IFNγ) which activate macrophages through classical activation leading to M1 cells (6). Lymphocytes from the adaptive immune system may also secrete cytokines that will direct macrophages to classic (M1) activation (16). M1 cells have enhanced antimicrobial and inflammatory properties as they are responsible for host defense, generate nitric oxide and are potent killers of invading organisms (6,16). Macrophages may also be alternatively activated (M2) by TH2 lymphocytes which produce the cytokines IL-4, IL-13 and IL-10 that produce M2 cells that work to reduce inflammation (6,16). M2 cells are produced in the late stages of inflammation where they promote vessel formation, work on tissue repair and remodeling to promote healing, and reduce microbial killing (6).

Macrophages are part of the first line of defense to potential pathogens that are inhaled. When inhaled particles enter the respiratory tract they may reach the lung alveoli where they are then ingested by alveolar macrophages (6). Alveolar macrophages have a

distinct phenotype compared to other macrophages (16). Alveolar macrophages work to clear particles (microbes, dust and pollutants) from the lung. These particles are then transported back to the bronchoalveolar junction and removed via mucus flow (6). The WNT/planar cell polarity (PCP) pathway is another pathway which directs macrophages to an M2 phenotype within inflamed lung tissue (17). Recent evidence suggests exposure to the pesticide fipronil alone or in combination with LPS alters this WNT/PCP pathway (17). How pesticide exposure alone, or in combination with LPS, may alter the lung profile and inflammatory response (including the recruitment of inflammatory cells such as macrophages), is of special interest to this research group.

If the inflammatory response is effective at efficiently removing the infection or injury, healing will begin in the affected area (16). However, if the pathogen is not removed or there is insufficient tissue repair, inflammation will not be resolved and persist, becoming a chronic condition (6). The macrophages and lymphocytes will continue to collect near the inflammatory site and form a granuloma as a result of monocyte recruitment, cell activation and TNF (6,16). Chronic granulomas have clinical significance as they may continue to grow and destroy normal tissue and may lead to conditions such as tuberculosis (6,18).

1.3.3 Eosinophils

Another polymorphonuclear, granulated leukocyte involved in the inflammatory response are eosinophils (6). Although classically associated with the destruction of parasites, eosinophils can be activated by IL-5 and other TH2 cytokines (6).

Previous studies looking at sex differences in the inflammatory immune response in airways found a significant difference in eosinophil numbers between male and female mice (19) with females having significantly greater airspace eosinophilia along with significantly higher eosinophil peroxidase levels (19). IL-4 and IL-5 levels, known eosinophil chemoattractants, were also elevated in female mice compared to male mice (19). These results were consistent with other studies comparing airway inflammation between male and female mice (20), indicating that eosinophils and associated cytokines are an important marker when comparing the male and female response following exposure to glyphosate and LPS.

1.3.4 Endothelium

Endothelium plays an important role in inflammation and the migration of inflammatory cells such as neutrophils (6). Endothelial cells line the walls of blood vessels and express P-selectin when they are signaled by PAMPs or DAMPs which binds to L-selectin on neutrophils (6). The vascular endothelium of lungs express TLR4 that recruit neutrophils into the lungs during LPS induced inflammation (21). Pandher *et al.* observed increased ICAM-1 and VCAM-1 staining in the large blood vessel endothelium in mice treated with glyphosate (22) indicating the potential for the increased recruitment of inflammatory cells into the lung following exposure to glyphosate.

1.3.5 Inflammatory Mediators

Immune system cells are able to synthesize and secrete numerous proteins called cytokines that regulate the immune response (6). The exposure of PAMPs to immune cells lead to cell signaling pathways that result in the production of three major proinflammatory cytokines: tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). TNF-α triggers IL-1 and IL-6 to be released, which in turn trigger steps further down in the inflammatory process (7). IL-1, IL-6, and TNF-α are proinflammatory cytokines whose presence lead to pathological changes during acute lung injury (23). These various components of the inflammatory response provide researchers and clinicians opportunities in which they can measure and assess the immune response during the many different inflammatory states (23).

Tumour necrosis factor (TNF) was first described in 1975 and was so aptly named due to the observation that it caused necrosis of tumours *in vitro* (24). Various immune cells such as activated monocytes, macrophages, activated endothelial cells, and activated T cells are all capable of TNF expression (25). TNF is a transmembrane 26 kDa protein induced by nuclear factor-κB (NF-κB), a family of transcription factors responsible for numerous cellular processes such as inflammation and immunity (26). When membrane TNF is cleaved by TNF-α-converting

enzyme (TACE), the soluble version of TNF is free to circulate in blood plasma and cause effect at various physiological sites (27). Some of these actions include adherence, migration, attraction and activation of leukocytes (6). There are two receptor molecules that TNF binds to (28); TNRF1 or p55/p60, found on most cells, and TNFR2 or p75/p80 found only on immune system cells (6,28). During the early stages of acute inflammation, TNF-α is released from mast cells and macrophages (7). TNF-α along with IL-1 acts to increase permeability in the vascular endothelial cells that line small blood vessels (6). TNF-α also stimulates neutrophils to migrate to sites of tissue damage and promotes their adherence to vascular endothelial cells (6). An increase of TNF-α amplifies inflammation by stimulating macrophages to synthesize other inflammatory mediators like NOX and COX-2 (6).

Interleukin-1 is produced when macrophages are stimulated at the CD14 and TLR4 receptor sites (6). When endotoxin is administered to model acute lung injury there is a characteristic response of neutrophil infiltration and an early increase of proinflammatory cytokines such as TNF-α and IL-1ß (29). Like TNF-α, IL-1ß increases adhesiveness in vascular endothelium cells along with promoting macrophage synthesis of NOS2 and COX-2 (6). The family of interleukins that are similar to IL-1 include IL-18, -33, -36, and IL-27 and all play a role in regulation of the innate immune response (6).

Another cytokine that plays a major proinflammatory role is IL-6. Il-6 is produced by macrophages, T cells and mast cells when they are stimulated by endotoxin, IL-1, and IL-6 (6,30). There is evidence that IL-6 facilitates the change from neutrophil-dominated early inflammation processes to macrophage-dominated response in later stages (6). Additionally, IL-6 has an anti-inflammatory role by inhibiting some TNF-α and IL-1 activities as well as promoting IL-10 production (6).

A20, or tumour necrosis factor α-induced protein (TNFAIP)3, is an important anti-inflammatory protein present in endothelial cells (31). A20 was previously known as nuclear factor κB inhibitory and antiapoptotic signaling protein, indicating its key role in regulating the NF-κB signaling pathways which are paramount to many cellular processes (31). In addition to protecting cells from TNF-induced cytotoxicity, A20 also inhibits NF- κB activation in the presence of IL-1, PRRS, and T and B cell antigen receptor activation (31). Recent studies have

identified A20 as a disease susceptibility gene following inflammatory and autoimmune pathology (31) affirming the role of A20 as a key regulator of inflammation. Currently there is little known about whether there are significant differences in the expression of A20 between the sexes.

1.4 Lipopolysaccharide

Lipopolysaccharides (LPS), also known as endotoxins, are a class of substances that are a major component of the outer (32) surface membrane of Gram-negative bacteria (33). LPS is comprised of a poly- or oligosaccharide portion plus a lipid A component which is believed to be the main cause of stimulating the innate immune system in eukaryotic organisms (33). LPS and Toll Like Receptor 4 (TLR4) are intimately linked. When LPS binds to the TLR4 receptor, the TLR4 signalling pathway is activated leading to cellular and molecular inflammatory changes and signalling events (34,35). These signalling events result in the translocation of nuclear factor – κB (NF-κB) transcription factor and inflammatory gene transcription (36).

A low dose of LPS is a standard method to evaluate the innate immune response during inflammation or to model acute lung injury (37) that induces a well characterized increase in cytokines. For example, TNF-α, IL-1ß, IL-6, IL-10, and interferon-γ (INF γ) may be measured along with other indicators of inflammation such as levels of leukocytes, C-reactive protein, heat shock proteins or LPS-binding proteins (37,38). Along with neutrophils, proinflammatory cytokines can be produced by monocytes and macrophages, endothelial cells and epithelial cells (39). LPS is recognized as a factor in the pathogenesis of asthma development and exposure can lead to airway inflammation and hyperresponsiveness (40). Exposure to LPS in agricultural workers leads to respiratory symptoms such as coughing, increased phlegm production, and shortness of breath (41–43).

When exposure to LPS occurs, monocytes, T cells, and B cells infiltrate the lungs (44,45). Exposure to LPS also leads to increased expression of ICAM-1 and VCAM-1 by endothelial cells which in turn leads to increased migration of neutrophils and eosinophils (45,46). Some studies indicate LPS may act as an adjuvant and enhance the immune response when the immune system is exposed to other antigens (32), such as pesticides. Given that more than one

inflammatory agent is often present during agricultural exposures, it is important to understand the role of combined exposures, such as LPS with another common agent, glyphosate.

1.5 Glyphosate

Worldwide, glyphosate-based herbicides (GBHs), such as Roundup, are the most commonly used herbicides (47) although the safety of these herbicides to non-target organisms is still under question (48). Glyphosate is a broad spectrum, nonselective, organophosphorus herbicide with high water solubility. It is an herbicide used ubiquitously in both industrialized and developing countries (49). In 2016, it was estimated that 8.6 billion kilograms of glyphosate has been used globally (49). Roundup® is the commercial name of the herbicide that is glyphosate formulated with polyoxyethylene amine (POEA), a surfactant that promotes the uptake and translocation of the product throughout the target plant (49) and penetrates across the cuticle of the plant (50). In 2015 glyphosate was re-classified as "probably carcinogenic to humans" by the World Health Organization's International Agency for Research on Cancer (51). Recently glyphosate has gained widespread media attention as lawsuits have been directed at Monsanto linking their products to cancers such as non-Hodgkin's lymphoma (52). However, a large, prospective cohort study of over 40 000 applicators using glyphosate found no association between it and cancer incidence (47).

In the European Union, glyphosate and aminomethylphosphonic acid (AMPA) levels were measured in agricultural top soils via liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and found that glyphosate and/or AMPA were present in 45% of top soils (53). A study in Iowa measured glyphosate and other pesticide residues in human urine via an immunoassay (enzyme-linked immunosorbent assay {ELISA}) (54).

1.5.1 Glyphosate Mode of Action

GBHs effectiveness as a herbicide is via the Shikimic acid pathway (Henderson, Gervais, Luukinen, Buhl, & Stone, 2010) and inhibition of the key plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (56) which is necessary for plant growth. This pathway is responsible for aromatic amino acid synthesis that are a part of essential metabolic processes in

plants, fungi and some bacteria (56). Since this pathway is not present in vertebrate cells it was believed these chemicals would have few deleterious effects on mammals. However, recent evidence shows GBHs do in fact act through various mechanisms to negatively impact mammalian biology (56).

1.5.2 Glyphosate Application in Agriculture

Glyphosate is applied to fields prior to planting for traditional agriculture crops and applied after planting on genetically modified glyphosate-resistant crops (57). It is also used in chemical summerfallow (chemfallow) in no-till agriculture, and for pre-harvest weed control. The Environmental Protection Agency (EPA) does not currently recommend any personal protective equipment (PPE) when using glyphosate, besides what is stated on product labels which recommends wearing long sleeves, pants, shoes and eyewear during product application (58,59). The Bayer data sheet does indicate a respirator may be necessary during application as well as recommending spraying in well ventilated areas (60). It is yet not clear if glyphosate has the potential to disrupt or provoke an inflammatory response. In addition to glyphosate exposure occurring during the application of glyphosate, exposure has the potential to occur in agricultural settings that involve grain handling such as animal feed and farming operations (61). Furthermore, glyphosate is rarely a single exposure; farmers may be exposed by their own use, or that of their neighbors over time. Glyphosate exposure is always combined with other types of field exposure, such as grain dust or other pollutants. The impact of these co-exposures, and the inflammatory capability of co-exposures is not well understood.

1.5.3 Glyphosate transformation in soil and health outcomes of exposures

Glyphosate, a herbicide intimately linked with canola production, has a significant association with increased rhinitis among pesticide applicators (62). As of 2018, there are 183 glyphosate containing products on the market in Canada (63). Those exposed to glyphosate report nose and throat irritation; however there is not yet any evidence that exposure to glyphosate is linked to an increase in asthma incidence (55).

Aminomethyl phosphonic acid (AMPA) is often the first decomposition product of glyphosate (64). The second generation glyphosate-resistant crops have the gene that codes for the enzyme glyphosate oxidase (65). The glyphosate is broken down into AMPA and a majority of the pesticide residues are thus AMPA and not the glyphosate itself. The breakdown of glyphosate in the soil is dependent on climate and soil type but can persist for up to 6 months (55). Glyphosate has been rarely reported in groundwater and when it is it is only present in very low concentrations (66). A study conducted by the EPA found 7 out of 27 877 groundwater samples positive for glyphosate with the highest concentration being 1.1 μg/L (55). In surface water glyphosate has a half-life of 7-14 days (50). However there is potential for glyphosate to be present in surface waters near fields where it is applied at a wide range of concentrations, 0.001-5153 μg/L (66).

Although glyphosate is considered of low oral toxicity to mammals (LD_{50} of 800-5000 mg/kg body weight) (67), researchers are curious about chronic effects and are using correlative research and experimental studies to investigate chronic exposure (68). A study in Colombia found that the aerial spraying of glyphosate leads to increased medical consultations with respiratory diagnosis by 0.465% (69). Controlled experiments are necessary as there have been disputes in the current literature over glyphosate exposure and how it may contribute to respiratory diseases (68).

There is limited data available on the *in vivo* inflammatory effects of glyphosate. Kumar *et al.* used aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) to collect glyphosate samples over a 24 hour period during spray season at 3 farms in Ohio. The samplers were used during the glyphosate spraying season over a 24-hour sampling period at a flow rate of 4 L/min (70). The glyphosate residues were analyzed from filters and an average amount of glyphosate/filter of 17.33 μg was recorded. This correlated to an average airborne concentration of 22.59 ng/m^3 (70). As these levels of glyphosate were measured during agriculturally relevant activities in which workers would be exposed, and as these levels were previously used by other researchers, this same concentration of glyphosate was utilized for our study. They also extracted endotoxin from the samplers and found an average airborne concentration of 4.87 EU/m^3. For intranasal instillation of glyphosate to mice either aerosolized

glyphosate from one of the farm samplers was used at 8.66 μg/ml in 30 μl of PBS daily for 7 days, or reagent grade glyphosate at 100 ng, 1 μg or 10 μg for 7 days (70). The results of this study showed mice exposed to glyphosate displayed an increase in cell count in lung and bronchial alveolar lavage, an increase in eosinophil and neutrophils, and increased production of IL-33, TSLP, IL-13 and IL-5 (70).

Another in vivo study looked at the effects of glyphosate in male rats. It was found that rats treated orally with higher doses of glyphosate had a significantly lower body weight compared to vehicle treated rats, indicating systemic effects of glyphosate (71). Proinflammatory cytokines IL-1ß, TNF-α and IL-6 were significantly elevated in rats exposed to glyphosate compared to control animals (71).

LPS and glyphosate have been shown to be present in the inhalable (less than 10 μm) components of agricultural aerosols (70). A recent study reported data from glyphosate exposure in combination with LPS over a 1, 5, or 10-day period in C57BL/6 male mice. An intranasal exposure at an agriculturally relevant concentration of glyphosate (1 μg/40 μl) resulted in a higher number of neutrophils and increased expression of Il-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid; and higher eosinophil peroxidase levels present in lung tissue compared to control animals (22). Additionally, lung histology showed repeated exposure to glyphosate resulted in leukocyte infiltration and increased expression of adhesion molecules ICAM-1 and VCAM-1 in the peribronchiolar, perivascular, and alveolar regions (22).

Taken together, it is evident that repeated, intranasal exposure to glyphosate has an impact on the immune system through increased expression of inflammatory mediators and potential systemic effects. Although the interaction of glyphosate plus LPS has been recently studied in a male mice model, it is not yet known how this exposure may impact females.

1.6 Agriculture Exposures & Respiratory Diseases

There is indisputable evidence that agriculture workers are subject to multiple exposures that may lead to adverse respiratory conditions (72) (73). It is well documented that various agriculture practices may contribute to respiratory diseases such as rhinitis, sinusitis, asthma, chronic bronchitis, hypersensitivity pneumonitis and chronic obstructive pulmonary

disease (COPD) (74). Symptoms like wheeze, cough, and increased phlegm are significantly higher in this occupational group (75,76). As well, there is a high association between work-related asthma and agriculture workers (62,77,78). There are many confounding factors that make these exposures difficult to assess. Different types of farming establishments exist, for example ranches for animal husbandry or those devoted only to grain production. Different farming establishments influences the pollutant profile to which workers are exposed. Other factors, such as climate, environment and common practices, may also vary (73).

Several different mechanisms may contribute to respiratory symptom reactions such as nonspecific airway irritation, allergic reactions to dust antigens, inflammatory reactions to known agents (for example endotoxins), damage to the airway mucosa or bronchial hyper-responsiveness (79). The mechanisms of how sex differences result from agriculture exposures is even less well documented but interest in this field is on the rise as women are becoming more involved in the agriculture industry.

1.7 Sex Differences & Respiratory Symptoms

It is an important distinction to recognize the difference between sex and gender. Sex is a biological variable influenced by an individual's hormones, chromosomes, and reproductive functions (80) while gender is a societal and cultural phenomenon that may affect behaviours and activities. While it is true that gender may influence aspects of health such as pathogen exposure or health-seeking behaviours (81), the interest of this study is how biological sex may alter the inflammatory response.

It is recognized that sex differences in respiratory diseases exist (82), although the underlying mechanisms are yet to be determined (68). Results are contradictory with some studies indicating an increased susceptibility to inflammatory lung disease in women compared to men (83), while other studies show females are able to mount a more robust and efficient early immune response leading to improved prognosis in surviving acute infections arising from a variety of pathogens (bacteria, viruses, trauma) compared to males (84–86). Previous research suggests there is a sex difference in the response to inhaled components such as cigarette smoke (87), or occupational exposures to dusts, gases and fumes (88) with the effects

on lung function being more detrimental for women. More women are being diagnosed with lung disease such as asthma, bronchitis, emphysema, pneumonia, and lung cancer at higher rates compared to men (89). Additionally, the prevalence rates for conditions such as asthma, COPD, and other inflammatory disease are increased in women compared to men (89). Various epidemiological studies suggests females experience a higher incidence of asthma compared to males (90). Asthma is a disease characterized by airway hyperresponsiveness to inhaled allergens or other various stimuli. Asthma prevalence is almost double in adult women vs. men (10.4% vs. 6.2 %) (91). However, pre-puberty asthma is more prevalent in boys and, once women reach menopause, asthma again becomes more severe in men (90). An explanation for the increased prevalence of asthma in women is an increase in inflammatory markers during both premenstrual and menstrual phases (92). Both epidemiology and experimental data indicate sex hormones may be one of the factors responsible for this disparity of lung diseases between the sexes (40). However, little research has been done to study sex differences in the innate immune response (37).

Various factors play a role on whether the inflammatory response will be more or less pronounced in males or females, including dose level or duration of exposure. One theory is that the presence of estrogen in females offers a protective effect against mounting an exaggerated innate immune response. Estrogen has been shown to provide a protective effect in acute lung injury models (93). Estrogen presence correlates with suppression of LPS induced acute lung injury via suppression of vascular cell adhesion molecules and proinflammatory mediators in mouse models (93). Estrogen has also been shown to inhibit the TLR4/TLR2 pathway and reduce expression of IL-1, IL-6, and TNF-α via inhibition of the NF-κB pathway (94,95) Females develop a more robust immune response to more quickly clear pathogens (81) but this efficiency may lead to increased prevalence of inflammatory and autoimmune diseases. Nearly 80% of those affected by autoimmune diseases are women (96,97). Another explanation for why females exhibit a more efficient immune response compared to males is cellular mosaicism, where the presence of two X chromosomes in females results in a better ability to respond to immune stimuli (98,99).

Card *et al.* wanted to find out if there was a sex difference in lung function and airway responsiveness in C57BL/6 mice, a strain commonly used to model lung disease (40). These investigators compared baseline lung functions and respiratory mechanics and found no significant differences between the sexes in naïve mice (40). When male mice were subjected to methacholine challenge they were significantly more responsive compared to same age C57BL/6 female mice (40). Male mice exposed to 50 µg oropharyngeal LPS via aspirating it into the respiratory tract displayed a severe hypothermic response 1-hour post exposure whereas the female hypothermic response was not significantly different compared to control treated mice. Card *et al.* also wanted to be assured that the observed effects were not specific to C57BL/6 mice, so their experiments were repeated using BALB/c mice, another strain often used to model inflammatory lung disease. The results observed in C57BL/6 mice were also observed in the BALB/c mice (40).

Male mice exposed to 50 µg oropharyngeal LPS had significantly higher levels of cells present in BAL fluid compared to both control animals and female mice exposed to LPS (40). LPS-treated female mice did not display significantly higher total BAL cell counts compared to saline treated female mice but did have higher levels of BAL neutrophils compared to control animals (40). BAL fluid cell populations were not statistically different between male and female control animals. Six hours post LPS exposure, TNF-α was significantly higher in males compared to females (~5000 pg/ml). IL-6 was increased compared to saline treated mice but was not significantly different between the sexes. The evaluation of lung sections displayed histopathological scores of LPS-treated female scores that were lower compared to LPS-treated male mice across parameters such as peribronchiolar and perivascular inflammatory cell influx. There was no difference found between LPS treated males or females compared to saline treated mice for whole-lung TLR4 protein levels via immunoblot analysis.

Card *et al.* found that castrated LPS-treated male mice displayed diminished hypothermia behaviour, decreased BAL fluid neutrophils and TNF-α levels, and decreased LPS-induced airway hyperresponsiveness compared to sham operated LPS-treated males. Ovariectomized LPS-treated female mice did not display significantly different levels of these inflammatory markers compared to sham operated LPS-treated females demonstrating that

male sex hormones must play a significant role in the lung's inflammatory response following LPS exposure (40). Additionally, LPS-treated female mice implanted with DHT pellets displayed a significant increase in their LPS inflammatory response and airway hyperresponsiveness equal to or greater to that of the LPS-treated males. (40).

Males are more likely to mount a TH1 response while females mount a TH2 type response (19) suggesting that estrogen enhances a TH2 response while testosterone promotes a TH1 response (19). In a study with C57BL/6 mice that investigated the potential for sex differences following exposure to multi-walled carbon nanotubes (MWCNTs) and crystalline silica (cSIO2), it was found there was a sex-based chronic pathology outcome related to the nano-sized MWCNT particles but not the micron-sized cSiO2 particles (19). Specifically, female mice mounted an eosinophilic response observed at 24 hours post MWCNT exposure, which continued through 7-days post exposure; while an eosinophilic response was not observed in male mice until 7-days post-exposure. Female mice also showed an increase in IL-4 and IL-5 cytokines at 7 day post MWCNT exposure (19). Studies indicate females may be less efficient at muco-ciliary clearance of pathogens as a result of lung anatomy and/or sex hormone-mediated regulation of epithelial cell function (100–102). Human female lungs are smaller compared to male lungs which correlate to a smaller number of alveoli (103). An increase in phagocytic cell migration was observed in the airway of female animals (19). This exaggerated TH2 type response observed in the females is consistent with other studies researching allergic-type inflammation.

Cai *et al.* found that an intraperitoneal injection of LPS at a dose of 1.5 mg/kg led to an increased sickness response in male CD1 mice compared to female mice of the same age. (Sickness symptoms = presence of 4 symptoms: huddling, piloerection, ptosis, and lethargy) (104). Sickness duration was increased in male mice. Male adult mice also displayed a significant decrease in body temperature compared to females of the same age. Male mice showed significantly more TNF-α compared to females. These cytokines were measured in the blood 10 hours following LPS injection. However, there was no significant difference between females and males for the cytokines IL-1B, IL-6, IL-10, IL-12, and IFNγ (104).The observation of increased TNF-α in male mice compared to female mice was correlated in a study by Mabley *et*

al. who used an intraperitoneal injection of 1 mg/kg intraperitoneally and measured after 90 minutes. This study also noted males produced more MIP-1 vs females (105). A study by Kuo *et al.* also observed an increase in TNF-α in male mice compared to female mice, along with an increase of IL-6 in male mice (106) This study concluded the proinflammatory cytokine response may be dose and time dependent. There was no sex difference observed in either TNF-α or IL-6 1 hour after LPS injection but there was at 3 hours post injection. These observations were made 3 hours following IP injection. This study used a range of LPS concentrations of 0.02 μg/g body weight to 5 μg/g body weight. These studies also noted that the difference in proinflammatory cytokines between the sexes is likely influenced by the sex hormones as the differences were not observed in young (<6 weeks old) mice.

A study conducted by Wichmann *et al.* found that testosterone can decrease macrophage function leading to suppression of the immune response (107). Reversely, estrogen can enhance the immune system by stimulating lymphocytes into maturity and increasing the production of antibodies (108)(109) which leads to a more vigorous cellular and humoral response in females that leads to a quicker recovery in females than males (110)(111). There may also be a sex differential component in the expression of TLR2 and TLR4 (112)(113). Endotoxin-challenged mononuclear cells from male humans demonstrated higher TLR4 expression while splenic lymphocytes from female mice infected with a virus showed higher TLR2 expression (112)(113). However, these differences in TLR4 and TLR2 expression may be due to the inflammatory response to a virus compared to a bacterium and not due to a difference between the sexes.

These results, along with the knowledge that male and female sex hormones can influence the timeframe in which different inflammatory mediators are resolved, may provide insight into why different studies have observed conflicting results in regard to the sex differential response to inflammatory stimuli and release of various inflammatory mediators.

Currently there is limited data on how the inflammatory response to pesticide exposures or other agriculture exposures and co-exposures may differ between the sexes in regard to lung inflammation.

1.8 Techniques for Studying Inflammation

1.8.1 Plethysmography

In addition to monitoring inflammatory mediators (those discussed in 1.3), various respiratory parameters can add meaningful information in the study of inflammation. Measures such as forced expiratory volume or total lung capacity can be obtained via spirometry or plethysmography in humans but may not be as useful in characterizing respiratory diseases in mice models (114). Although invasive measures such as ventilators to measure pulmonary function and lung physiological parameters in mice models are very sensitive and specific, there are limitations to this invasive technique that may encourage researchers to choose the noninvasive approach of whole body plethysmography (114). Along with requiring an experienced individual to run the ventilator, invasive methods are time consuming and once the lung mechanism measurements are obtained, the mice are dead and unavailable for follow up tests (114). Conversely, whole body plethysmography is a noninvasive method where several mice may be monitored simultaneously and parameters such as respiratory frequency, tidal volume, peak flows, and enhanced pause (PenH) can be recorded. Additionally, due to the noninvasive nature of WBP, these mice can then be used for further experiments (114). Enhanced pause values, or PenH, allow for measuring an animal's airway hyperresponsiveness (AHR) and was first described by Hamelmann *et al.* (115). They discovered that increased AHR following methacholine challenge correlated with IgE serum level increase, eosinophil lung infiltration increase along with increased lung resistance determined by the use of an invasive method (115). Along with PenH, WBP can record respiratory parameters such as the frequency of breaths per minute; the minute volume (the amount of air inhaled in one minute), and the estimated peak expiratory flow rate (PEFB) a parameter often used to quantify asthma severity (114).

According to DeLorme and Moss, single or double chamber plethysmography are useful apparatus for the objective measure of physiologic changes in the airways depending on the specific objectives and data of the project (116). Single chamber plethysmography gives more accurate analysis of airway reactivity while double chamber plethysmograph provides more accurate physiological data (i.e. tidal volume and respiratory rate) (116).

1.8.2 Multiple-image radiography (MIR)

Multiple-image radiography (MIR) is the acquisition of multiple images utilizing synchrotron x-rays and refraction of x-rays at the interface of tissue and air that generates contrast to quantify lung optical properties (117). This method is a variation of diffraction enhanced imaging (DEI) and can be used, for example, to quantify lung optical properties of acute lung injury following LPS instillation (117). MIR has the capability to produce more accurate images compared to DEI (118). Due to the high number of air-tissue interfaces present in the lungs, they are an excellent candidate for MIR imaging (117) where it is possible for the investigator to make subtle assessments of the lungs following exposure to agents such as LPS and/or glyphosate. In a study utilizing MIR to evaluate changes in the lungs over a period of time following LPS exposure in male C57BL/6 mice, it was found that refraction and scatter were reduced following LPS exposure which represented increase in fluid buildup, or higher levels of edema in the lungs (119). MIR provides quantitative information on the status of the lungs following acute lung injury (117) and is a non-invasive technique that could be utilized to study pulmonary pathologies.

1.9 Summary & Gaps in Current Literature

The overall goal of this project was to study the sex differential inflammatory effects of a short-term exposure to LPS plus glyphosate in C57BL/6 mice. The inflammatory potential of exposure to glyphosate in addition to LPS is still being unraveled. While Pandher *et al.* worked to establish the respiratory inflammatory potential of exposure to LPS plus glyphosate over various time points (22), that work was conducted in male mice. It is yet to be established how this common agriculture exposure may alter the respiratory inflammatory effects in females. Pandher *et al*. measured biological indicators of inflammation following repeated exposure to LPS plus glyphosate such as cytokines important to the innate and adaptive immune response (22), but how these inflammatory mediators respond to the LPS plus glyphosate exposure in female mice has not yet been established. Proinflammatory cytokines such as TNF-α and IL-6 have been previously measured following exposure to a low dose of LPS (40,120), but have not

yet been measured after a repeated low dose of LPS plus glyphosate in female mice. Furthermore, it is not known if there is a sex differential inflammatory response to these exposures, thus we evaluated the inflammatory effects of a 5-day co-exposure to LPS plus glyphosate in female mice and then compared the female response to results obtained from the same exposure in male mice. Finally, this study aims to establish that multiple image radiography (MIR) is an appropriate way to measure inflammatory changes to lung structure following these agriculture exposures, and how this technique compares to results obtained by other assays. To do this, we compared differences in physiological markers (using whole body plethysmography) and structural measures (using multiple image radiography) in female mice following 5-day exposures to LPS, glyphosate, or LPS plus glyphosate.

Chapter 2: Hypotheses and Objectives

Objective 1: To determine if in female mice the inflammatory effects from a repeated exposure to lipopolysaccharide (LPS) and glyphosate is significantly different compared to an exposure to LPS or glyphosate alone.

Research Hypothesis 1: Glyphosate will interact with LPS to induce molecular and respiratory inflammatory effects that are significantly different compared to each single exposure in female mice.

Objective 2: To determine if LPS plus glyphosate has additive or synergistic respiratory inflammatory effects that differ between the sexes.

Research Hypothesis 2: Interactions between LPS and glyphosate will induce molecular respiratory inflammatory expressions that are significantly different between males and females.

Objective 3: To observe if there are differences in physiological markers (using whole body plethysmography and histology) and structural measures (using multiple image radiography) in female mice exposed to LPS plus glyphosate.

Research Hypothesis 3: Glyphosate will interact with LPS to induce physiological and structural respiratory inflammatory effects that are significantly different compared to each single exposure in female mice.

Chapter 3: Lung Inflammation after Repeated Exposure to LPS and Glyphosate in Female Mice

3.1 Abstract

Agricultural industries are rich with airborne pollutants including dust and pesticides. Workers in the agriculture industry are at risk for developing respiratory effects from work exposures. Very little is known of the effects of agricultural exposures on females. It is unclear if exposure to glyphosate (an herbicide prevalent in crop production) causes lung inflammation from inhalation. Furthermore, agriculture workers are exposed to a mixture of pollutants, not just a single agent. Little data exists on the inflammatory potential of mixed exposures common in agriculture, in particular, lipopolysaccharide (LPS), a component of grain dust, and glyphosate. The objective of this work was to understand the inflammatory potential of single and combined exposures to glyphosate and LPS in female mice.

C57BL/6 female mice were intranasally treated with glyphosate (1µg), LPS (0.5µg), combined LPS + glyphosate (LPS: 0.5 µg + glyphosate: 1µg), or HBSS for 5 days. Following treatments, mice were euthanized and bronchoalveolar lavage fluid (BAL) and lung tissue were collected. BAL was collected and analyzed for cellular changes. Lung tissues were stained to observe structural changes.

Mice exposed to both LPS and glyphosate for 5 days had significantly higher airway restriction compared to the group treated with only glyphosate, and the control group. The pro-inflammatory cytokines/chemokines TNF-α, KC, IL-6, MCP-1, and MIP-2 were significantly higher in combined LPS and glyphosate treated mice compared to those treated with either glyphosate alone, or controls, but not different from the LPS only group. Levels of myeloperoxidase expression was significantly higher in mice treated with LPS or LPS plus glyphosate, compared to other treatment groups. Histology revealed greater recruitment of cells into alveolar regions in the lungs of the mice from the combined LPS and glyphosate treated group as compared to other treatments.

Female mice, exposed to the combination of LPS and glyphosate for 5 days showed an increase in inflammatory mediators compared to control animals, or those treated with only

glyphosate. These observations were a result of LPS driving the inflammatory response by interacting with glyphosate in the combined exposure.

3.2 Background

The evidence is mounting that workers in the agriculture industry are at risk for developing deleterious respiratory effects from workplace exposures including grain dust and pesticides. Glyphosate; an herbicide with prevalent use in agricultural, may affect the lungs. Agriculture workers are commonly exposed to more than one pollutant at a time; if there is an interaction between glyphosate and lipopolysaccharide (a component of grain dust) and how this interaction contributes to respiratory inflammation has yet to be determined. This study is the first to study the potential additive and/or synergistic inflammatory effects of LPS and glyphosate in female mice.

Lipopolysaccharides (LPS), also known as endotoxins, are a class of substances that are a major component of the outer (32) surface membrane of Gram-negative bacteria (33). A low dose of LPS is a standard method to evaluate the innate immune response during inflammation or to model acute lung injury (37) that induces a well characterized increase in cytokines. For example, TNF-α, IL-1ß, IL-6, and IL-10 may be measured along with other indicators of inflammation such as levels of leukocytes, C-reactive protein, heat shock proteins or LPS-binding proteins (37). Some studies indicate LPS may act as an adjuvant and enhance the immune response when the immune system is exposed to other antigens such as pesticides (32). There is mounting evidence that exposures to pesticides, in combination with LPS, produces an inflammatory response that is significantly increased compared to the response measured from exposure to the pesticide alone (32)(121).

Worldwide, glyphosate-based herbicides (GBHs), such as Roundup, are the most commonly used herbicides (47). Glyphosate is an herbicide used ubiquitously in both industrialized and developing countries (49). In Canada, there are 183 products on the market that contain glyphosate (63). Glyphosate, an herbicide intimately linked with canola production, has been shown to have a significant association with increased rhinitis among pesticide applicators (62). Those exposed to glyphosate report nose and throat irritation; however, there

is not yet any evidence that exposure to glyphosate is linked to an increase in asthma incidence (55).

There is limited data available on the in vivo inflammatory effects of glyphosate. Kumar *et al.* used aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) to collect glyphosate samples over a 24 hour period during spray season at 3 farms in Ohio. The glyphosate residues were extracted from suspensions eluted from the filters and glyphosate levels were determined to be at an average airborne concentration of 22.59 ng/m^3 (70). Kumar *et al.* also extracted endotoxin from the samplers to determine an average airborne concentration of 4.87 EU/m^3. For intranasal instillation of glyphosate, female mice were given either aerosolized glyphosate from one of the farm samplers used (8.66 μg/ml in 30 μl of PBS) daily for 7 days, or reagent grade glyphosate at 100 ng, 1 μg or 10 μg for 7 days (70). The results of this study found female mice exposed to either glyphosate-rich air samples or reagent grade glyphosate experienced an increase in cell count in lung and bronchial alveolar lavage, an increase in eosinophil and neutrophils, and increased production of IL-33, TSLP, IL-13 and IL-5 (70).

Previous studies looked at a glyphosate exposure in combination with LPS over a 1, 5, or 10-day period in male mice to measure the respiratory inflammatory response. An intranasal exposure at an agriculturally relevant concentration of glyphosate (1 μg/40 μl) resulted in a higher number of neutrophils and increased expression of IL-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid; and higher eosinophil peroxidase levels present in lung tissue compared to control animals (22). Additionally, lung immunohistochemistry showed repeated exposure to glyphosate results in leukocyte infiltration and increased expression of adhesion molecules ICAM-1 and VCAM-1 in the peribronchiolar, perivascular, and alveolar regions (22).

As the study mentioned above, and much of the current literature to date, focuses on exposure effects of male mice; this study will focus on the inflammatory effects on female mice exposed to common agricultural exposures: LPS and glyphosate.

Objective: To determine if in female mice the inflammatory effects from a repeated exposure to lipopolysaccharide (LPS) and glyphosate is significantly different compared to an exposure to LPS or glyphosate alone.

Research Hypothesis**:** Glyphosate will interact with LPS to induce molecular and respiratory inflammatory effects that are significantly different compared to each single exposure in female mice.

3.3 Materials and Methods

Animals

C57BL/6 female mice (Charles River Laboratory, Quebec, Canada) aged 8-10 weeks were used. The mice were housed in ventilated cages in the Laboratory Animal Services Unit (LASU) at the University of Saskatchewan where commercial rodent chow and water was available *ad libitum*. The animals were acclimatized to the whole-body plethysmography (WBP) chambers (Buxco FinePointe Whole Body Plethysmography 4-site system; Data Sciences International, Minneapolis, MN) for 20 minutes per day at least twice prior to the start of treatments. Experiment protocols were approved by the Animal Ethics Research Board of the University of Saskatchewan (AUP 20190017). All animal work was performed in accordance with the Canadian Council on Animal Care guidelines.

Study Design

Twenty mice were randomly divided into 4 groups (n=5/group). Nasal instillation was used to administer either LPS, glyphosate, LPS + glyphosate (LG), or Hank's Balanced Salt Solution (HBSS) (control). The control group received 40 μl of HBSS (Life Technologies, Grand Island, NY, without calcium, pH 7.4). The LPS group received LPS in HBSS (0.5 μg/40μl) (1 mg/ml; *E. coli* serotype 0111:B4, Sigma, St Louis, MO). The glyphosate group received GLY in HBSS (1 μg/40 μl) (0.85 M; analytical grade PESTANAL standard, Sigma, St Louis, MO). The LG group received both LPS and glyphosate in HBSS (0.5 μg LPS + 1 μg glyphosate/40 μl). Mice were lightly anesthetized using isoflurane and treated intranasally with 40 μl of the treatment

solution. Treatments were given daily for 5 days to mimic a typical work week. Following each exposure, mice were allowed to recover from anesthesia in a separate container before being placed back in their respective cages. After exposure on the 5th day, mice were placed back in the cages for 4 hours. Following the four-hour period, mice were placed in the WBP chambers for 20 minutes to collect respiratory data. Once WBP analysis was complete, mice were euthanized and blood, BAL, and lung tissue were collected and analyzed (Figure 3.1).

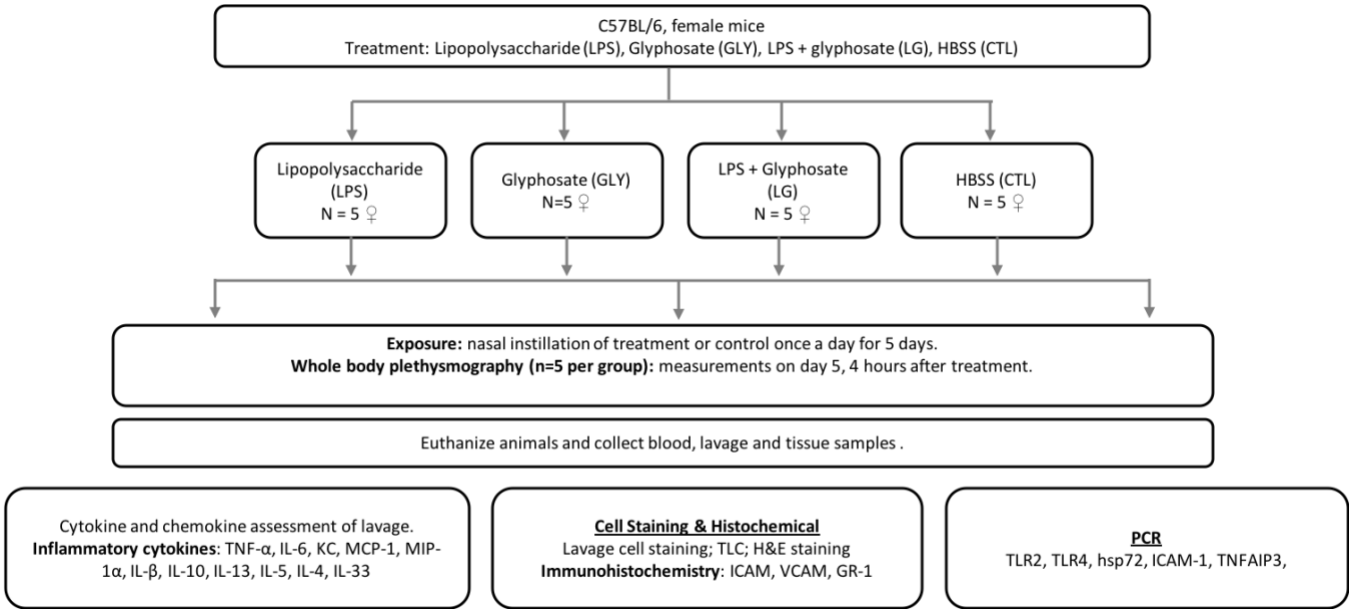

Figure 3.1: Experimental Design

Sample Collection and Analysis

Whole Body Plethysmography

Following treatment on day 5, four hours after treatment, mice were placed into whole body plethysmography chambers to monitor the respiratory changes according to the manufacturer's protocol. Mice were placed into calibrated chambers and respiratory measures (enhanced pause {PenH}, average frequency of breath, minute volume, and peak expiratory volume) were recorded every 2 seconds for 20 minutes; these results were averaged per

animal. Animals were continuously monitored for any indications of pain or distress. Data collection was done using FinePointe software (Data Sciences International, Wilmington, NC).

Blood and Hormone Analysis

The blood was collected from mice via cardiac puncture. Whole blood with EDTA was stored in -80°C until further analysis. Blood samples were sent to Eve Technologies (Calgary, AB) for quantification of estradiol, testosterone, cortisol, progesterone, triiodothyronine (T3), and thyroxine (T4) using the steroid/thyroid hormone 6-plex discovery assay.

Bronchoalveolar Lavage

The bronchoalveolar lavage (BAL) fluid was collected by washing the lungs three times with 0.5 mL HBSS. BAL fluid was centrifuged at 1000 x g for 10 minutes at 4^0C and the supernatant was stored at 80^0C for future analysis. The cell pellet was resuspended in HBSS and kept on ice. Total leukocyte counts (TLC) were measured using a hemocytometer and expressed as an absolute number. Total protein concentration in BAL fluid was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). The plate was read at 562nm on a BioTek SynergyHT plate reader (BioTek, Winooski, VT).

Lung Tissue Collection

Following BAL fluid collection, the right lung was tied at the primary bronchus, removed, flash-frozen in liquid nitrogen and stored in -80°C for mRNA and protein analysis. The left lung was inflated with 200 µL of 4% paraformaldehyde (PFA), removed and fixed in 4% PFA for 16 hours at 4°C followed by two overnight washes with 70% ethanol. The fixed tissues were processed through a series of alcohols in an Intelsint RVG/1 Histology Vacuum tissue processor (Intelsint; Turin, Italy), followed by embedding in paraffin using a Tissue Tek II tissue embedder (Sakura Finetek; Nagano, Japan).

Eosinophil peroxidase, and Myeloperoxidase Levels

Lung homogenates were prepared using 2 mm Zirconia beads (BioSpec, Bartlesville, OK) in tubes containing 500 µL RIPA Lysis Buffer containing 1X Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA) in a BioSpec Mini-Beadbeater-24 homogenizer (BioSpec, Bartlesville, OK) twice for 2 minutes, with cooling on ice for 5 minutes in between rounds. Myeloperoxidase (MPO) was quantified using a Mouse Myeloperoxidase DuoSet ELISA (R&D Systems, Minneapolis, MN). Eosinophil Peroxidase (EPO) was quantified using a Mouse Eosinophil Peroxidase/EPX ELISA assay (Lifespan Biosciences, Seattle, WA). Plates for MPO and EPO quantification were read at 450 nm on a BioTek SynergyHT plate reader (BioTek, Winooski, VT).

Lung Histology

Sections of 5 µm thickness were cut from the embedded lung tissue using an Microm 350S microtome (Microm, Germany). Tissue sections were stained with Hematoxylin and Eosin according to the Histology Core Facility's protocol (https://healthsciences.usask.ca/facility-services/histology-core-facility.php) and coverslipped using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL USA). These slides were imaged on an Aperio CS2 virtual microscopy system (Leica Biosystems, Concord ON Canada) as well as a bright field microscope equipped with an Infinity 5-5 Microscope camera (Teledyne Lumenera, ON Canada) at 10, 40 and 100x magnification.

Immunohistochemistry

Lung sections were incubated with primary antibodies followed by appropriate secondary antibody. Briefly, lung sections were rehydrated through a series of xylene and descending grades of alcohol. Lung sections were covered with 0.5% hydrogen peroxide in methanol for 20 minutes to quench tissue peroxidase activity. The sections were covered with 2 mg/ml of pepsin in 0.01 N hydrochloric acid (HCl) for 45 minutes to unmask antigens. Next the sections were covered in 1% bovine serum albumin (BSA) for 30 minutes to block non-specific binding sites. The sections were incubated overnight at 4^0 C with the primary antibody in 1X TBS + 1% BSA. The following primary antibodies and concentrations were used:

ICAM-1 (dilution 1:100; rabbit monoclonal anti-mouse ICAM-1, ab79707, Abcam Inc., ON Canada). Following overnight incubation, the sections were covered with anti-rabbit secondary antibody (Agilent Dako, Mississauga, ON). Sections were covered with NovaRed solution (Vector laboratories, Burlington, ON Canada) for 1-5 minutes and then counter-stained with methyl green nuclear stain for 5 seconds. Sections were coverslipped using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL, USA). Von Willebrand Factor (dilution 1:200; rabbit monoclonal anti-mouse vWF, ThermoFisher Scientific, Waltham, MA, USA) was used as a positive control. Additionally, controls of staining without primary antibody, secondary antibody, or both, were completed.

ELISA

Quantification of TNF-α, IL-6, KC, MCP-1, MIP-2, IL-1β IL-10, IL-13, IL-5, IL-4, IL-33 in BAL fluid was determined using a Custom ProcartaPlex Luminex Immunoassay (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's specification for magnetic bead-based ELISAs. Plates were read using a Bioplex 200 system (Bio-Rad, Mississauga, ON) and Bioplex Manager Software (Bio-Rad, Mississauga, ON).

RNA purification, cDNA synthesis and Real-time PCR

RNA was extracted from lung tissue using Qiagen RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA USA) according to manufacturer's instructions for tissue extraction. The concentration of total RNA was quantified for each sample using a Take3 plate and BioTek Synergy HT plate reader (BioTek, Winooski, VT USA). cDNA was generated using iScript Reverse Transcription Supermix (BioRad, Hercules, CA USA) using 0.5 µg mRNA according to the manufacturer's instructions.

Real-time PCR reactions were carried out in duplicate in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA). Expression of mouse TLR2 (Mm00442346_m1), TLR4 (Mm00445273_m1), HSPa1a (Mm01159846_s1) ICAM-1(Mm00516023_m1), and TNFAIP3 (Hs00234713_m1) (Life Technologies, Grand Island, NY USA) were assessed. The reaction was first incubated at 50°C for 2 minutes, at 95°C for 10 minutes, then 40 cycles of 15 seconds at

95°C, then finally 1 minute at 60°C. To determine the relative quantification of each target gene, the ΔΔCt method was used.

Statistical Analysis

GraphPad Prism 9 (Graph-pad Software, San Diego, CA) was used to complete statistical analysis and preparation of graphs. Data are presented as mean + standard deviation (SD). For cytokine levels below the predicted limit of detection a value less than the lowest detected value, or a value equal to half the lowest level of quantification (LLOQ) was used. Statistical significance was determined using one-way ANOVA with a follow-up Tukey test for multiple comparisons. If the assumption of equal variance using the Brown's Forsythe test was not met, a non-parametric Kruskal Wallis (KW) test was used. A p-value of ≤0.05 was considered significant.

3.4 Results

Whole Body Plethysmography

For the parameters of Peak Expiratory Volume and Minute Volume there were no significant differences between any of the treatment groups (Figure 3.2). Enhanced pause (PenH), was significantly higher in the combined (LG) group compared to the glyphosate (GLY) and control (CTL) groups, but not different from the LPS group. The average breath frequency of the LG group was significantly lower compared to the CTL group. There were no significant differences between the LPS and LG groups for any of the WBP parameters.

Hormone Analysis

There were no significant differences between any of the treatment groups for the hormone panel analysis of estradiol, testosterone, cortisol, progesterone, triiodothyronine (T3), and thyroxine (T4) (Figure 3.3).

Leukocyte Counts in Bronchoalveolar Lavage Fluid

There were no significant differences between any of the treatment groups for total leukocyte counts present in the bronchoalveolar lavage fluid (Figure 3.4).

Protein Expression in Lungs

There were no differences in vascular permeability between any of the treatment groups (Figure 3.5A).

For eosinophil peroxidase concentration, the combined treatment group (LG) was significantly lower compared to only the control (CTL) treatment group (Figure 3.5B).

The myeloperoxidase concentration in the LPS and LG groups was significantly higher compared to the myeloperoxidase concentration of the CTL group (Figure 3.5C). Both the LPS and LG groups were also higher compared to the glyphosate treatment group, but not significantly so. There were no significant differences between the LPS and LG groups for either myeloperoxidase or eosinophil peroxidase expression.

Lung Histology

Representative images from treatment groups showed leukocyte infiltration in the perivascular, peribronchial, and alveolar regions of the animals treated with glyphosate and LPS, whereas robust leukocyte infiltration was present in these regions in the combined (LG) treatment group (Figure 3.6). Disruption and thickening to the alveolar epithelium was also present in the LPS and combined treatment groups. Increased thickening of the bronchial epithelium was present in the lungs of the LG group.

ICAM-1 Staining

Images from the treatment groups showed typical ICAM expression in the alveolar regions (Figure 3.7). LPS and combined (LG) treatment groups showed increased ICAM expression in the perivascular region compared to sections from the glyphosate and control animals.

GR-1 Staining

Images from the treatment groups show increased GR-1 expression in the combined (LG) treated animal (Figure 3.8) in the bronchial epithelium compared to sections from the other treatment groups.

Cytokines in BAL

There were no significant differences between any of the treatment groups for the expression of IL-10, IL-13, IL-33, IL-4, and IL-5 (Figure 3.9). The combined (LG) treatment group showed a significantly higher expression of KC, IL-1β, IL-6, MCP-1, MIP-2, and TNF-α compared to the glyphosate (GLY) and control (CTL) groups (Figure 3.9). There was no significant difference between the LG group and the lipopolysaccharide (LPS) group for any of these cytokines.

ICAM, TLR2, TLR4, HSP, and A20 expression in lungs

There was no significant difference between any of the treatment groups for the expression of ICAM, TLR2, TLR4, and HSP (Figure 3.10). The glyphosate (GLY) treatment group showed a significantly higher expression of A20 compared to the combined (LG) treatment group (Figure 3.10E). There was no significant difference between the LPS and LG groups for any of these markers.

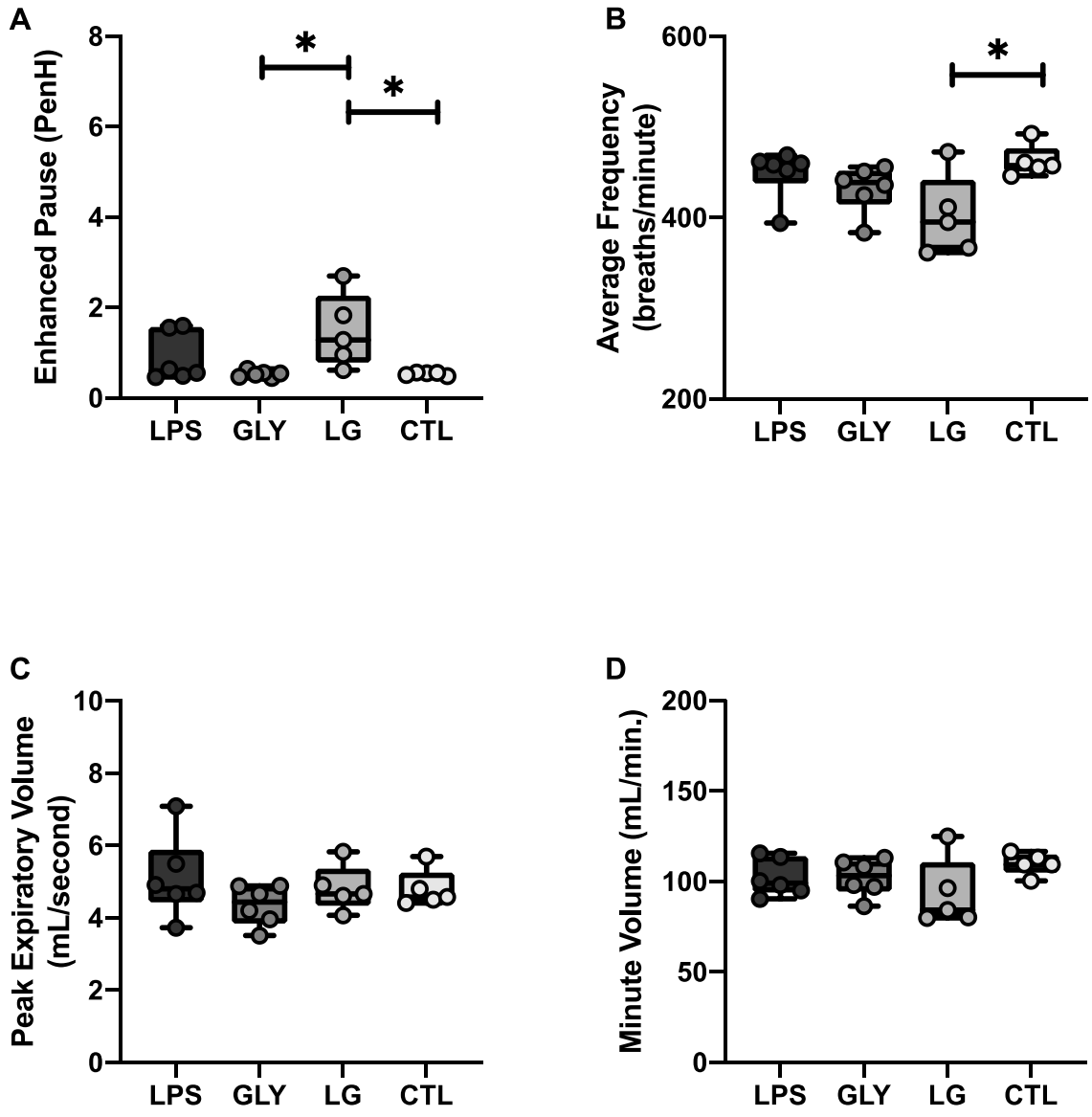

Figure 3.2: Whole body plethysmography (WBP) data presented as mean + SD (n=5-6). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. WBP data was averaged for 20 minutes and presented as mean + SD. * indicates significance level of $p < 0.05$.

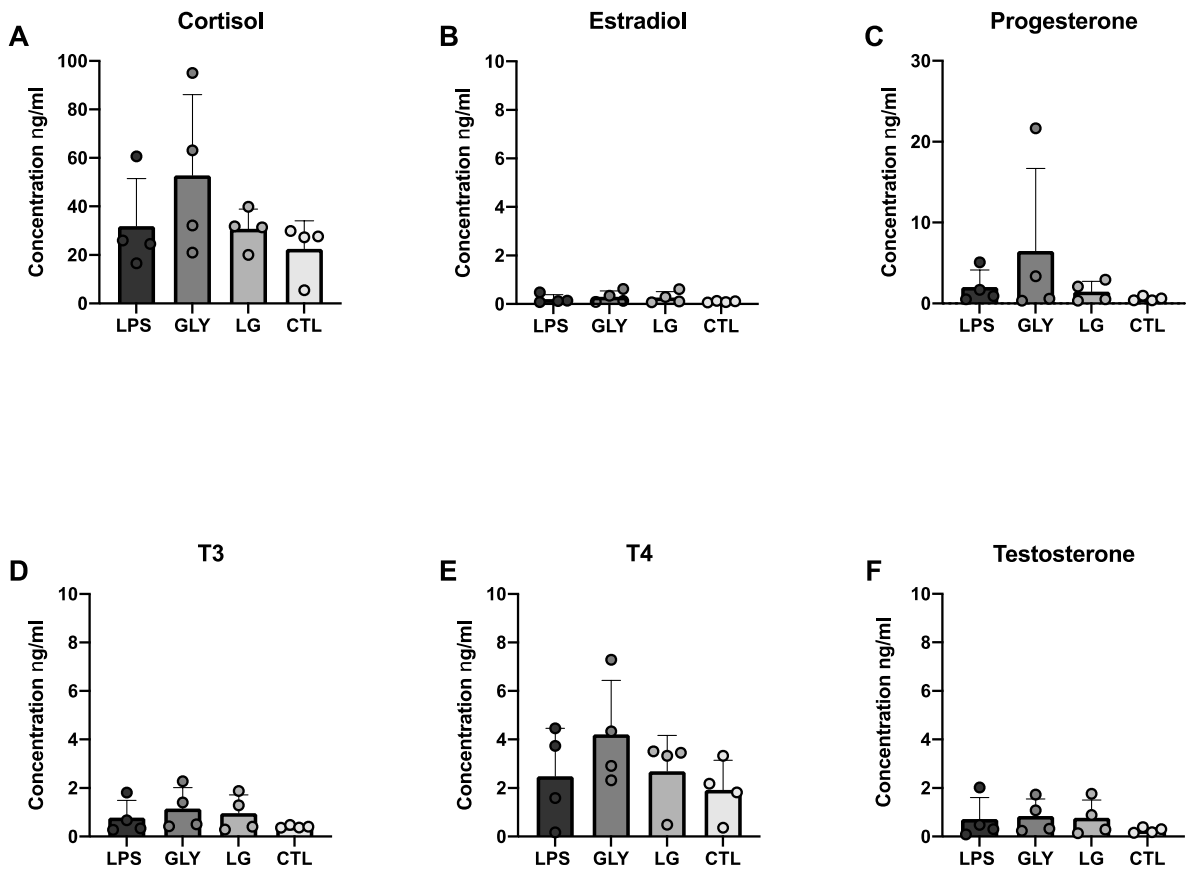

Figure 3.3: Hormone analysis from mouse blood samples. Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5-6). No significant difference was detected between any of the treatment groups.

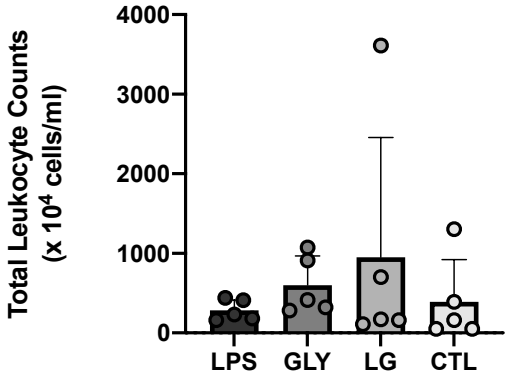

Figure 3.4: Total leukocyte counts present in bronchoalveolar lavage fluid. Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5). No significant difference was detected between any of the treatment groups.

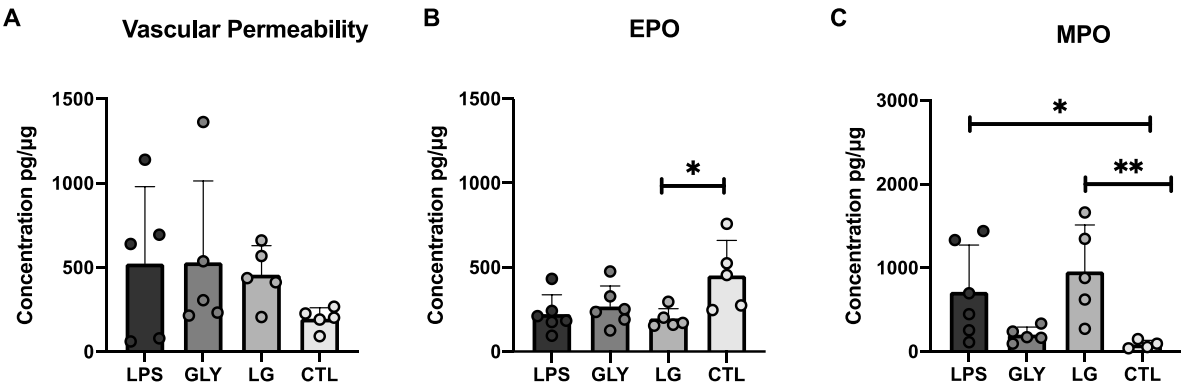

Figure 3.5: Protein concentration data presented as mean + SD (n=5-6). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. * indicates significance level of $p < 0.05$. ** indicates significance level of $p < 0.01$.

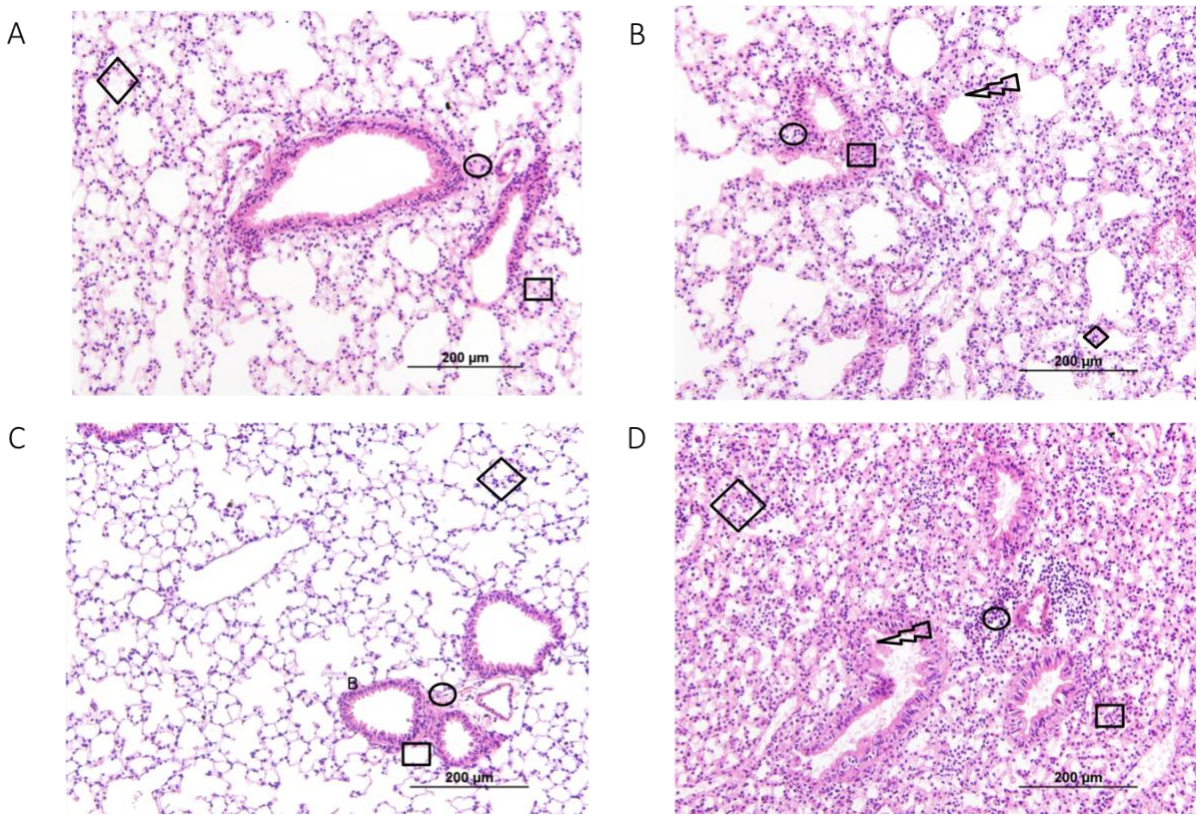

Figure 3.6: H&E staining of female mice lungs of control animals (A) or treated with lipopolysaccharide (B), glyphosate (C), or LPS plus glyphosate (D) for 5 days. Images show leukocyte infiltration in perivascular (square), peribronchiolar (circle), and alveolar (diamond) regions. Lightning bolt – epithelium disruption. Magnification: 40x objective.

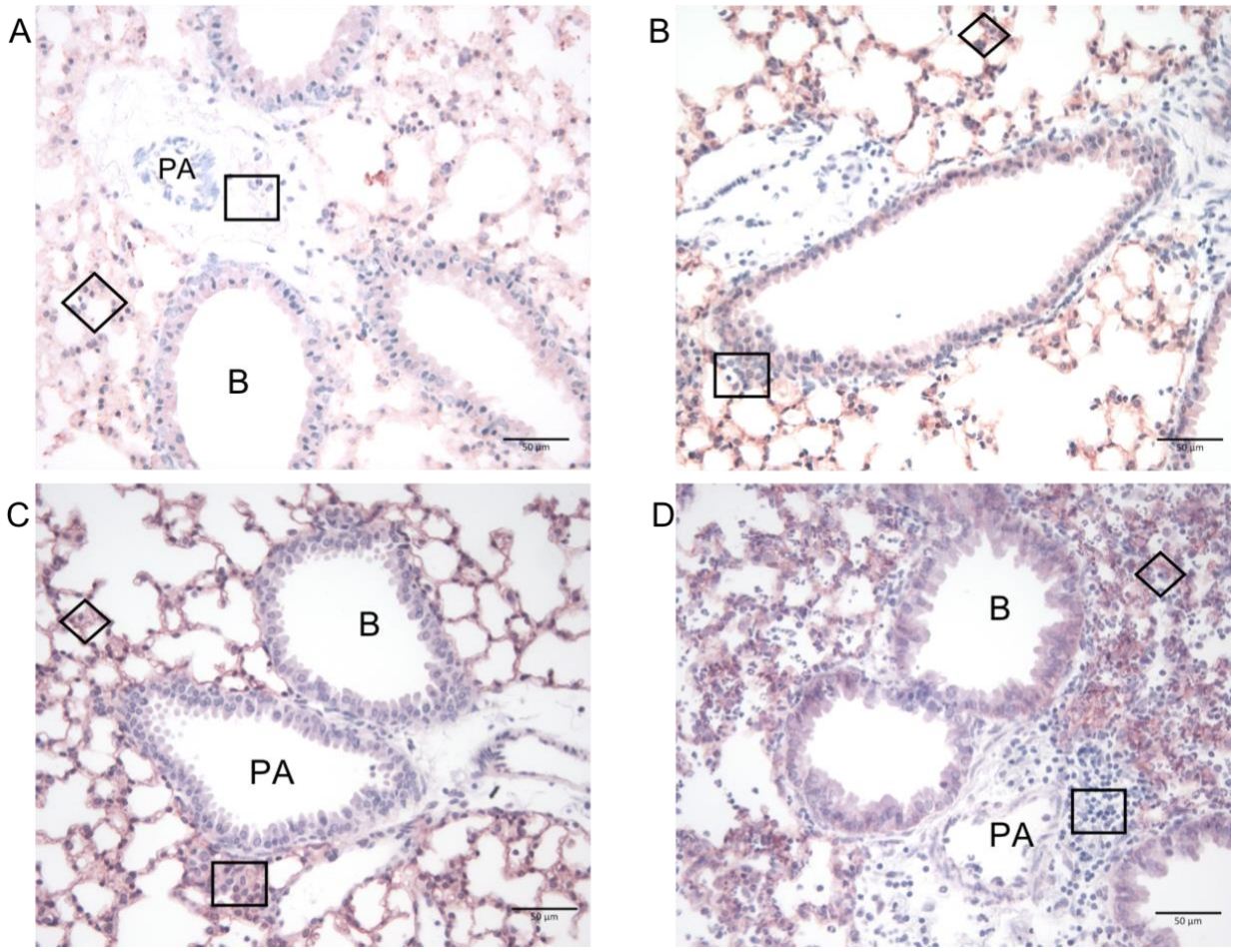

Figure 3.7: ICAM expression in female mice lungs of control animals (A), or treated with lipopolysaccharide (B), glyphosate (C), or LPS plus glyphosate (D) for 5 days. Images show ICAM expression in perivascular (square) and alveolar (diamond) regions. Magnification: 40x objective. B – bronchus; PA – Pulmonary Artery

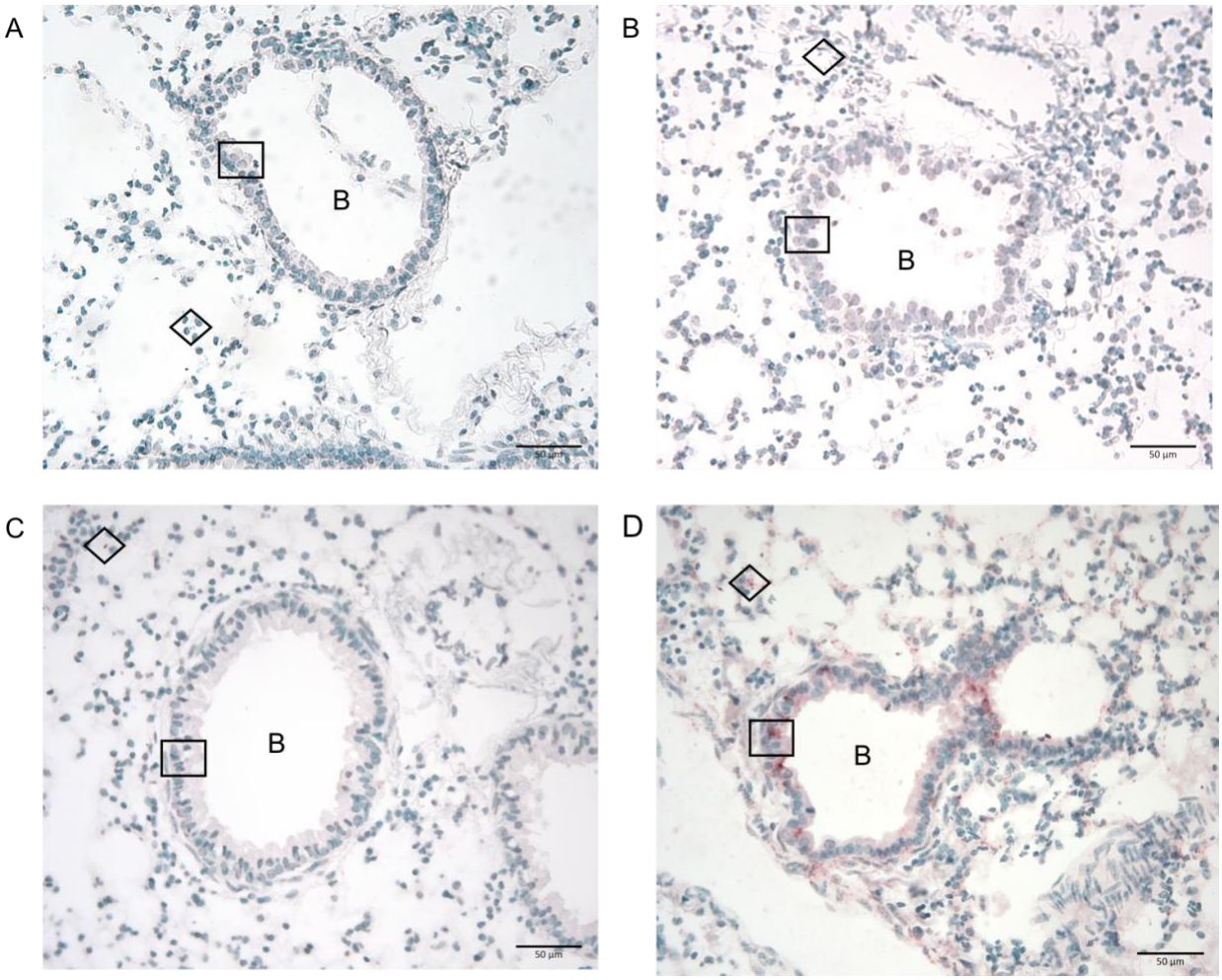

Figure 3.8: GR-1 (neutrophil marker) expression in female mice lungs of control animals (A) or treated with lipopolysaccharide (B), glyphosate (C), or LPS plus glyphosate (D) for 5 days. Images show expression in bronchial epithelium (square) and alveolar (diamond) regions. Magnification: 40x objective. B – bronchus

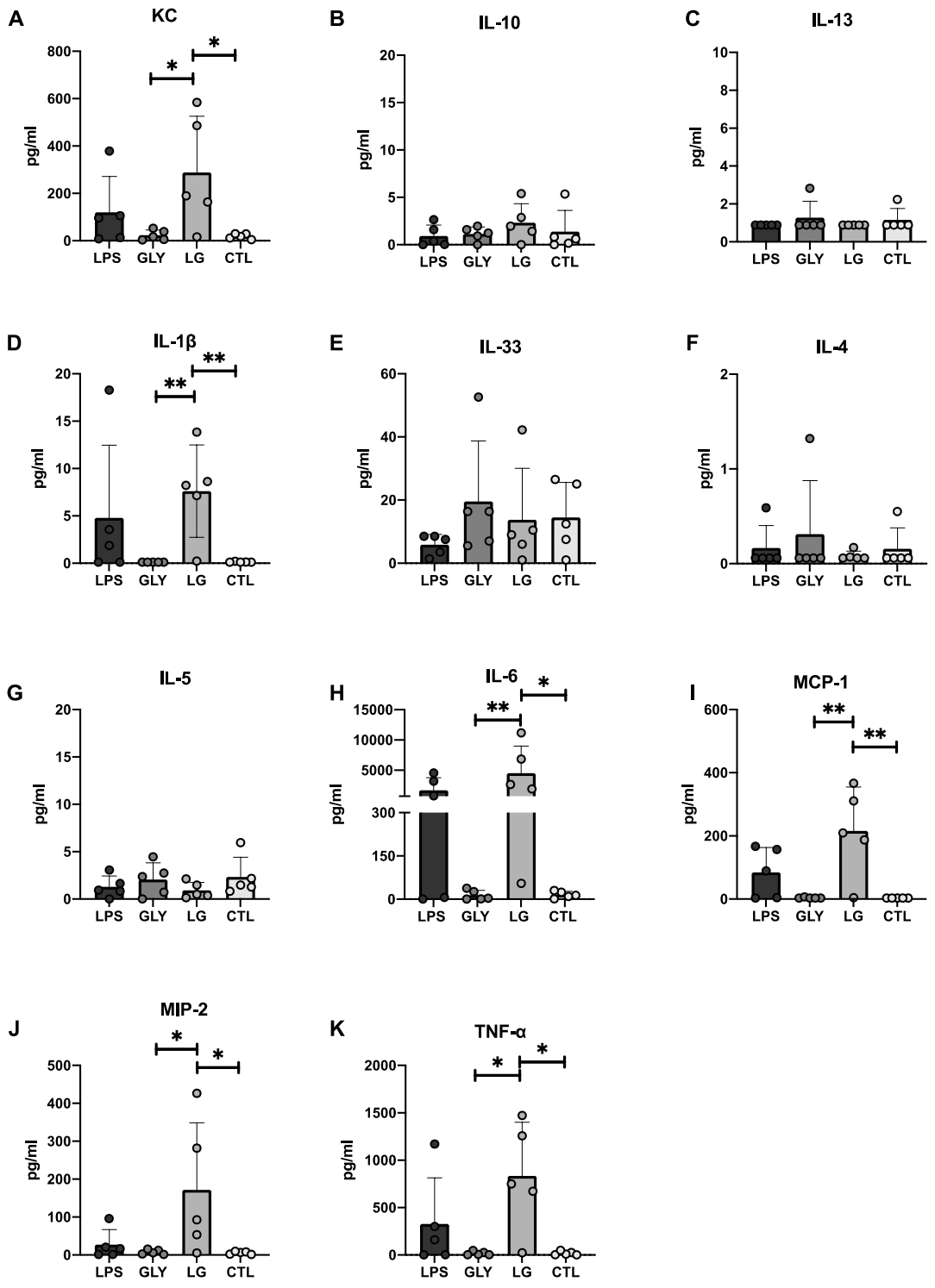

Figure 3.9: Cytokine levels (pg/ml) in bronchoalveolar lavage fluid. Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5). * indicates significance level of $p < 0.05$. ** indicates significance level of $p < 0.01$.

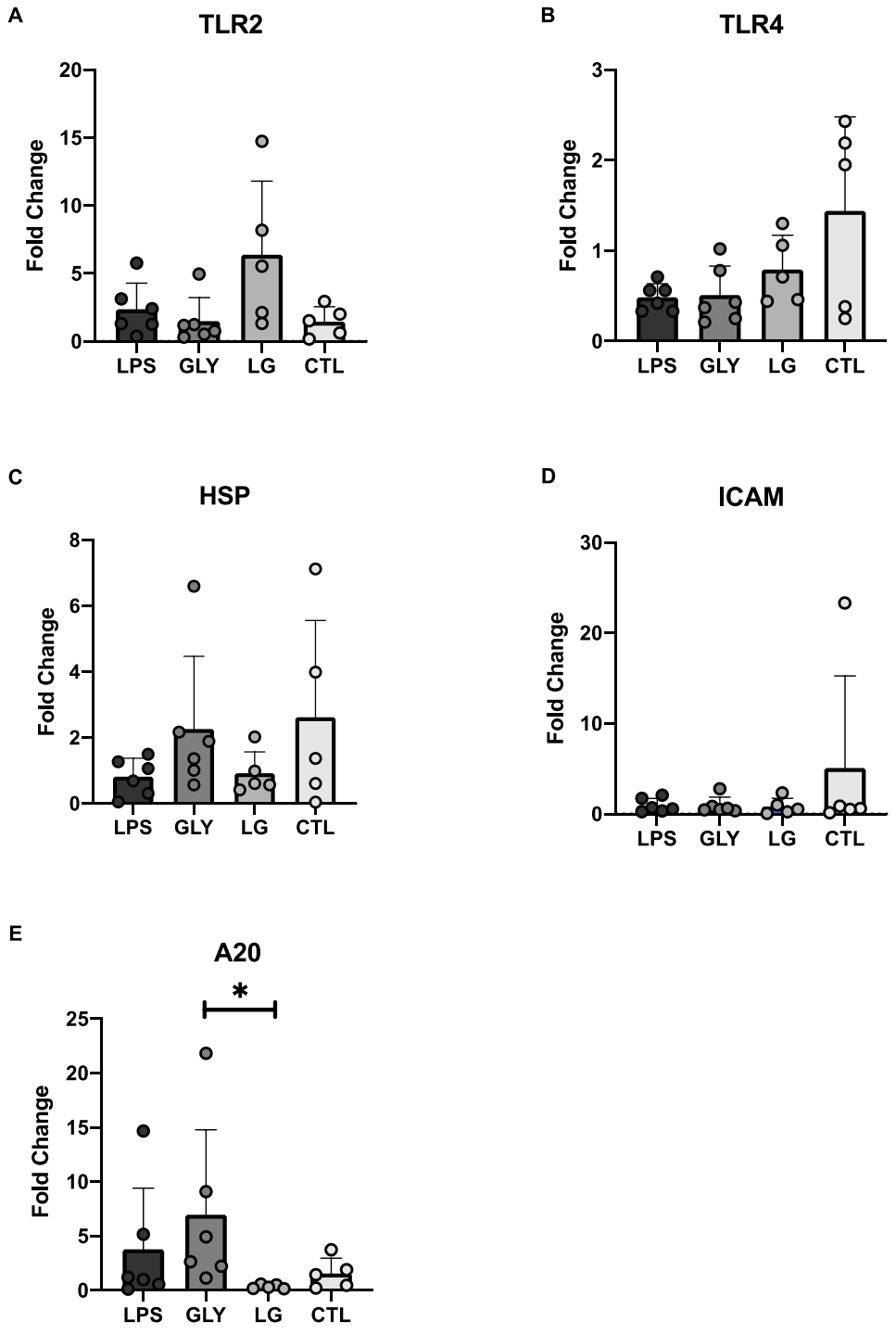

Figure 3.10: Real time PCR of ICAM, TLR2, TLR4, HSP, and A20 from mice lung tissue presented as fold changes. Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5-6). * indicates significance level of $p < 0.05$.

3.5 Discussion

The female inflammatory respiratory response to common agricultural exposures such as LPS and glyphosate has yet to be determined. Female workers are prevalent in the agriculture industry and while it is known that the female inflammatory response differs compared to the male response (81), the research to date has been primarily concerned with that of the male response to agricultural exposures. This is the first study which evaluates the inflammatory response of female mice exposed to a combination of LPS and glyphosate and revealed female mice exposed to a combined exposure demonstrated an increase in inflammatory mediators compared to control animals, or those treated with only glyphosate. The results show that in female mice LPS is driving the inflammatory response and the addition of another inflammatory agent, glyphosate, impacts but does not have a robust alteration to inflammatory effects after 5 days of exposure.

The WBP results show mice that received LPS and LPS plus glyphosate had higher PenH compared to the other groups. Whole body plethysmography (WBP) is a noninvasive technique to evaluate various respiratory parameters such as respiratory frequency, tidal volume, peak flows, and enhanced pause (PenH). Enhanced pause, or PenH, is one such parameter that is considered to be a marker for airway resistance in the lungs. Airway resistance is a well-known response to LPS in female mice (122). However, the addition of glyphosate to the LPS resulted in higher PenH as compared to the control group, suggesting that the addition of another inflammatory agent resulted in greater airway restriction. In addition, the average breath frequency was significantly lower in the group receiving the combination of LPS and glyphosate as compared to the control group. However, LPS administration has been shown to increase breathing frequency (123). This indicates the addition of the glyphosate caused the combined treatment group to experience more difficulty in maintaining a normal rhythm of breath compared to control animals. Previous studies have also observed decreases in respiratory function following LPS exposure at a dose similar to the one used in our study and used both male and female mice (40). Our findings reinforce that a low dose exposure to LPS can significantly inhibit respiratory function and the addition of glyphosate further compounds these respiratory effects.

The results reveal that myeloperoxidase expression is significantly higher in the LPS and LPS plus glyphosate treated mice. It has well been established that exposure to LPS leads to the recruitment and activation of neutrophils (124). As myeloperoxidase expression is a surrogate marker for the presence of neutrophils, our results corroborate this. The higher levels of neutrophils present in the combined (LPS plus glyphosate) treated animals is also evident in our immunohistochemistry results as there is higher levels of GR-1 expression present in the bronchial epithelium of the combined treatment group. However, TLR4, a ligand well documented to be involved in neutrophil recruitment, was not upregulated in these treatment groups though MPO expression was. Given that these results were after 5 days of exposure, inflammatory resolution may have already began as females are known to respond quickly to LPS (35).

The inflammatory mediator results show that female mice exposed to the combination of LPS and glyphosate could be differentiated from mice exposed only to LPS. There were no significant differences between any of the treatment groups for the expression of ICAM, TLR4, and HSP. However, this is expected given that in female mice inflammatory resolution has been shown within 36 hours after exposure (35), and these were five-day exposures. Of interest is the TLR2 expression, which was higher in the combined treatment group. TLR2 has been shown to be an important PRR in asthma-like inflammatory responses. Furthermore, exposure to the combined treatment resulted in significantly higher expression of proinflammatory cytokines (KC, IL-1β, IL-6, MCP-1, MIP-2, and TNF-α). These cytokines are important proinflammatory mediators and suggest a potentiation in inflammation when glyphosate and LPS are co-exposed. Additionally the H&E and immunohistochemistry results adds additional support of potentiation between LPS and glyphosate as representative slides from this treatment group displayed robust leukocyte infiltration, thickening of the alveolar epithelium and bronchial epithelium, and increased ICAM expression in the perivascular region; these effects were not observed in the animals treated only with LPS. The macrophage inflammatory protein (MIP-2) expression also reveals potentiation in inflammatory response when glyphosate and LPS are combined in an exposure. MIP-2 is a proinflammatory chemokine that attracts macrophages and facilitates the neutrophilic inflammatory response. MIP-2 production from eosinophils has

also been shown to be an important chemokine in allergic lung inflammation (125). To the best of our knowledge, there has been no previous studies investigating how glyphosate and LPS alter the expression of macrophage inflammatory protein. These results add additional evidence to the work of Pandher *et al.*, who found that in male mice the same concentration of LPS plus glyphosate in a 5 day exposure led to an asthma like phenotype that was distinguished by higher neutrophil numbers and higher expression of TLR2 (121).

These results further justify that the combination of glyphosate and LPS is driving an inflammatory response that is different than LPS alone. It has previously been demonstrated that female mice are more easily able to overcome endotoxemia (126). In our study, considering that for many proinflammatory cytokines, the levels remained significantly higher in the LPS plus glyphosate treatment group but not in the LPS alone group, it is possible the presence of glyphosate is interacting with the LPS and prolonging the expression of these proinflammatory cytokines. This indicates the effects from the combined treatment group take longer for the inflammatory system to resolve as compared to single exposures. Based on the various inflammatory mediators measured, the systemic effects of the combined exposure appear to be resolving; however, the increased thickening of the bronchial epithelium evident in the histology results supports the respiratory system and the lung architecture sustained effects from the combined exposure.

Hormone levels in female mice are known to contribute to regulating the inflammatory response (93). In our study there was no difference in hormone expression between any of the treatment groups. Exposure to pesticides is often associated to hormone disruption. As no significant differences between any of the treatment groups for the hormone panel analysis of estradiol, testosterone, cortisol, progesterone, triiodothyronine (T3), and thyroxine (T4) (Figure 2.2) were observed; this adds confidence to our study design as the effects we observed in the respiratory system were not a result of the agents we used impacting the endocrine system. As hormone levels are known to fluctuate throughout lifetime, especially in females, another study evaluating if the results observed in our 6-8 week old female mice changes during different time periods (such as during pregnancy or post menopause) would add considerable understanding to this area of study.

The sample size of 5 per treatment group was used as it was the smallest number we could use to be able to discern significant differences between treatment groups while still minimizing the number of animals used in our study. Increasing the sample size of each treatment group would increase the power and robustness of our statistical analysis and would further justify that the results we observed were a real phenomenon. In this study the concentrations of LPS and glyphosate used are based on those found in the real world adding validity to our study. The concentration of glyphosate used is one that has been used in previous studies in the same strain of mouse making for ease of comparison between results.

A difficulty we encountered was that differential cell counts (DLC) were not able to be performed due to issues with the protocol used. As the results from our total cell count did not show any differences between treatment groups, having the DLCs may have provided valuable insight as to whether specific cell populations were driving the differences between treatment groups observed in other inflammatory mediators. Based on the myeloperoxidase expression results it is likely there would be higher neutrophil counts for the combined treatment LPS treatment group.

3.6 Conclusion

This research is the first to show how exposure to two common agricultural respiratory contaminants, LPS and glyphosate, impacts the lungs of female mice following a 5 day exposure. Female mice, exposed to LPS and glyphosate for 5 days showed higher levels of inflammatory mediators compared to control animals, or those treated with only glyphosate. While the effects observed in the combined treatment group compared to the animals exposed to just LPS were not significantly different, inflammatory mediators such as proinflammatory cytokines were elevated in LPS plus glyphosate group, indicating that while female mice are able to ameliorate the effects of LPS after 5 days, the addition of the glyphosate molecule makes it more difficult for the animals to return to baseline levels such as those observed in the LPS only treated animals.

CHAPTER 4: Differences in Inflammatory Response Between the Sexes Following an Agriculture Respiratory Exposure

4.1 Abstract

There is increasing evidence that workers in the agriculture industry are at risk for developing deleterious respiratory effects from workplace exposures. Common exposures include dust containing high levels of lipopolysaccharide, and pesticides, commonly glyphosate. Dust exposure has been well studied in this industry. Agriculture workers are commonly exposed to more than one pollutant at a time, exposure to the combination of dust and pesticides being very common. Studies more commonly describe the response of males to agricultural exposures. Very little is known about the female inflammatory response to common agricultural exposures and no studies to-date have evaluated the sex differential responses to the combined exposure to common agricultural exposures of lipopolysaccharide and glyphosate.

C57BL/6 female and male mice were intranasally treated with glyphosate (1µg), LPS (0.5µg), combined glyphosate + LPS (glyphosate: 1µg + LPS: 0.5µg), or HBSS for 5 days. Following treatments, mice were euthanized and bronchoalveolar lavage fluid (BAL) and lung tissue were collected. BAL was collected and analyzed for cellular changes. Lung tissues were stained to observe structural changes.

Mice exposed to both LPS and glyphosate daily for 5 days showed a significantly different respiratory inflammatory response between males and females. The males showed higher levels of inflammatory mediators including proinflammatory cytokines, myeloperoxidase, TLR2, TLR4, and ICAM-1 expression compared to female mice. Histology revealed male mice have a much more robust leukocyte infiltration as compared to female mice exposed to the combination of LPS and glyphosate after a 5-day exposure.

This is the first study to elucidate the differences in inflammatory mediators between the sexes following a short-term agriculture respiratory exposure to lipopolysaccharide and glyphosate.

4.2 Background

It has been previously established that agriculture workers are exposed to numerous potential pollutants, including grain dust and pesticides. The majority of the research to-date has focused on the respiratory and inflammatory effects in male animals and workers. Female workers make up approximately 25% of the agricultural working population. Very little is known about the female response to common agricultural exposures. Agriculture workers are commonly exposed to more than one pollutant at a time; how the interaction between glyphosate and lipopolysaccharide (a component of grain dust) will differentially affect the sexes is not known. This study is the first to evaluate sex differential inflammatory responses from exposure to common agricultural components LPS and glyphosate.

Evidence demonstrates that across species, females have better outcomes in surviving acute infections arising from a variety of pathogens (bacteria, viruses, trauma) compared to males (127)(128). It has been demonstrated that females are more efficient and more likely to recover from sepsis (126). An efficient innate immune response is comprised of quickly clearing the pathogen and avoiding damage to tissues that can arise as a result of the body being in a prolonged state of inflammation. Conversely, increased efficiency mean females are more predisposed to developing autoimmune diseases such as lupus or rheumatoid arthritis (129).

Females mount a more robust early immune response leading to improved prognosis (126). These improved outcomes in females may be due to sex differences in immune cells leading to increased efficiency in innate immunity (127) and cell mediated immune responses. Female mice and rats possess higher numbers of mononuclear leukocytes present in the pleural and peritoneal cavities (128) and macrophages derived from female mice demonstrate increased phagocytic activity (128). In a mouse model of streptococcal peritonitis, female mice expressed lower levels of proinflammatory cytokines, lower levels of neutrophils, and better control of the infection (128). Additionally, female mice possess double the level of resident CD4 T cells compared to males (128).

Evidence suggests the presence of estrogen in females offers a protective effect against mounting an exaggerated innate immune response (93). It has also been proposed that females mount a TH2 type response (19) suggesting that estrogen enhances a TH2 response while

testosterone promotes a TH1 response (19). However, conflicting studies suggest that estrogen will actually enhance the response of the immune system by stimulating lymphocytes into maturity and increasing the production of antibodies (108)(109).

Lipopolysaccharides (LPS), also known as endotoxins, are a class of substances that are a major component of the outer (32) surface membrane of Gram-negative bacteria (33). LPS is an ubiquitous component of agricultural environments where it is present in high concentrations and has been shown to be a powerful inflammatory agent (33,124). Previous studies have shown that C57BL/6 male mice, when exposed to oropharyngeal LPS, mount a greater immune response compared to female mice characterized by an increase of cells in the bronchoalveolar lavage (BAL) fluid, higher histological scores for peribronchial and perivascular cell influx, and higher levels of TNF-α 6 hours post exposure (40).

Agriculture workers are seldom exposed to just one irritant; thus, the goal of this study is exploring how a combination of molecules may alter the inflammatory response. Worldwide, glyphosate-based herbicides (GBHs) are the most commonly used herbicides (47). Glyphosate is an herbicide used ubiquitously in both industrialized and developing countries (49). Glyphosate has been shown to have a significant association with increased rhinitis among pesticide applicators (62). Currently, there has been minimal work done to evaluate how a respiratory glyphosate exposure may differentially impact the sexes.

Work done by Kumar *et al.* and by Pandher *et al.* have previously described how glyphosate exposure alters the respiratory inflammatory response (22,70). Additionally, Pandher *et al.* were the first to unravel the inflammatory potential of an exposure to LPS plus glyphosate in male mice. However, no work has yet been done on evaluating the differences between male and female mice exposed to both LPS plus glyphosate. This is the first study to evaluate the differences in the inflammatory respiratory response between the sexes following a short-term agriculture respiratory exposure.

Objective**:** To determine if glyphosate plus LPS has additive or synergistic respiratory inflammatory effects that differ between the sexes.

Research Hypothesis**:** Interactions between LPS and glyphosate induce molecular respiratory inflammatory expressions that are significantly different between males and females.

4.3 Materials and Methods

Animals

C57BL/6 female and male mice (Charles River Laboratory, Quebec, Canada) aged 8-10 weeks were used. The mice were housed in ventilated cages in the Laboratory Animal Services Unit (LASU) at the University of Saskatchewan where commercial rodent chow and water was available *ad libitum*. The animals were acclimatized to the whole-body plethysmography (WBP) chambers (Buxco FinePointe Whole Body Plethysmography 4-site system; Data Sciences International, Minneapolis, MN) for 20 minutes per day at least twice prior to the start of treatments. Experiment protocols were approved by the Animal Ethics Research Board of the University of Saskatchewan (AUP 20160106 {males} and AUP 20190017 {females}). All animal work was performed in accordance with the Canadian Council on Animal Care guidelines.

Study Design

Twenty female and twenty male mice were randomly divided into 4 groups (n=5/group) including: control (Hank's Balanced Salt Solution {HBSS}, lipopolysaccharide (LPS), glyphosate (GLY), and lipopolysaccharide plus glyphosate (LG). The control group received 40 μl of HBSS (Life Technologies, Grand Island, NY, without calcium, pH 7.4). The LPS group received LPS in HBSS (0.5 μg/40μl) (1 mg/ml; *E. coli* serotype 0111:B4, Sigma, St Louis, MO). The glyphosate group received glyphosate in HBSS (1 μg/40 μl) (0.85 M; analytical grade PESTANAL standard, Sigma, St Louis, MO). The LG group received LPS and glyphosate in HBSS (0.5 μg LPS + 1 μg glyphosate/40 μl). Mice were lightly anesthetized using isoflurane and treated intranasally with 40 μl of the treatment solution. Treatments were given daily for 5 days. Following each exposure, mice were allowed to recover from anesthesia in a separate container before being placed back in their respective cages. After exposure on the 5th day, mice were placed back in the cages for 4 hours. Following the four-hour period, mice were placed in the WBP chambers

for 20 minutes to collect respiratory data. Once WBP analysis was complete, mice were euthanized and blood, BAL, and lung tissue were collected and analyzed (Figure 4.1). Identical experimental designs were used for the male and female mice and completed in 2019 (females) and 2018 (males - Pandher *et al.* (22)).

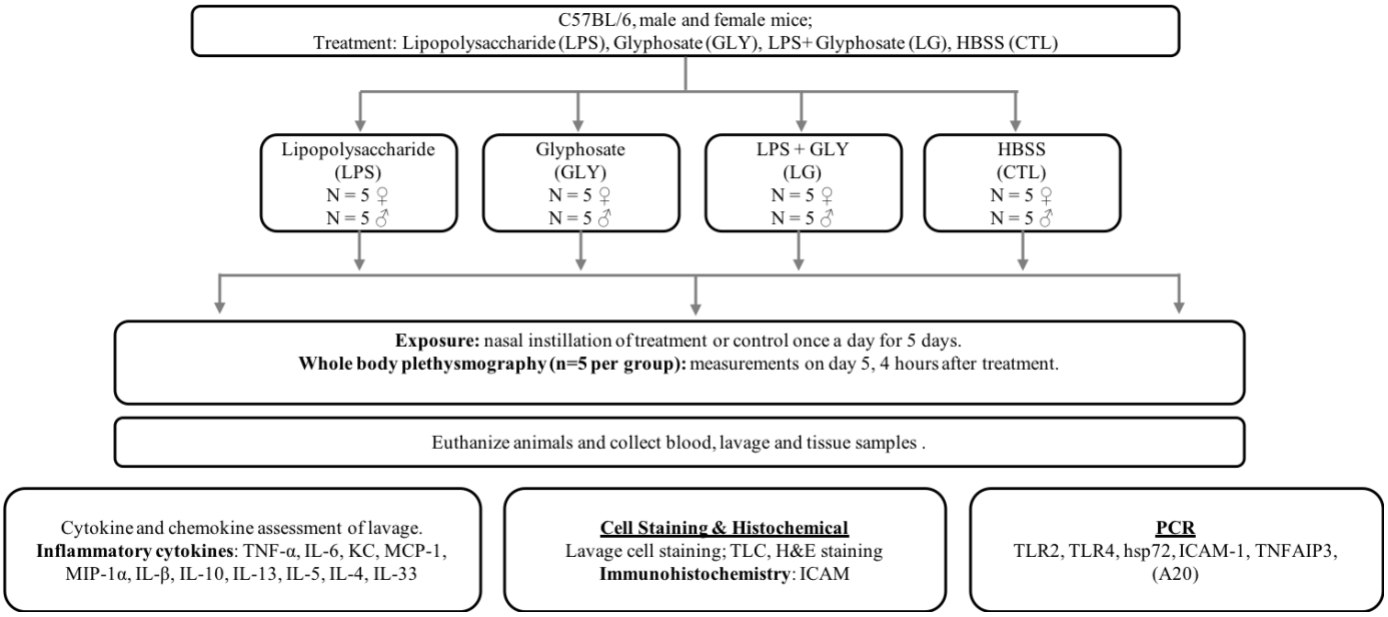

Figure 4.1: Experimental Design

Sample Collection and Analysis

Whole Body Plethysmography

Following treatment on day 5, four hours after treatment, mice were placed into whole body plethysmography chambers to monitor the respiratory parameters according to manufacturer's protocol. Mice were placed into calibrated chambers and respiratory measures (enhanced pause {PenH}, average frequency of breath, minute volume, and peak expiratory volume) were recorded every 2 seconds for 20 minutes using FinePointe software (Data Sciences International, Wilmington, NC). Results were averaged for each animal for each measure. Animals were continuously monitored for any indications of pain or distress.

Blood

Blood was collected from mice via cardiac puncture.

Bronchoalveolar Lavage

The bronchoalveolar lavage (BAL) fluid was collected by washing the lungs three times with 0.5 mL HBSS. BAL fluid was centrifuged at 1000 x g for 10 minutes at 4^0C and the supernatant was stored at 80^0C for future analysis. The cell pellet was resuspended in HBSS and kept on ice. Total leukocyte counts (TLC) were measured using a hemocytometer and expressed as an absolute number. Total protein concentration in BAL fluid was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). The plate was read at 562nm on a BioTek SynergyHT plate reader (BioTek, Winooski, VT).

Lung Tissue Collection

Following BAL fluid collection, the right lung was tied at the primary bronchus, removed, flash frozen in liquid nitrogen and stored in -80°C for mRNA and protein analysis. The left lung was inflated with 200 μL of 4% paraformaldehyde (PFA), removed and fixed in 4% PFA for 16 hours at 4°C followed by two overnight washes with 70% ethanol. The fixed tissues were processed through a series of alcohols in an Intelsint RVG/1 Histology Vacuum tissue processor (Intelsint; Turin, Italy), followed by embedding in paraffin using a Tissue Tek II tissue embedder (Sakura Finetek; Nagano, Japan).

Eosinophil peroxidase and Myeloperoxidase Levels

Lung homogenates were prepared using a BioSpec Mini-Beadbeater-24 homogenizer (BioSpec, Bartlesville, OK). 2 mm Zirconia beads (BioSpec, Bartlesville, OK) were added to tubes containing 20-25 mg of lung tissue in 500 μL RIPA Lysis Buffer containing 1X Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA). Tubes were sealed and homogenized twice for 2 minutes each, with cooling on ice for 5 minutes in between rounds. Myeloperoxidase (MPO) was quantified using a Mouse Myeloperoxidase DuoSet ELISA (R&D Systems, Minneapolis, MN). Eosinophil Peroxidase (EPO) was quantified using a Mouse

Eosinophil Peroxidase/EPX ELISA assay (Lifespan Biosciences, Seattle, WA). Plates for MPO and EPO quantification were read at 450 nm on a BioTek SynergyHT plate reader (BioTek, Winooski, VT).

Lung Histology

Sections of 5 µm thickness were cut from the paraffin embedded lung tissue using an Microm 350S microtome (Microm, Germany). Tissue sections were stained with Hematoxylin and Eosin according to the Histology Core Facility's protocol (https://healthsciences.usask.ca/facility-services/histology-core-facility.php) and coverslipped using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL USA). These slides were imaged on an Aperio CS2 virtual microscopy system (Leica Biosystems, Concord ON Canada) as well as a bright field microscope equipped with an Infinity 5-5 Microscope camera (Teledyne Lumenera, ON Canada) at 10, 40 and 100x magnification.

Immunohistochemistry

Briefly, lung sections were rehydrated through a series of xylene and descending grades of alcohol. Lung sections were covered with 0.5% hydrogen peroxide in methanol for 20 minutes to quench tissue peroxidase activity. The sections were covered with 2 mg/ml of pepsin in 0.01 N hydrochloric acid (HCl) for 45 minutes to unmask antigens. Next the sections were covered in 1% bovine serum albumin (BSA) for 30 minutes to block non-specific binding sites. The sections were incubated overnight at 4^0 C with primary antibody in 1X TBS + 1% BSA. The following primary antibodies and concentrations were used: ICAM-1 (dilution 1: 100; rabbit monoclonal anti-mouse ICAM-1, ab79707, Abcam Inc., ON Canada) rat anti-CD45R/B220 (Pan-B cell marker, dilution 1:200, clone RA3-6B2; ThermoFisher Scientific, Waltham, MA USA), rat anti-CD3 (Pan-T cell marker, dilution 1:100, clone 17A2; ThermoFisher Scientific, Waltham, MA USA) VCAM-1 (dilution 1:100; rabbit monoclonal anti-mouse VCAM-1, ab134047, Abcam Inc., ON Canada). Following overnight incubation, the sections were covered 1:200 with anti-rabbit secondary antibody (Agilent Dako, Mississauga, ON) or 1:200 anti-rat secondary antibody (ThermoFisher Scientific, Waltham, MA, USA) in 1X TBS + 1% BSA. Sections were covered with

NovaRed solution (Vector laboratories, Burlington, ON Canada) for 1-5 minutes and then counter-stained with methyl green nuclear stain for 5 seconds. Sections were dehydrated and coverslipped using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL, USA). Von Willebrand Factor (dilution 1:200; rabbit monoclonal anti-mouse vWF, ThermoFisher Scientific, Waltham, MA, USA) was used as a positive control. Additionally, controls of staining without primary antibody, secondary antibody, or both were completed.

ELISA

Quantification of TNF-α, IL-6, KC, MCP-1, MIP-2, IL-1β IL-10, IL-13, IL-5, IL-4, IL-33 in BAL fluid was determined using a Custom ProcartaPlex Luminex Immunoassay (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's specification for magnetic bead-based ELISAs. Plates were read using a Bioplex 200 system (Bio-Rad, Mississauga, ON) and Bioplex Manager Software (Bio-Rad, Mississauga, ON).

RNA purification, cDNA synthesis and Real-time PCR

RNA was extracted from lung tissue using Qiagen RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA USA) according to manufacturer's instructions for tissue extraction. The concentration of total RNA was quantified for each sample using a Take3 plate and BioTek Synergy HT plate reader (BioTek, Winooski, VT USA). cDNA was generated using iScript Reverse Transcription Supermix (BioRad, Hercules, CA USA) using 0.5 µg mRNA according to the manufacturer's instructions.

Real-time PCR reactions were carried out in duplicate in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA). Expression of mouse TLR2 (Mm00442346_m1), TLR4 (Mm00445273_m1), HSPa1a (Mm01159846_s1) ICAM-1(Mm00516023_m1), and TNFAIP3 (Hs00234713_m1) (Life Technologies, Grand Island, NY USA) were assessed. The reaction was first incubated at 50°C for 2 minutes, at 95°C for 10 minutes, then 40 cycles of 15 seconds at 95°C, then finally 1 minute at 60°C. To determine the relative quantification of each target gene, the ΔΔCt method was used.

Statistical Analysis

GraphPad Prism 9 (Graph-pad Software, San Diego, CA) was used to complete statistical analysis and preparation of graphs. Data are presented as mean + standard deviation (SD). For cytokine levels below the predicted limit of detection a value less than the lowest detected value, or a value equal to half the lowest level of quantification (LLOQ) was used. Statistical significance was determined using one-way ANOVA with follow-up Tukey test for multiple comparisons. If the assumptions of equal variance using the Brown's Forsythe test was not met, a non-parametric Kruskal Wallis (KW) test was used. A p value of ≤0.05 was considered significant for all tests performed.

4.4 Results

Whole Body Plethysmography

Average breath frequency was significantly lower in the male LPS group compared to the female LPS group (Figure 4.2B). Peak expiratory volume was significantly higher in the male lipopolysaccharide (LPS) group compared to the female LPS group (Figure 4.2C). The peak expiratory volume of the male combined (LG) treatment group was also significantly higher compared to the females (Figure 4.2C). There were no significant differences between any of the treatment groups for the PenH (Figure 4.2A) and minute volume (Figure 4.2D), although the PenH of the male LPS and LG treatment groups did appear higher compared to the respective treatment groups in the female mice.

Leukocyte Counts in Bronchoalveolar Lavage Fluid

There was no significant difference between the females and males for any of the treatment groups for total leukocyte counts present in the bronchoalveolar lavage fluid (Figure 4.3A).

Protein, EPO, and MPO expression in Lungs

There was no significant difference between the females and males for any of the treatment groups for protein levels in the bronchoalveolar lavage fluid (vascular permeability (Figure 4.3B).

There were no significant differences between the male and female treatment groups for EPO expression (Figure 4.3C). MPO concentration of the male combined (LG) group was significantly higher compared to the female combined LG group (Figure 4.3D).

Lung Histology

Comparison of representative images from male and female H&E stained mice lungs showed leukocyte infiltration in the perivascular, peribronchial, and alveolar regions of the animals treated with only glyphosate or LPS, whereas robust leukocyte infiltration was present in these regions in the combined (LG) treatment groups (Figure 4.4). Disruption and thickening of the alveolar epithelium was present in the LPS and combined treatment groups in both male and female mice. There was robust leukocyte accumulation in the alveolar regions in the male LG combined treatment group that was not present in the female LG group. Increased thickening of the bronchial epithelium was also evident in lungs of the both the male and female LG combined group.

ICAM-1 Staining

Images from the combined (LG) treatment group and control group in male and female mice lungs showed typical ICAM expression in the alveolar regions (Figure 4.5). LG treatment groups showed increased ICAM expression in the perivascular region compared to the control animals. LG-treated male mice showed intense staining in the perivascular region not present in the LG-treated female mice.

Cytokines in BAL

There was no significant difference between any of the female and male treatment groups for the expression of KC, TNF-α, IL-6, MIP-2, IL-1β, and MCP-1 (Figure 4.6A). For these proinflammatory cytokines the male LG treatment groups were higher than the female LG

treatment groups, though not significantly so. The exception was MIP-2 where the female LG treatment group was higher compared to the male LG treatment group, though not significantly.

There was no significant difference between any of the female and male treatment groups for the expression of IL-5, IL-4, IL-10, and Il-33 (Figure 4.6B). The expression of IL-13 was significantly higher in the male glyphosate group compared to the female glyphosate group. For the expression of IL-10 and IL-5 the male mice treated with either LPS alone or the LG treatment group was higher compared to the female LPS and LG groups, though not significantly so.

ICAM, TLR2, TLR4, HSP, and A20 expression in lungs

There was no significant difference between any of the female and male treatment groups for the expression of HSP (Figure 4.7). For the expression of A20, the male LG group had a significantly higher fold change (15x) as compared to the female LG group (1x fold change). For the expression of TLR4, TLR2, and ICAM the male LG, LPS, and GLY treatment groups were significantly higher than the respective female treatment groups, however, the fold-change differences were consistently highest in the LG combined exposures. The greatest fold-change differences occurred for TLR2 LG combined exposures, where on average the male mice had a 70x fold change whereas the female mice experienced on average a 5x fold change.

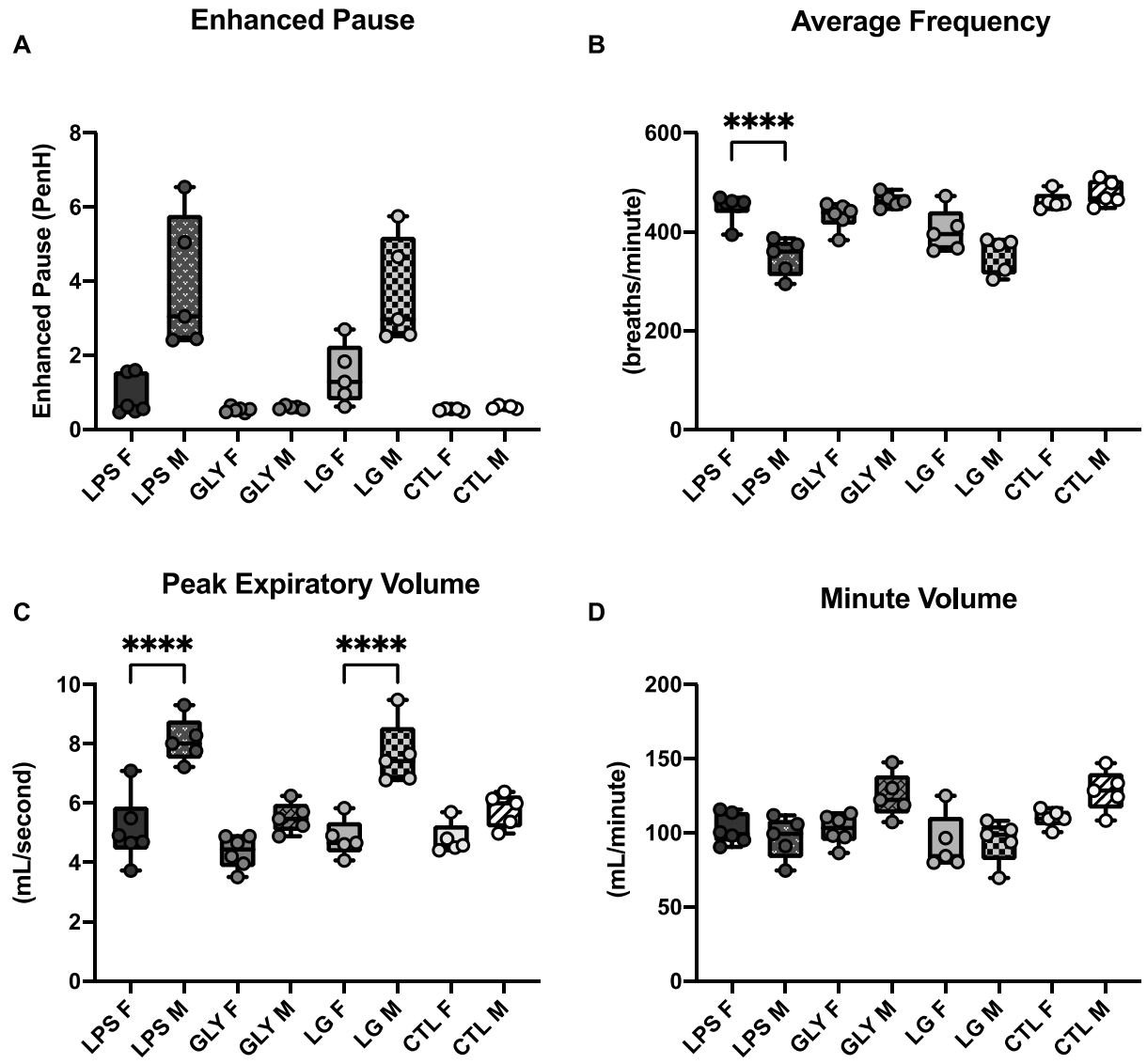

Figure 4.2: Whole body plethysmography data presented as mean + SD (n=5-6). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. **** indicates significance level of $p < 0.0001$.

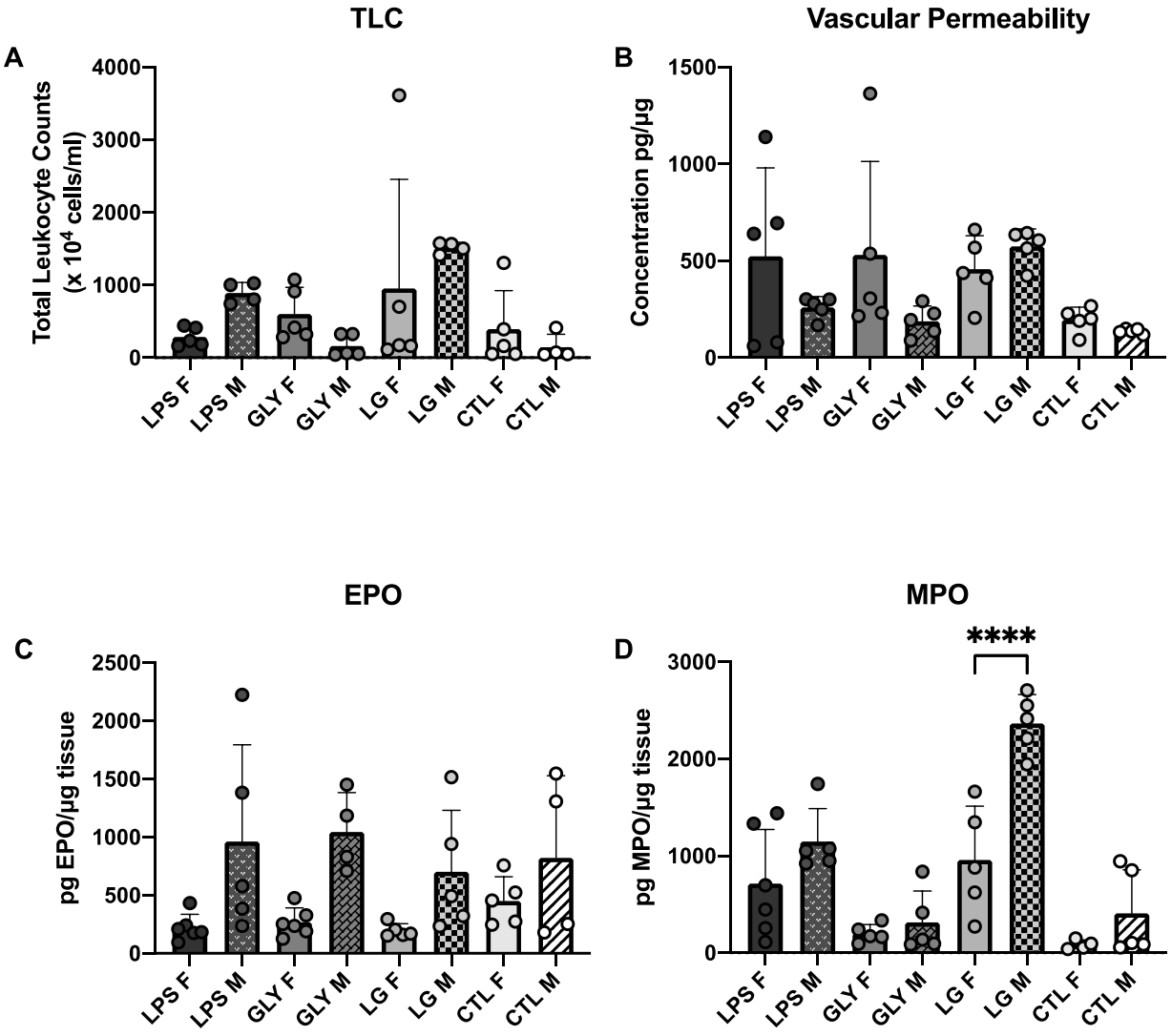

Figure 4.3: (A) Total leukocyte counts present in bronchoalveolar lavage fluid after a 5-day exposure presented as mean + SD (n=4-5). (B) Total protein concentrations in bronchoalveolar lavage fluid presented as mean + SD (n=5-6). (C) Eosinophil peroxidase and (D) myeloperoxidase levels present in mouse lung tissue presented as mean + SD (n=5-6). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL). **** indicates significance level of $p < 0.0001$.

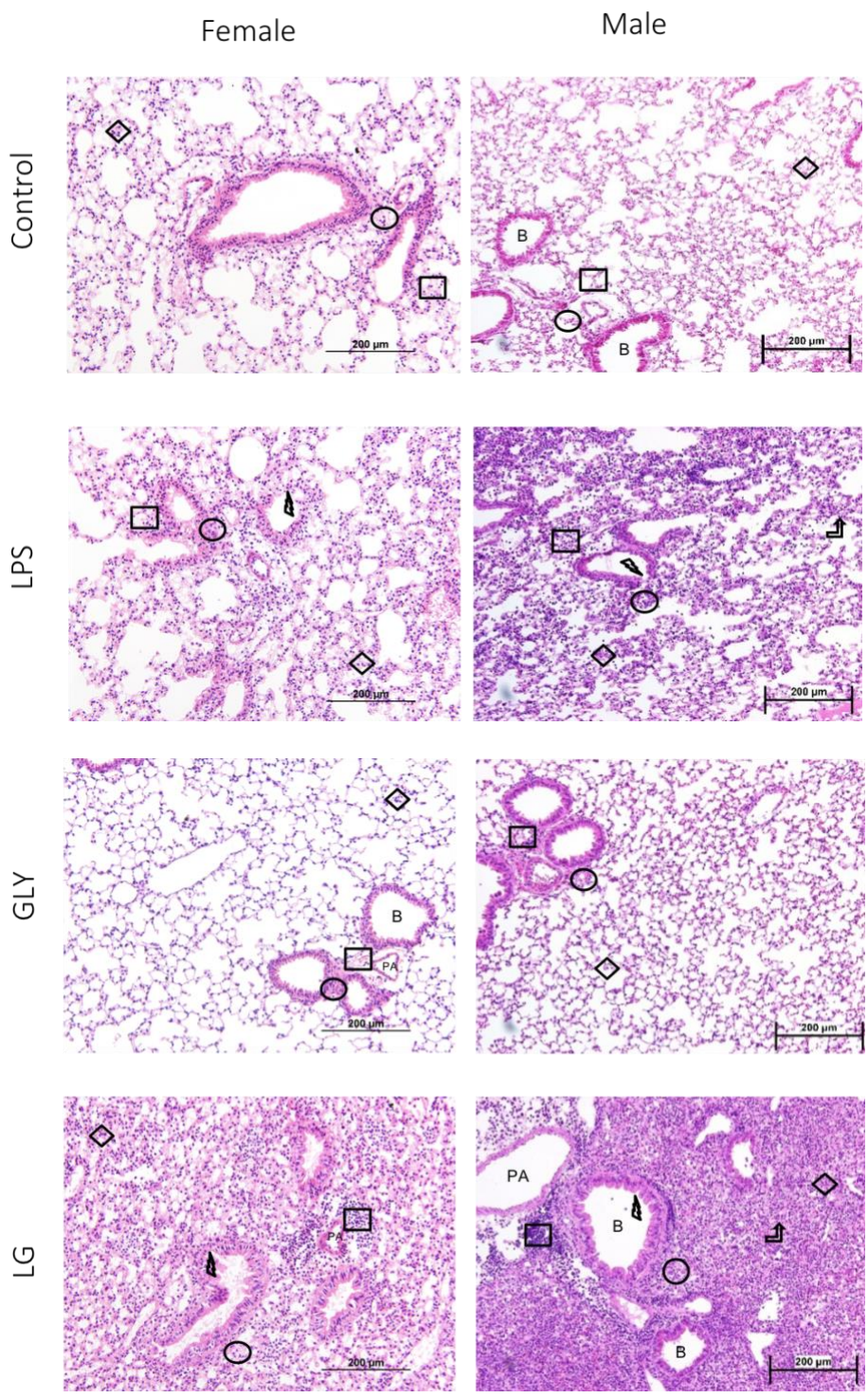

Figure 4.4: H&E staining of male and female mice lungs of control animals, or treated with lipopolysaccharide, glyphosate, or LPS plus glyphosate for 5 days. Images show leukocyte infiltration in perivascular (square), peribronchial (circle), and alveolar (diamond) regions. Magnification: 40x objective. B – bronchus, PA – pulmonary artery, Lightning bolt – disruption of the epithelium; bent arrow – alveolar septal thickening.

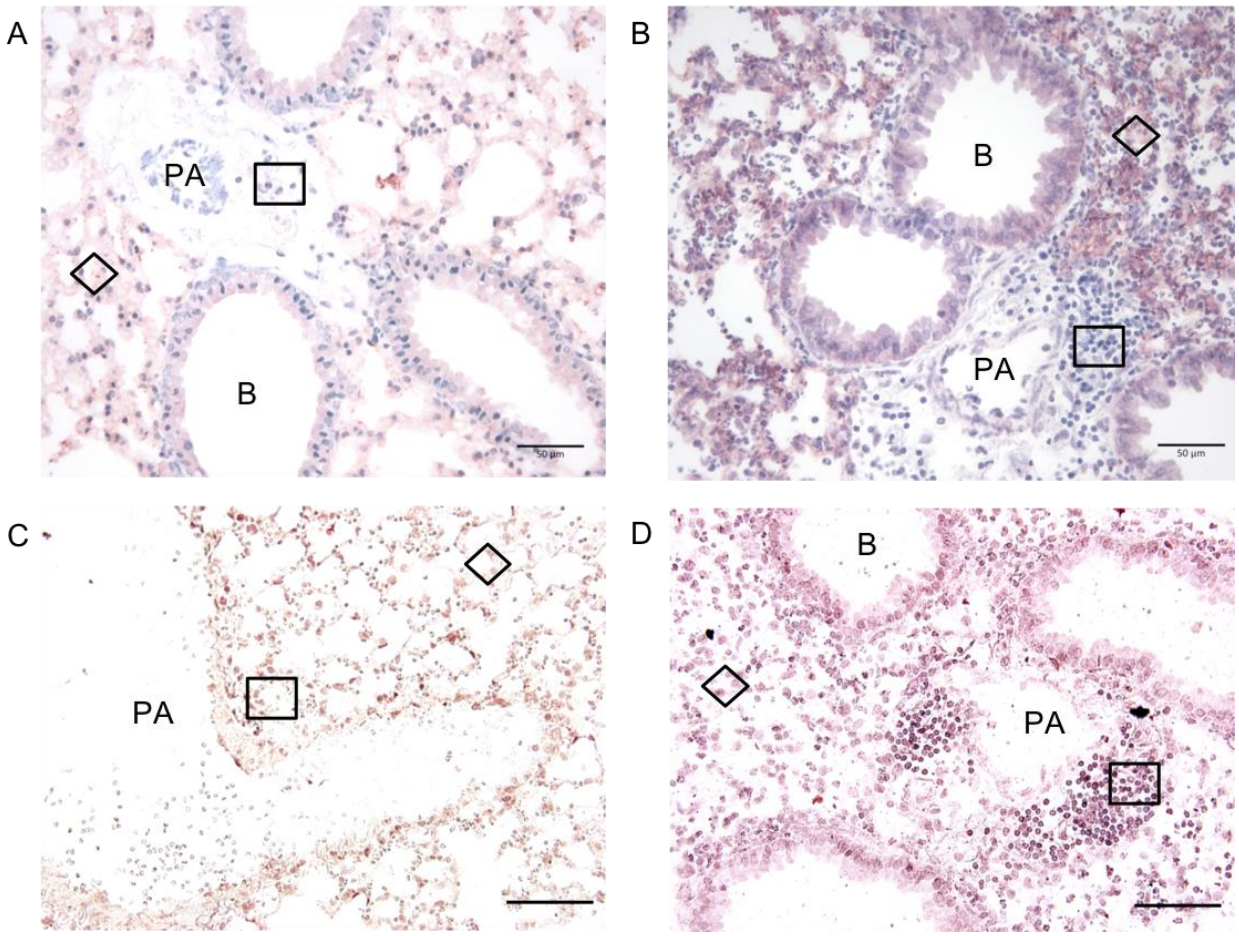

Figure 4.5: ICAM-1 staining of female (A and B) and male (C and D) mice lungs treated with either lipopolysaccharide plus glyphosate (B and D) for 5 days, or control animals (A and C). Images show staining in perivascular (square), and alveolar (diamond) regions. Magnification 40x objective. B – bronchus, PA – pulmonary artery.

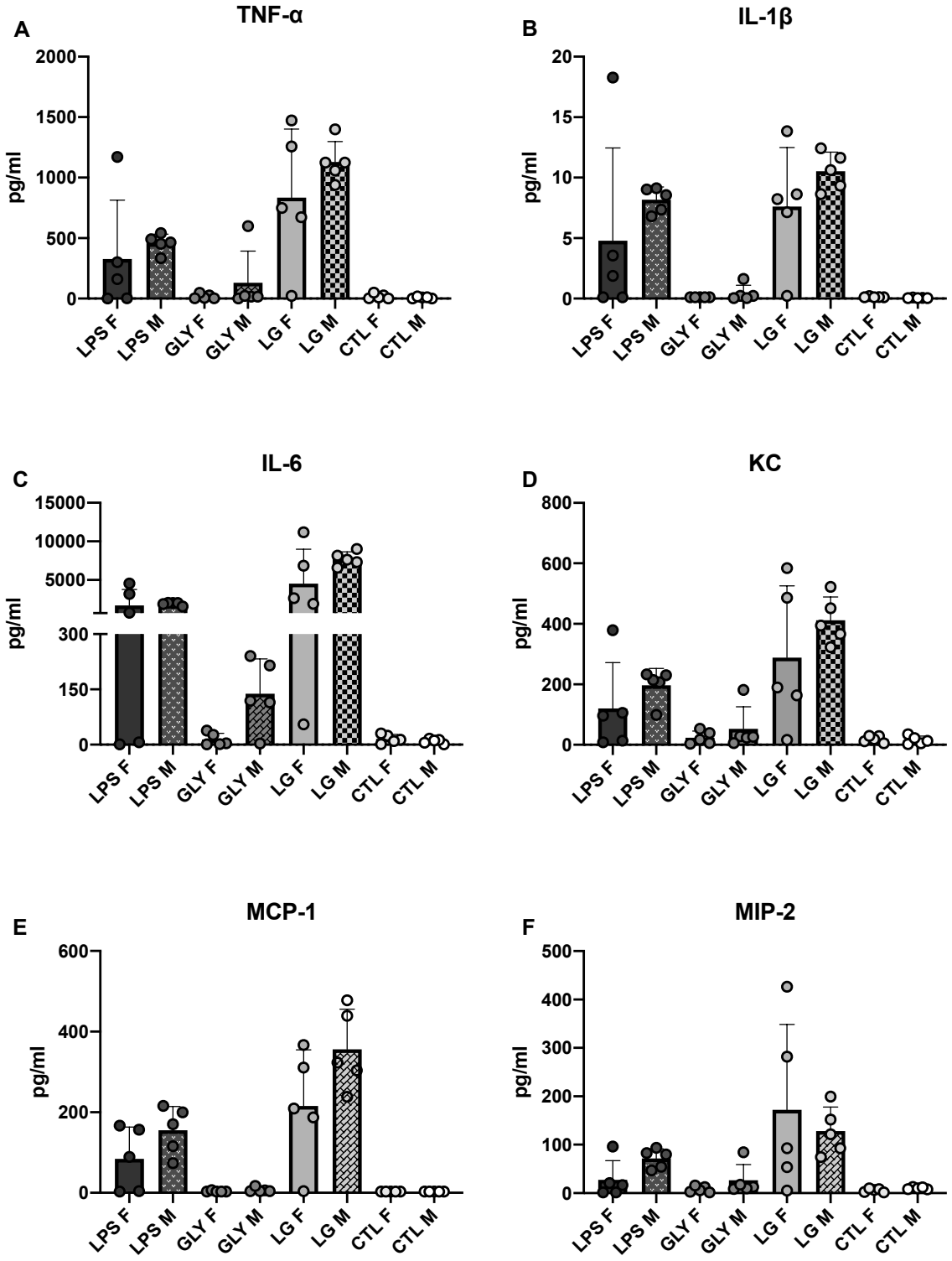

Figure 4.6A: Cytokine levels in bronchoalveolar lavage fluid (pg/ml). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5-6). No significant differences were detected between the female and male treatment groups.

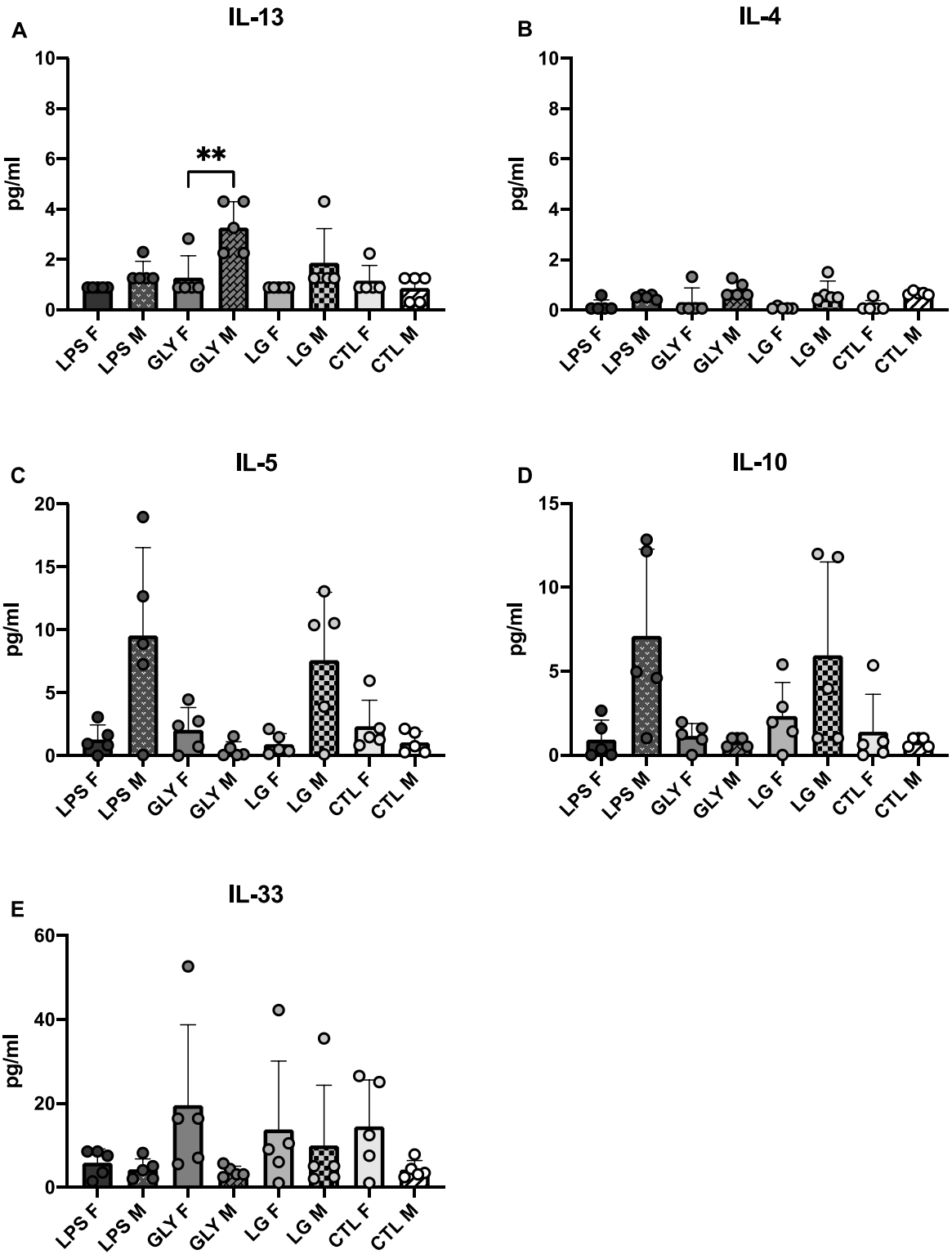

Figure 4.6B: Cytokine levels in bronchoalveolar lavage fluid (pg/ml). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5-6). ** indicates significance level of $p < 0.01$.

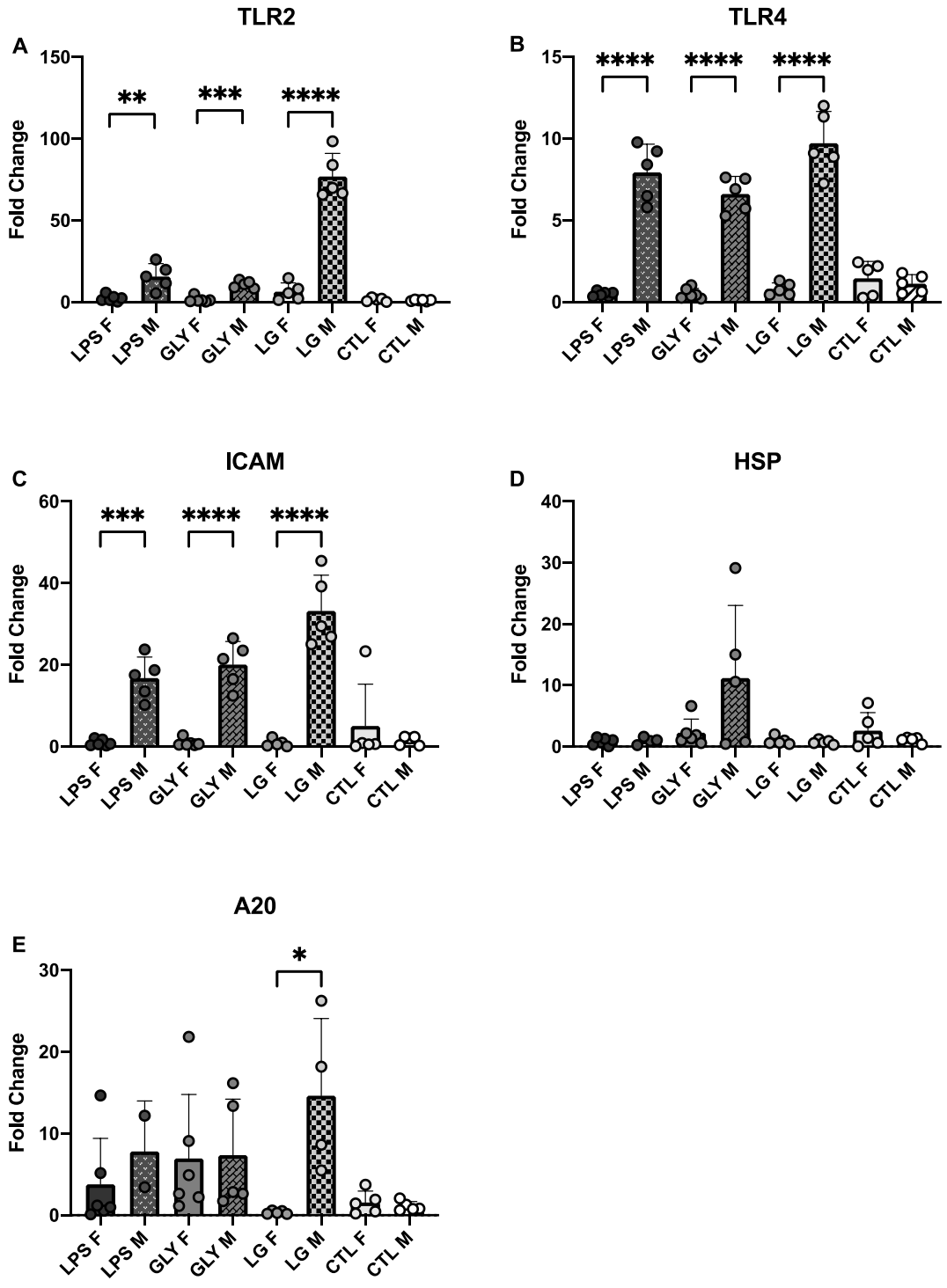

Figure 4.7: Real time PCR data from mice lung tissue presented as fold change of ICAM, TLR2, TLR4, HSP, and A20. Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=5-6). * indicates significance of $p < 0.05$. ** indicates significance level of $p < 0.01$ *** indicates significance level of $p < 0.001$. **** indicates significance level of $p < 0.0001$

4.5 Discussion

Female workers play an important role in the agriculture industry, yet the majority of research done to date has focused on the male response to common agriculture exposures. This is the first study to compare the differential inflammatory response between the sexes exposed to an environmentally relevant combination of lipopolysaccharide (LPS) and glyphosate. Following a 5 day exposure to a combination of LPS plus glyphosate in age matched female and male C57BL/6 mice, we observed an altered respiratory inflammatory response in male mice as compared to female mice receiving the same treatments either due to females not mounting a robust an immune response as the males, or because the inflammatory response has already begun to resolve in the female mice while persisting in the male mice.

Sex differences in airway responsiveness with exposure to LPS have been shown previously (40), and is consistent with our findings. The male mice treated with either LPS or LPS plus glyphosate exhibited the highest PenH, a measure of airway resistance. It has been previously established that naïve C57BL/6 mice do not show any differences between the sexes for baseline respiratory function (40); however following methacholine challenge, male mice displayed significantly higher airway responsiveness (40). This finding is not a strain specific, but a sex specific phenomenon, the experiment was replicated with BALB/c mice and established again that males were more sensitive to methacholine challenge (40). As enhanced pause (PenH) is a surrogate marker for airway resistance, this finding correlates to our results that there was a sex differential response in respiratory function for mice exposed to either LPS alone or LPS + glyphosate.

Neutrophils appear to be an important distinguishing feature in the differential inflammatory response between male and female mice exposed to the combination of LPS and glyphosate. The male mice treated with LPS plus glyphosate displayed significantly higher myeloperoxidase expression compared to the female mice receiving the same exposure. Myeloperoxidase expression is a surrogate marker for presence of neutrophils, indicating greater neutrophil recruitment in the male mice compared to females. As active neutrophils may eventually contribute to damage of the lung tissue if the activation is prolonged (130), the increased MPO expression seen in the male LPS plus glyphosate treatment group also

correlates to the increase of leukocytes and increased damage of the lung tissue present in the histology of the male LPS plus glyphosate mice that was not present to the same extent in the histology of the female LPS plus glyphosate mice. These histology results are in accordance with other studies evaluating LPS exposure, where LPS-treated females exhibited less severe inflammation based on levels of proinflammatory cytokines and lower histopathological scores (40). Additionally, for the male mice, when glyphosate was added to the LPS exposure (LG), the neutrophil response was higher, and there was greater cellular activity in the histology, suggesting an enhanced inflammatory response following exposure to the combination LPS plus glyphosate (22) that was not present in the female mice.

The robust positive ICAM staining in perivascular space in male mice treated with both LPS plus glyphosate along with the qPCR results of increased ICAM expression in male treatment groups provides further evidence of why a sex differential response is being observed. ICAM expression has been previously shown to play an important role in lung inflammation progression following LPS exposure and contributes to neutrophil adhesion and migration (46,131). Interestingly, the presence of estrogen has been previously been shown to decrease the expression of ICAM in mouse models (132) which may provide partial explanation of why ICAM was significantly higher in the male mice treatment groups for both the immunohistochemistry and PCR results. However, there was no significant difference between the male and female control animals for ICAM expression. Additionally, estrogen has been shown to inhibit the adhesion of inflammatory cells to endothelial cells following LPS exposure (132). When ICAM is upregulated by proinflammatory cytokines, there is an increase in leukocyte migration towards inflammatory sites; this finding provides an explanation of why there are higher levels of MPO expression and higher levels of leukocytes visible in the histology images of the male mice treated with LPS and especially LPS plus glyphosate compared to the females.

Proinflammatory cytokines are an indicator of inflammatory response to LPS. Previous work done in evaluating the sex differential response in LPS treated mice shows that typically, the expression of proinflammatory cytokines, especially TNF-α and IL-6 is higher in male mice compared to same age female mice (40,104–106). The male and female response differed for

several of the proinflammatory cytokines; for the male mice the LPS plus glyphosate treated group displayed significantly higher expression of IL-6, KC and TNF-α compared to all other male treatment groups, including the LPS treated male mice (121). *In vitro* studies indicate estrogen can inhibit the expression of IL-1, IL-6 and TNF-α following LPS stimulation via the inhibition of the NF-κB pathway (94,95). Conversely, within the female mice the proinflammatory expressions did not differ between the LPS and LPS plus glyphosate group for any of these cytokines (Paper 1). In the sex differential comparison of proinflammatory response the levels of MCP-1, IL-6, KC, TNF-α, and IL-1β were not significantly different between the males and female mice receiving the same treatments. Additionally, for LPS and LPS plus glyphosate treated groups there were no significant differences between the sexes for IL-4, IL-5, IL-10, or IL-33. IL-10 and IL-5 expression was higher in LPS and LPS plus glyphosate treated males compared to females although not significantly so. In the combined LPS and glyphosate exposure the male mice showed significantly higher A20 and TLR2, TLR4, and ICAM1 expression as compared to the female mice. The male LPS plus glyphosate group had a 15x fold change in A20 as compared to the female LPS plus glyphosate group which experienced only a 1x fold change from exposure to the combination of LPS and glyphosate. For the expression of TLR4, TLR2, and ICAM the male LPS, GLY, and LPS plus GLY treatment groups were significantly higher than the respective female treatment groups. Furthermore, the fold-change differences between the sexes were consistently highest in the male LPS plus glyphosate combined exposures where there was a 70x fold change in TLR2 males exposed to the combination of LPS and glyphosate, whereas the female mice experienced an average of 5x fold change in TLR2 expression. It has been shown that human male peripheral blood neutrophils express higher levels of TLR4 and release greater levels of TNF-α compared to female neutrophils following LPS exposure (133) which may explain why the male mice observed here displayed significantly higher levels of TLR4 and higher levels of TNF-α compared to females following LPS and LPS plus glyphosate exposures. Similarly, male immune cells express higher levels of TLR4 compared to females resulting in higher levels of proinflammatory cytokine production (134). These results offer an explanation on why females are better able to overcome sepsis diagnosis compared to males (133). These differences may arise due to the protective effects of estrogen (93).

Previous studies have established that human females possess a predominantly TH2 cytokine profile (IL-4 and IL-10) while males possess a TH1 cytokine profile (IFN-γ and IL-2) (135). It is believed that this TH2 cytokine profile of females may be a contributing factor in the prevalence of autoimmune diseases in females (135).

A20 or TNFAIP3 is an important inflammatory mediator in models of allergic asthma via inhibiting activation of NF-κB. In our studies the LPS plus glyphosate combined male mice showed significantly higher A20 expression compared to LPS plus glyphosate female mice as well as higher A20 expression compared to all other treatment groups. Because of the regulatory role of A20 (136), this indicates the high levels of A20 could be working to dampen the strong immune response exhibited by the male mice who received the combined treatment. As the immune response did not appear to be as robust in the female mice, even in the combined treatment group, this could be why the levels of A20 were not significantly higher in the females. A20 can also be induced by NF-κB, a pathway that can be inhibited by estrogen (94,95), offering another explanation of why the male combined treatment group exhibits higher A20 expression compared to the females. To further explore these differences in A20 expression levels, we propose conducting time point comparison studies to see if A20 expression in the female mice peaks before the 5 days measured in this current study.

The role of estrogen and other sex hormones are continuously being researched on how they influence lung function and inflammatory mediators (93,107,137,138) and results can vary depending on various factors such as experimental models and end point analysis (40). In the future, using ovariectomized females and castrated males exposed to the same exposures for 5 days would add great information on how the role of sex hormones can reduce or enhance inflammatory mediators following an agriculture respiratory exposure.

The X chromosome contains many genes that directly or indirectly contribute to the regulation of immune function (139). Libert *et al.* propose the presence of two X chromosomes in females are advantageous when dealing with pathogens but can also increase susceptibility to autoimmune diseases (139). The idea of “cellular mosaicism” is one way to explain why this is so: the possession of two X chromosomes results in higher physiological diversity when exposed to microbial infections (98,99). The X chromosome may also contain yet undiscovered

genes that may contribute to the superiority of female immunity (139). Females are able to more quickly clear pathogens because of their stronger innate and adaptive immune responses (81) and this observation is true across multiple species. An explanation for this phenomenon is one of evolutionary importance: in return for reduced immune function for males, they trade off for characteristics that increase reproductive success (140).

4.6 Conclusion

This study is the first to show that following a short term agricultural respiratory exposure to common irritants LPS and glyphosate, female C57BL/6 mice display a different inflammatory respiratory response compared to male C57BL/6 mice. This is demonstrated by less severely damaged lung architecture present in histology images from female animals across treatment groups; significantly lower levels of inflammatory markers such as myeloperoxidase expression, ICAM, TLR4, and TLR2 expression; and lower levels of proinflammatory cytokine expression in female mice as compared to male mice. This is the first study to validate that a significant difference exists between the male and female immune response following a short-term agriculture respiratory exposure to LPS and glyphosate.

Chapter 5: Multiple Image Radiography as a Tool to Evaluate a Short-Term Agriculture Respiratory Exposure in Female Mice

5.1 Abstract

Agricultural industries are rich with airborne pollutants including dust and pesticides. Workers in the agriculture industry are at risk for developing respiratory effects from work exposures. Very little is known of the effects of agricultural exposures on females. It is unclear if exposure to glyphosate, an herbicide prevalent in crop production, causes lung inflammation from inhalation. Furthermore, agriculture workers are exposed to a mixture of pollutants, not just a single agent. Little data exists on the inflammatory potential of mixed exposures common in agriculture, in particular, lipopolysaccharide (LPS), a component of grain dust, and glyphosate. The objective of this work was to compare physiological markers and structural measures to see if in female mice the combined exposure effect is significantly different than LPS or glyphosate alone.

C57BL/6 female mice were intranasally treated with glyphosate (1µg), LPS (0.5µg), combined glyphosate (1µg) + LPS (0.5µg), or HBSS for 5 days. On day 5 mice were transported to the Canadian Light source Synchrotron (CLS) for image acquisition at the Biomedical Imaging and Therapy - Bending Magnet (BMIT-BM). Following treatments, mice were euthanized and bronchoalveolar lavage fluid (BAL) and lung tissue were collected. BAL was collected and analyzed for cellular changes. Lung tissues were stained to observe structural changes.

Mice exposed to both LPS and glyphosate for 5 days showed compromised lung tissue revealed in the MIR images. Histology revealed the lungs of the mice treated with both LPS and glyphosate displayed greater recruitment of leukocytes into alveolar regions and disruption to the bronchial epithelium as compared to other treatments groups. These results reveal that female mice exposed to the combination of LPS and glyphosate displayed physiological and structural effects that were different from mice exposed to LPS or glyphosate alone.

5.2 Background

Workers in the agriculture industry are at risk for developing detrimental respiratory effects from workplace exposures including grain dust and pesticides. Agriculture workers are

commonly exposed to more than one pollutant at a time; whether there is an interaction between glyphosate and lipopolysaccharide (a component of grain dust) and how this interaction contributes to respiratory inflammation has yet to be determined. This study is one of the first to compare physiological markers and structural measures to see if in female mice the combined exposure effect is significantly different than LPS or glyphosate alone.

Lipopolysaccharides (LPS), also known as endotoxins, are a class of substances that are a major component of the outer (32) surface membrane of Gram-negative bacteria (33). A low dose of LPS is a standard method to evaluate the innate immune response during inflammation or to model acute lung injury (37) that induces a well characterized increase in cytokines. Some studies indicate LPS may act to enhance the immune response when the immune system is exposed to other compounds such as pesticides (32). There is mounting evidence that exposures to pesticides, in combination with LPS, produces an inflammatory response that is significantly increased compared to the response measured from exposure to the pesticide alone (121).

Worldwide, glyphosate-based herbicides (GBHs), such as Roundup, are the most commonly used herbicides (47). Glyphosate is an herbicide used ubiquitously in both industrialized and developing countries (49). In Canada, there are 183 products on the market that contain glyphosate (63). Glyphosate, an herbicide intimately linked with canola production, has been shown to have a significant association with increased rhinitis among pesticide applicators (62). Those exposed to glyphosate report nose and throat irritation; however, there is not yet any evidence that exposure to glyphosate is linked to an increase in asthma incidence (55). There is limited data available on the *in vivo* inflammatory effects of glyphosate. One study found female mice exposed to either glyphosate-rich air samples or reagent grade glyphosate experienced an increase in cell count in lung and bronchial alveolar lavage, an increase in eosinophil and neutrophils, and increased production of IL-33, TSLP, IL-13 and IL-5 (70).

Multiple-image radiography (MIR) is the acquisition of multiple images utilizing synchrotron x-rays and refraction of x-rays at the interface of tissue and air that generates contrast to quantify lung optical properties (117). This method is a variation of diffraction enhanced imaging (DEI) and can be used to quantify the optical properties of acute lung injury

following LPS instillation (117). Due to the high number of air-tissue interfaces present in the lungs, they are an excellent candidate for MIR imaging (117) where it is possible for the investigator to make assessments of subtle changes in the lungs following exposure to agents such as LPS and/or glyphosate. The number of air-tissue boundaries (alveoli) present in the lung are relative to the MIR parameter ultra-small-angle-x-ray scatter (USAXS) which may also be referred to as width (141). In a study utilizing MIR to evaluate changes in the lungs over a period of time following LPS exposure in male C57BL/6 mice, it was found that refraction and scatter were reduced following LPS exposure which represented increase in fluid buildup, or higher levels of edema in the lungs (119).

To the best of our knowledge there have been no studies that have used Multiple Image Radiography (MIR) to evaluate the effects of a short term agriculture respiratory exposure. This study is the first to compare physiological markers and structural measures to see if in female mice the combined exposure effect is significantly different than LPS or glyphosate alone.

Objective: To observe if there are differences in physiological markers (using whole body plethysmography and histology) and structural measures of X-ray optical properties (using multiple image radiography) in female mice exposed to LPS plus glyphosate.

Research Hypothesis: Glyphosate will interact with LPS to induce physiological and structural respiratory inflammatory effects that are significantly different compared to each single exposure in female mice.

5.3 Materials and Methods

Animals

Female C57BL/6 mice (Charles River Laboratory, Quebec, Canada) aged 8-10 weeks were used. The mice were housed in ventilated cages in the Laboratory Animal Services Unit (LASU) at the University of Saskatchewan where commercial rodent chow and water was available *ad libitum.* Mice were acclimatized to the whole body plethysmography (WBP) chambers (Buxco FinePointe Whole Body Plethysmography 4-site system; Data Sciences

International, Minneapolis, MN) for 20 minutes per day at least twice prior to the start of treatments. Experimental protocols were approved by the Animal Ethics Research Board of the University of Saskatchewan (AUP 20190017). All animal work was performed in accordance with the Canadian Council on Animal Care guidelines.

Study Design

Twelve mice were randomly divided into 4 groups (n=3/group). Nasal instillation was used to administer either Lipopolysaccharide (LPS), glyphosate (GLY), LPS + glyphosate (LG), or control (CTL) (Hank's Balanced Salt Solution {HBSS}). The control group received 40 μl of HBSS (Life Technologies, Grand Island, NY, without calcium, pH 7.4). The LPS group received LPS in HBSS (0.5 μg/40μl) (1 mg/ml; *E. coli* serotype 0111:B4, Sigma, St Louis, MO). The glyphosate group received GLY in HBSS (1 μg/40 μl) (0.85 M; analytical grade PESTANAL standard, Sigma, St Louis, MO). The LG group received both LPS and glyphosate in HBSS (0.5 μg + 1 μg/40 μl). Mice were lightly anesthetized using isoflurane and treated intranasally with 40 μl of the treatment solution. Treatments were given daily for 5 days. Following exposure, mice were allowed to recover from anesthesia in a separate container before being placed back in their respective cages. After exposure on the 4th day, mice were placed back in the cages for 4 hours. Following the four-hour period, mice were placed in the WBP chambers for 20 minutes to collect respiratory data (Figure 5.1).

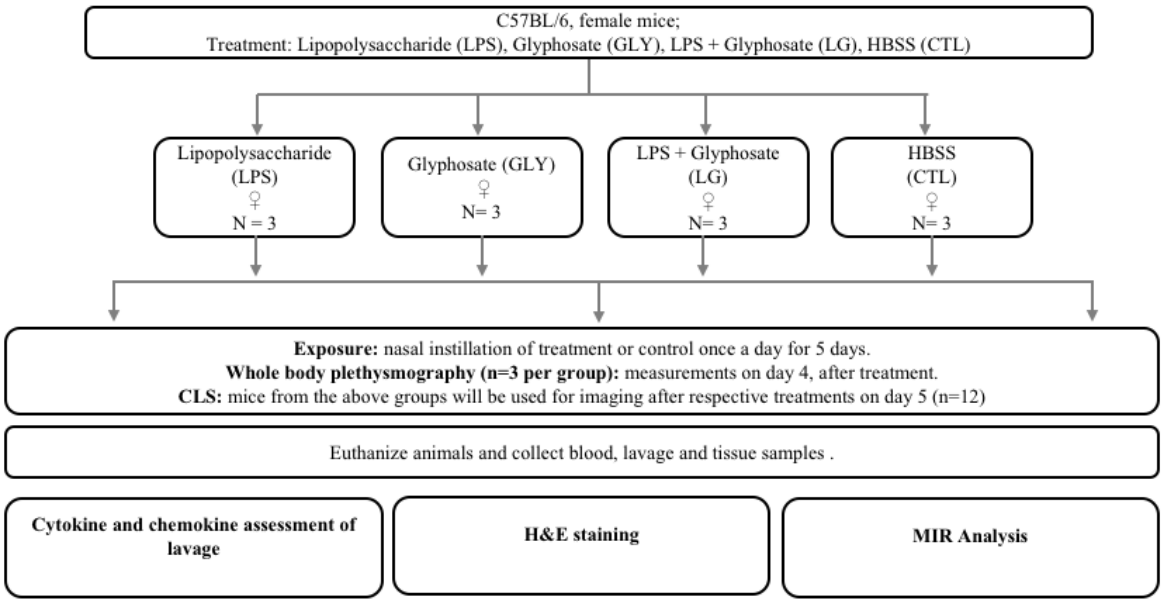

Figure 5.1: Experimental Design

Whole Body Plethysmography

Following treatment on day 4, four hours post treatment, mice were placed in whole body plethysmography chambers to monitor the respiratory changes as per manufacturer's protocol. Mice were placed in calibrated chambers and respiratory measures (enhanced pause {PenH}, average frequency of breath, minute volume, and peak expiratory volume) were recorded ever 2 seconds for 20 minutes, these results were averaged per animal. Animals were continuously monitored for any indications of pain or distress. Data collection was done using FinePointe software (Data Sciences International, Wilmington, NC).

Multiple Image Radiography

Following treatment on day 5 day, mice were transported to the Canadian Light Source Synchrotron for multiple image x-radiography. Three shifts of beam time were approved at Canadian Light Source Synchrotron (June 14, 2019). These shifts were used to establish a protocol and "proof of principle" and familiarize the research team with use of the synchrotron.

Mice were imaged at the Biomedical Imaging and Therapy - Bending Magnet (BMIT-BM) beamline of the CLS located at the University of Saskatchewan, Canada, using the protocol previously described by Aulakh *et al.* (117). Briefly, all objects were positioned in hutch POE-2 of the BMIT beamline, 24 m downstream from the synchrotron source. The vertical and horizontal beam size is 4.0 mm and 250.0 mm, respectively. Si (2,2,0) planes were used in the double crystal monochromator to select 30 keV with a matching analyzer positioned between the mouse and the detector. A Photonic Science SCMOS detector (Photonic Science, UK), was placed 1.0 m downstream. This detector has a 25 X 25 µm pixel size with a field of view of approximately 75 mm (horizontal) x 50 mm (vertical). Due to the limited beam time, 3 mice were imaged in sequential vertical fields of view with approximately 24.5 µm surface dose per image. Each field of view with a MIR data-set contains 14 images with angular settings of the analyzer crystal and the acquisition time was 0.55 s/image.

The mice were anesthetized using an intraperitoneal (IP) injection of a combination of 100 mg/kg ketamine (Ketalar®, Pfizer, US) and 20 mg/kg xylazine (Rompun®, Bayer, US). Anesthesia was maintained with further 0.05-0.1ml intraperitoneal injections with the same anesthetic combination using return of the pedal reflex as a guide to anesthetic depth. Mice were mounted on a plexi-glass holder and transferred to the imaging hutch. An infra-red heat lamp to maintain body temperature at 37°C was used and body temperature was continuously monitored using a rectal probe. Eye lubrication gel was used to protect the corneas. Pulse oximetry was used to measure hemoglobin oxygen saturation (SpO_2) by placing a probe on the right hind paw. The mice were maintained at normal body temperature and SpO_2 were monitored throughout the study. The breathing rate was calculated by counting the number of diaphragmatic movements during one minute from the control room using a camera directed towards mice.

Sample Collection and Analysis

Following image acquisition, mice were euthanized, and blood, BAL, and lung tissue were collected and analyzed.

Blood

The blood was collected from mice via cardiac puncture. Whole blood was stored in 80^0C until further analysis.

Bronchoalveolar Lavage

The bronchoalveolar lavage (BAL) fluid was collected by washing the lungs three times with 0.5 mL HBSS. BAL fluid was centrifuged at 1000 x g for 10 minutes at 4^0C and the supernatant was stored at 80^0C for future analysis.

Lung Tissue Collection

Following BAL fluid collection, the right lung was tied off at the primary bronchus, removed, flash frozen in liquid nitrogen, and stored in -80^0C. The left lung was inflated with 200 µl of 4% paraformaldehyde (PFA; Sigma, St. Louis, MO), removed and fixed in 4% PFA for 16 hours at 4°C followed by 2 overnight washes with 70% ethanol. The fixed tissues were processed through a series of alcohols in an Intelsint RVG/1 tissue processor (Intelsint; Turin, Italy) followed by embedding in paraffin using a Tissue Tek II tissue embedder (Sakura Finetek; Nagano, Japan).

Lung Histology

Sections of 5 µm thickness were cut from the embedded lung tissue using a Microm 350S microtome (Microm, Germany). Tissue sections were stained with Hematoxylin and Eosin according to the Histology Core Facility's protocol (https://healthsciences.usask.ca/facility-services/histology-core-facility.php) and coverslipped using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL USA). These slides were imaged on an Aperio CS2 virtual microscopy system (Leica Biosystems, Concord ON Canada) as well as a bright field microscope equipped with an Infinity 5-5 Microscope camera (Teledyne Lumenera, ON Canada) at 10, 40 and 100x magnification.

ELISA

Quantification of TNF-α, IL-6, KC, MCP-1, MIP-2, IL-1β IL-10, IL-13, IL-5, IL-4, and IL-33 in BAL fluid was determined using a Custom ProcartaPlex Luminex Immunoassay (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol for magnetic bead-based ELISAs. Plates were read using a BioPlex 200 plate reader (Bio-Rad, Mississauga, ON) and Bioplex Manager Software (Bio-Rad, Mississauga, ON).

MIR Image Analysis

The MIR analysis steps were to dark correct the object and flat field images by subtracting images acquired with the beam shutter closed from them. The analyzer angles where the 14 images for the object and flat images were taken was determined by fitting to a known analyzer rocking curve. The 14 image sets were then parameterized by fits to a Gaussian function.

This resulted in three Gaussian parameters; amplitude, center angle and width (USAXS). These fits were done with every pixel in the acquired object and flat field images. With these parameters, an intensity image could be created for the object and the matching flat. The ratio of these formed transmission images and taking a negative logarithm results in a radiograph. The difference between the object center angle and the flat center angle images results in the object' refraction angle image. The scatter width (sometimes called an ultra-small angle x-ray scattering or USAXS image) is created by subtracting, in quadrature, the flat scatter width image from the object scatter width image. Finally, for image sets where multiple exposures were taken to cover a larger vertical size of the object, the images were stitched together to form the final images.

The USAXS image was then used to select the outline of the lung and separate it from the rest of the image. As USAXS squared parameter (μrad^2) is directly proportional to thickness of scattering interfaces, this parameter was chosen to plot the median USAXS parameter across groups.

Statistical Analysis

GraphPad Prism 9 (Graph-pad Software, San Diego, CA) was used to complete statistical analysis and preparation of graphs. Data are presented as mean + standard deviation (SD). For cytokine levels below the predicted limit of detection a value less than the lowest detected value, or a value equal to half the lowest level of quantification (LLOQ) was used. Statistical significance was determined using a Kruskal Wallis (KW) test. A p value of ≤0.05 was considered significant.

5.4 Results

Whole Body Plethysmography

For the parameters of PenH, Average Frequency, Peak Expiratory Volume, and Minute Volume, there were no significant differences between any of the treatment groups (Figure 5.2). One mouse in the combined treatment group (LG) did exhibit higher PenH and much lower breath frequency and minute volume levels compared to the other mice in the LG treatment group.

MIR Image Analysis

Ultra-small-angle X Ray Scatter (USAXS) images show that lungs possess the maximum number of alveolar air interfaces in the central regions that are color coded in green, yellow, orange or red to represent high USAXS parameter (>17 μrad^2). Note the concentric reduction in USAXS parameter (<17 μrad^2) along the lung circumference as a result of reduction in the number of alveolar interfaces (Fig. 5.3.1A). The histograms (Fig 5.3.2) show there is reduced scatter in the LPS plus glyphosate animal, especially in the left lung (Figure 5.3.2.H) represented by the increase of pixel count fraction that were below the median of 14 μrad^2 in control lungs. There were no significant differences between any of the treatment groups for median lung USAXS parameter in either the right or left lung lobes. There is a decrease in USAXS values in the LPS (median USAXS of 7.554 μrad^2; range 3.59-18.16 μrad^2) and LPS plus glyphosate (median USAXS of 12.04 μrad^2; range 8.14-17.86 μrad^2) animals compared to the control (median USAXS of 14.67 μrad^2; range 13.71-21.44 μrad^2) and glyphosate (median USAXS of 18.17 μrad^2; range 3.59-16.58 $\mu rad^2 \mu rad^2$) animals. This is represented by the predominance of

darker (black or blue colored) lung regions in the LPS (57.3% of the pixels in the left and 44.6% of the pixels in the right lung are below 14 μrad^2, Fig. 5.3.2 C, D) and LPS plus glyphosate (97.4% of the pixels in the left and 73.1% of the pixels in the right lung are below 14 μrad^2) lungs (Figure 5.3.2 G, H) compared to the control (53.5% of the pixels in the left and 46.4% of the pixels in the right lung are below 14 μrad^2, Fig. 5.3.2 A, B) lungs, which is reflected in the USAXS occupied lung pixel fractions for lungs (Fig. 5.3.2). Interestingly, glyphosate treated left lung displayed predominantly higher USAXS (μrad^2) pixel values which are pseudo-colored in green, yellow, orange or red and occupied 67.1% of the pixels in left lung above 14 μrad^2 (Fig. 5.3.2F). The lung transmittance and radiographic absorption are indicative of the attenuation characteristics. We observed a reduction in right lung transmittance in the glyphosate group (median transmittance 0.527; range 0.519-0.541)) compared to control (median transmittance 0.5745; range 0.558-0.591)) group ($p=0.0132$ for overall differences and $p<0.05$ for glyphosate vs control, Fig. 5.4C). There was a simultaneous increase in right lung radiographic absorption in the glyphosate group (median absorption 0.6410; range 0.614-0.657) compared to control group (median absorption 0.5545; range 0.526-0.583)) ($p=0.0123$ for overall differences and $p<0.05$ for glyphosate vs control, Fig. 5.4E). There weren't any significant differences observed in the transmittance (Fig. 5.4D) or absorption in glyphosate treated left lung (Fig. 5.4F)

Lung Histology

Representative images from treatment groups showed moderate leukocyte infiltration in the perivascular, peribronchiolar, and alveolar regions of the mice treated with glyphosate. Robust leukocyte infiltration was present in these regions in the LPS and LPS plus glyphosate treated animals (Figure 5.5). Increased thickening of the bronchial epithelium is visible in the lungs of the LPS and LPS plus glyphosate treated mice. Disruption of the bronchial epithelium is present in the LPS plus glyphosate treated mice along with thickening and disruption of the alveolar epithelium.

Cytokines in BAL

There were no significant differences between any of the treatment groups for expression of any of the cytokines (Figure 5.6).

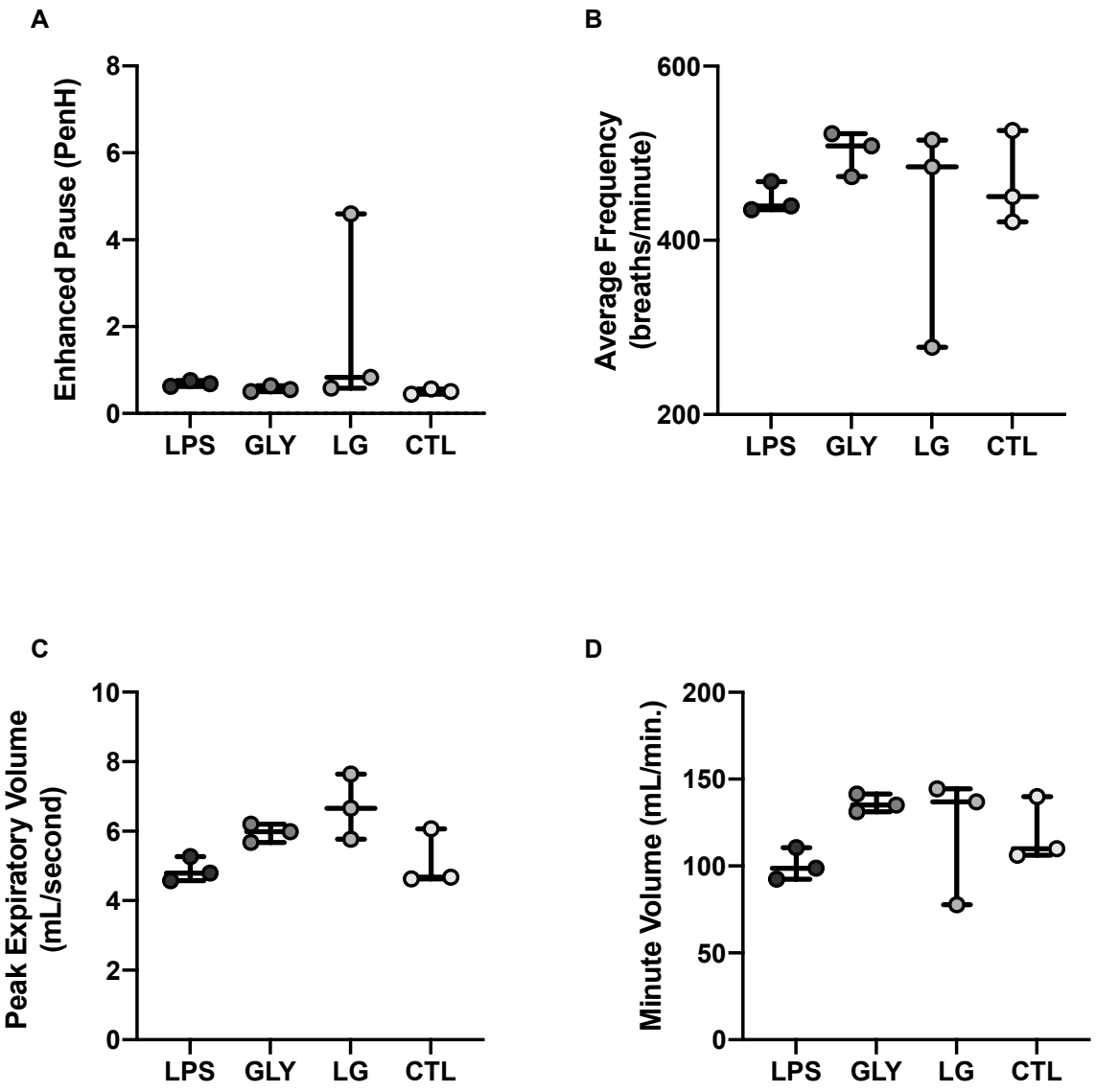

Figure 5.2: Whole body plethysmography data presented as mean + SD (n=3). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. WBP data was collected on day 4.

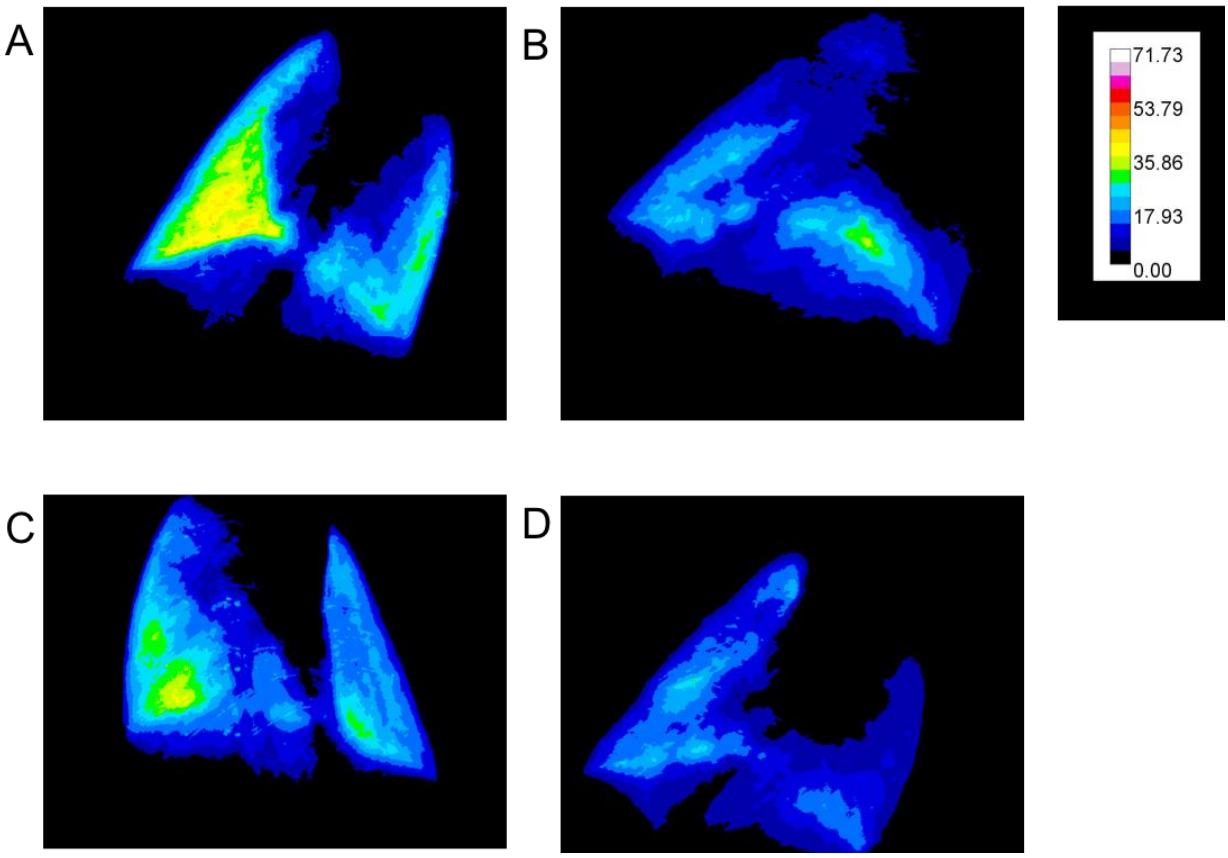

Figure 5.3.1: Comparison of Ultra X-ray Scatter images of female mice lungs for control animals (A) or treated with Lipopolysaccharide (B), Glyphosate (C), or Lipopolysaccharide plus glyphosate (D) for 5 days.

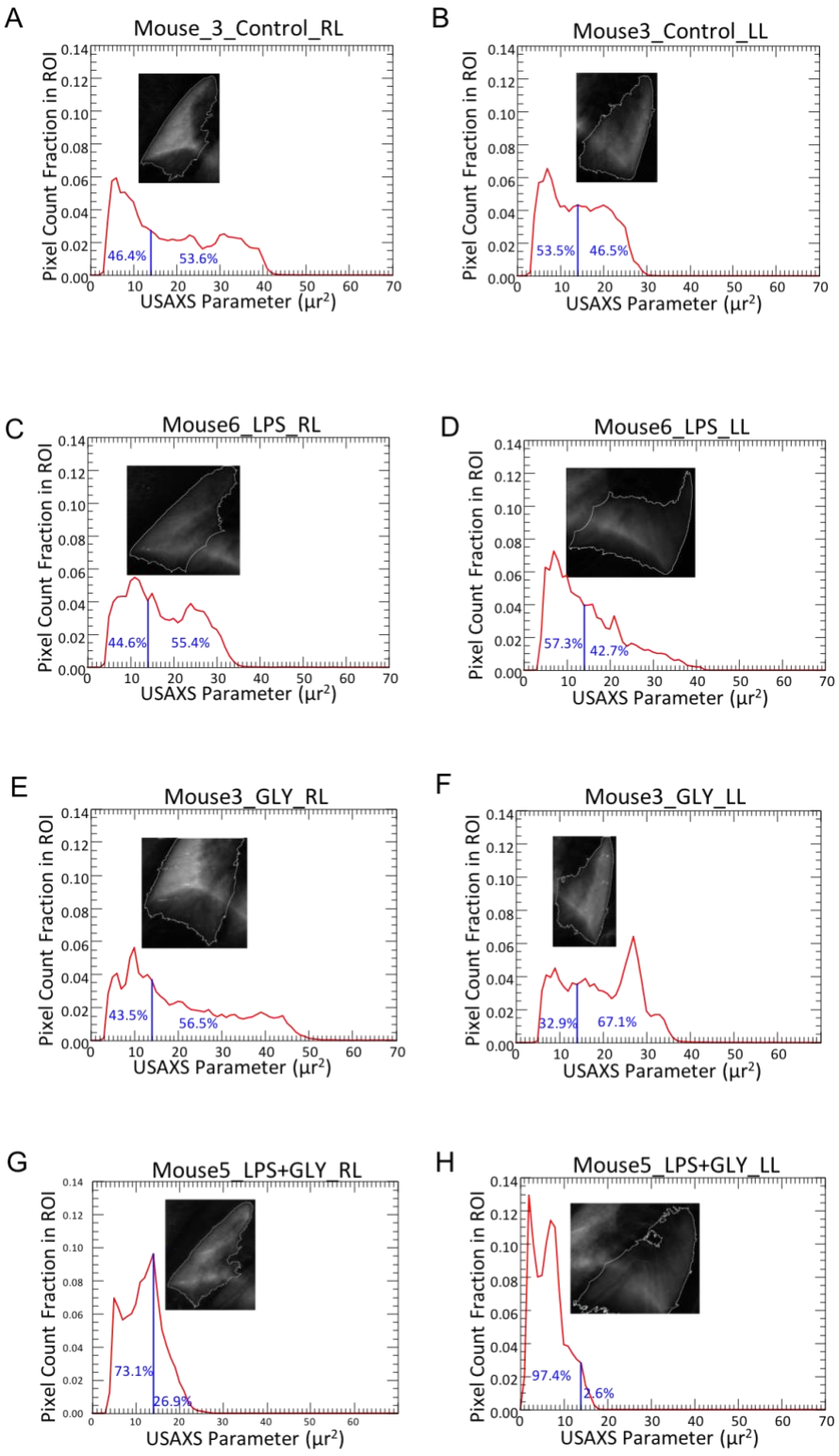

Figure 5.3.2: Comparison of Ultra X-ray Scatter histograms of female mice lungs for control animals (A, B) or treated with Lipopolysaccharide (C,D), Glyphosate (E,F), or Lipopolysaccharide plus glyphosate (G,H) for 5 days. The left column shows the right lung (A,C,E,G) while the right column shows the left lung (B,D,F,H). The median USAXS parameter is 14.

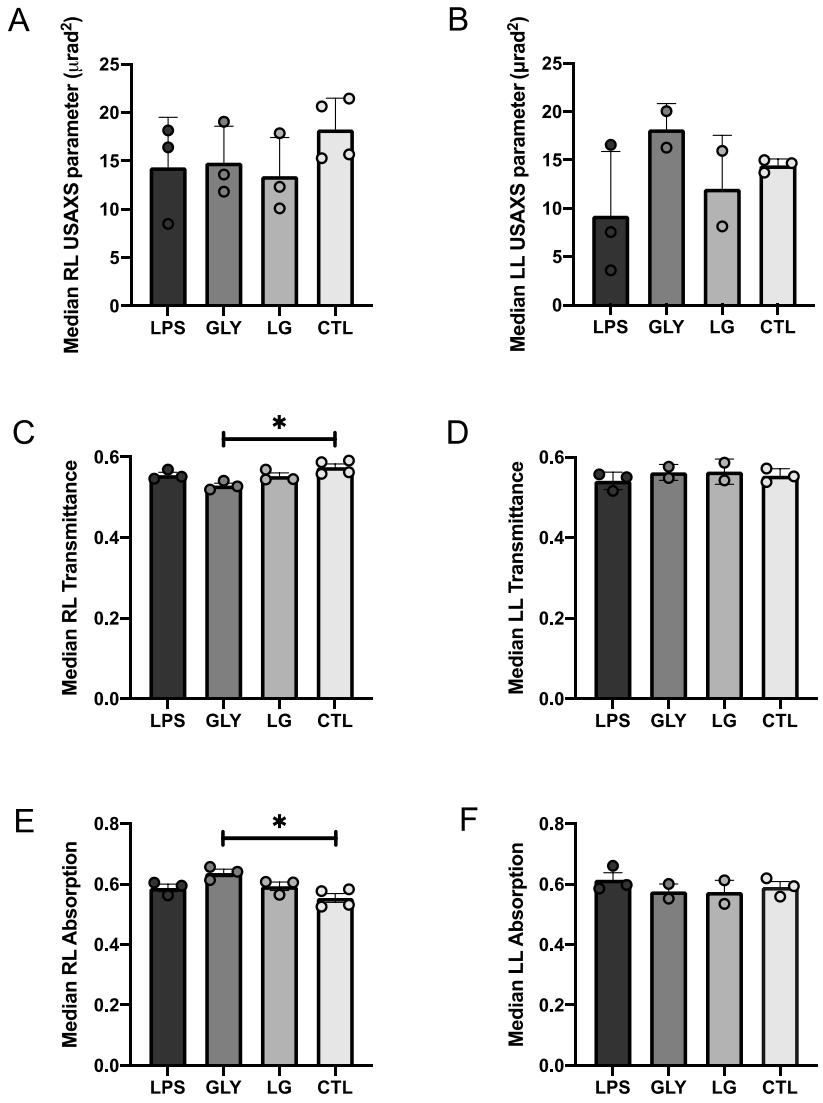

Figure 5.4: Median lung USAXS squared present in the right lung (A) and left lung (B). Median lung transmittance in the left (C) and right (D) lung. Median radiographic absorption in the left (E) and right (F) lung. Female mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=2-4). * represents $p<0.05$

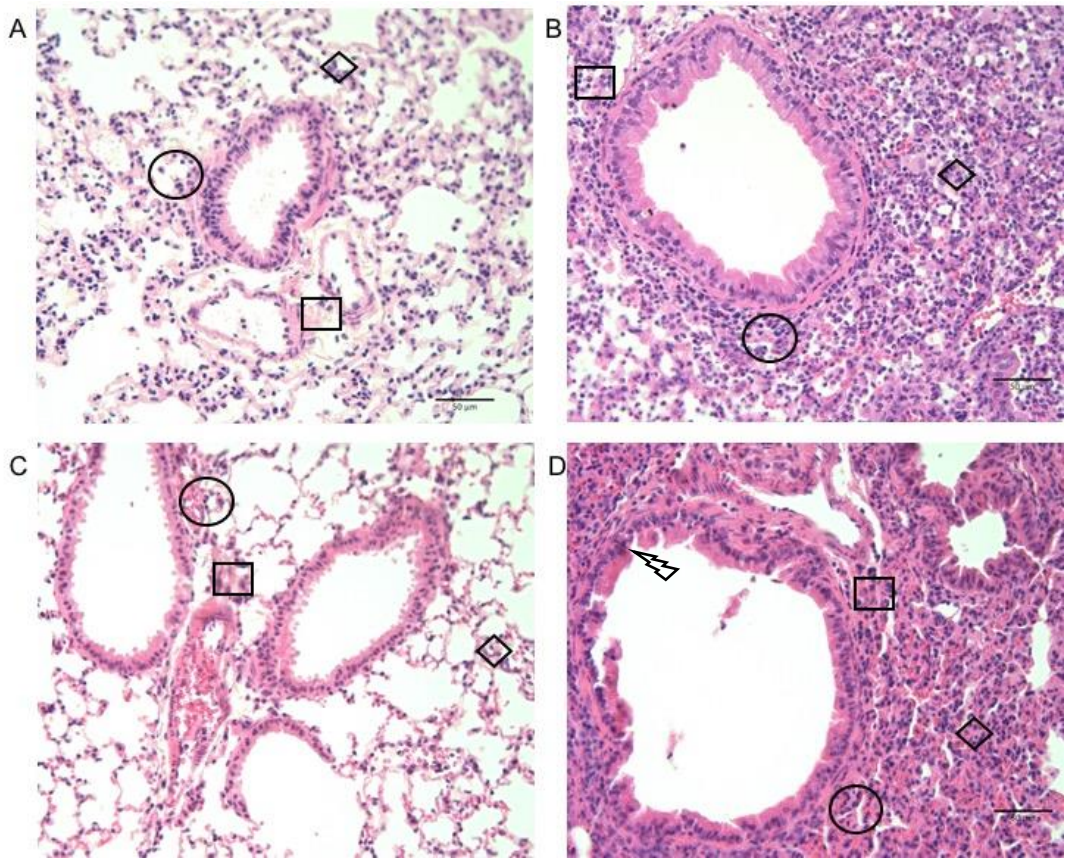

Figure 5.5: H&E staining of female mice lungs for control animals (A) or treated with Lipopolysaccharide (B), Glyphosate (C), or Lipopolysaccharide plus Glyphosate (D) for 5 days. Images show leukocyte infiltration in perivascular (square), peribronchiolar (circle), and alveolar (diamond) regions. Lightning bolt indicates disruption of the bronchial epithelium. Magnification: 40x objective.

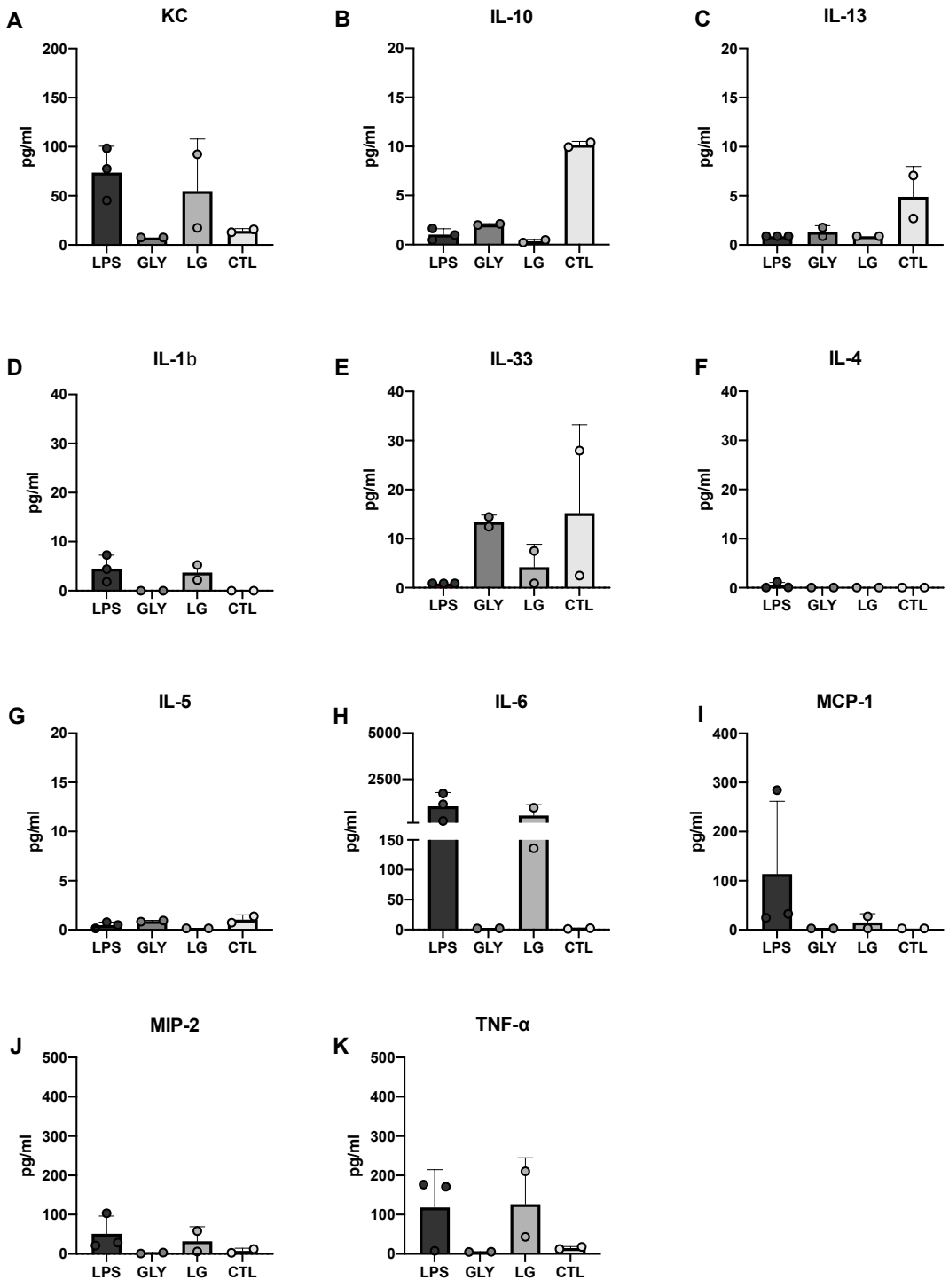

Figure 5.6: Cytokine levels in bronchoalveolar lavage fluid (pg/ml). Mice were exposed to lipopolysaccharide (LPS), glyphosate (GLY), LPS plus glyphosate (LG) or control (CTL) for 5-days. Presented as mean + SD (n=3).

5.5 Discussion

The female inflammatory response to common agricultural exposures such as LPS and glyphosate is still being determined. This is the first study which evaluates the inflammatory response of female mice exposed to a combination of LPS and glyphosate comparing physiological markers (using whole body plethysmography and histology) and structural measures (using multiple image radiography {MIR}) to see if in female mice the combined exposure effect is significantly different than LPS or glyphosate alone. This paper provides further evidence that in female mice LPS is driving the inflammatory response and the addition of another inflammatory agent, glyphosate, impacts these inflammatory effects after 5 days of exposure. Furthermore, this study utilises an imaging technique, MIR, to study these inflammatory effects.

In the width or USAXS image, scatter is related to the number of boundaries or interfaces of air and tissue (141). In a normal lung, there are more interfaces, or alveoli, present in the middle of the lung and these alveoli or interfaces gradually decrease towards the periphery. In a compromised lung, there will be less scatter visualized in the centre of the lung indicating fluid presence and disruption of the alveoli. The LPS and LPS plus glyphosate treated lungs exhibit less scatter represented by black or blue color-coded areas, especially in the apical regions of the lung. The black portions of the lung, where lung tissue is be expected to be, are indicative of loss of air-tissue interfaces due to fluid in the lung region or a compromised lung region. For X-ray interactions, tissue and fluid will behave the same and not result in x-ray USAXS. Although total scatter in the lung can be quantified using the measurement "rawintdens" of the USAXS parameter (μrad^2), we did not include it in our current analysis owing to non-uniformity in the lung regions imaged across mice. We ascribe two main reasons for the high variation in the USAXS parameter. Firstly, not all animals were imaged uniformly. Some of the mice lungs were imaged partially leading to variation in the region that was accounted for in the analysis. MIR is direction sensitive; during analysis we realized that some mice were oriented at an angle compared to others which were in a vertical orientation, which attributed to different distribution patterns of USAXS. Secondly, the beam and detector

displayed non-uniform flat and dark normalized images, which is a technical issue with the MIR image acquisition set-up.

An interesting observation from the MIR analysis was the reduction in lung transmittance and an increase in absorption in glyphosate treated mice compared to control group as both these parameters are related. This observation suggests that glyphosate either induces fluid accumulation or tissue fibrosis which warrants carefully planned future studies in order to confirm the relevance of these observations. Additionally, the USAXS histograms also suggest abnormal fluid accumulation or cellular infiltration, as there was abnormal scatter present in the glyphosate treated mice. However, there is an insufficient sample size to deduce these changes from our current histological and plethysmography results.

Kaur *et al.* observed statistically significant changes in LPS treated mice lungs across various parameters of the width/USAXS images (119). Although the influence of LPS and LPS plus glyphosate is visible in the actual images, these differences did not come through in the statistical analysis. However, this study used a small sample size and the variable mouse positioning which led to increased variability across measurements. Additionally, the decrease in scatter for some animals was varied between left and right lung lobes, and variation was observed even regionally within the same lobe (i.e. apex vs. base of lung). In future studies, as the procedural processes improve, we are confident these qualitative changes observed will translate into quantitative changes that are statistically significant and biologically relevant.

The compromised lungs of the LPS and LPS plus glyphosate treated mice are also evident in the histology images. There was severe disruption to the bronchial epithelium present in the LPS plus glyphosate treated mice not present in other treatment groups. The bronchial epithelium is a primary defense mechanism in maintaining normal airway function (142) and acts to initiate the immune response through the releases of cytokines and activation of inflammatory cells (142). Disruption of this structure can increase permeability of the airway and alter the air flow in the lungs. Based on the H&E images along with the MIR images, it is apparent the respiratory system and the lung architecture sustained substantial effects following the 5 day combined exposure to LPS and glyphosate.

This was a pilot study to orient the research team to the BMIT-BM beamline at the CLS and refine the MIR technique. There was a steep learning curve associated with the study and throughout this process multiple steps have been streamlined that will be invaluable moving forward with similar experiments. These improvements include refining the experimental procedure and productivity during the actual beamline shifts, and the stacking and analysis of the field of view images acquired. Now that these processes have been developed, future studies can benefit from using an increased sample size, as well as imaging male mice in order to further explore the sex differential analysis presented in paper 2.

5.6 Conclusion

This was the first study to compare results obtained from multiple techniques; whole body plethysmography, multiple image radiography, and histology, to evaluate a common short-term agriculture respiratory exposure in C57BL/6 female mice. Taken together, these results reveal that female mice exposed to the combination of LPS and glyphosate displayed physiological and structural effects that were different from mice exposed to LPS or glyphosate alone.

Chapter 6: Discussion

6.1 Overall Discussion

Agriculture workers, in Saskatchewan and worldwide, are exposed to numerous potential pollutants, including grain dust and pesticides. Although female workers make up approximately 25% of the agricultural working population, the majority of current research on the respiratory and inflammatory effects in agriculture has been conducted on male animals and workers; very little is known about the female response.

An efficient innate immune response is comprised of quickly clearing the pathogen to avoid tissue damage which can arise when the body stays in a prolonged state of inflammation. Evidence demonstrates that across species, females mount a more robust and efficient early immune response leading to improved prognosis in surviving acute infections arising from a variety of pathogens (bacteria, viruses, trauma) compared to males (126–128). Conversely, increased efficiency (clearing the pathogen quickly) means that females are more predisposed to developing autoimmune diseases such as lupus or rheumatoid arthritis (129).

The presence of multiple inflammatory agents is common in agricultural workplaces. How the respiratory inflammatory response alters when subjected to more than one inflammatory agent at a time is of special interest. Two of the most common agricultural exposures are glyphosate and lipopolysaccharide (a component of grain dust). How these common inhaled exposures differentially affect the respiratory inflammatory response of the sexes is not known. This study is the first to evaluate sex differential inflammatory responses from exposure to common agricultural components LPS and glyphosate.

Lipopolysaccharides (LPS), also known as endotoxins, are a class of substances that are a major component of the outer (32) surface membrane of Gram-negative bacteria (33). LPS is an ubiquitous component of agricultural environments where it is present in high concentrations and has been shown to be a powerful inflammatory agent (33,124). A low dose of LPS has been shown to induce an innate immune response that releases proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (143) and is used to model acute lung injury (37,144). Some studies suggest LPS may enhance the immune response when the immune system is exposed to other antigens such as pesticides (32).

Worldwide, glyphosate-based herbicides (GBHs) are the most commonly applied herbicides (47) in both industrialized and developing countries (49). Glyphosate has been shown to have an association with increased rhinitis among pesticide applicators (62). There has been minimal work to evaluate respiratory glyphosate exposures, and in particular, how exposure to glyphosate, when paired with LPS, impacts the respiratory inflammatory response between the sexes.

Studies by Kumar *et al.* and Pandher *et al.* have described how inhaled glyphosate exposure can alter the respiratory inflammatory response (22,70). Pandher *et al.* were the first to unravel the inflammatory potential of an exposure to LPS plus glyphosate in male mice. However, no work has yet been done on evaluating the response of female mice, nor the differences between male and female mice exposed to a combination of LPS and glyphosate.

C57BL/6 female mice were intranasally treated with glyphosate (1µg), LPS (0.5µg), combined LPS + glyphosate (LPS: 0.5 µg + glyphosate: 1µg), or HBSS for 5 days. The whole body plethysmography parameter PenH revealed mice exposed to both LPS and glyphosate exhibited significantly higher airway restriction compared to the group treated with only glyphosate, and the control group, indicating that these concentrations of LPS plus glyphosate has the ability to significantly inhibit respiratory function after 5 days of exposure. This result has potential to impact the real-world findings of this research: biologically relevant levels of these contaminants were used, demonstrating that after just one 5-day work week, agriculture workers could experience altered breathing functions.

Proinflammatory cytokines/chemokines were significantly higher in combined LPS and glyphosate treated mice compared to those treated with either glyphosate alone, or controls, but not significantly higher from the LPS only group. Levels of myeloperoxidase expression, a surrogate marker for neutrophil activation, was significantly higher in mice treated with LPS or LPS plus glyphosate, compared to other treatment groups. Histology revealed greater recruitment of cells into alveolar regions in the lungs and disruption to the bronchial epithelium in the combined LPS and glyphosate treated group compared to other treatment groups. MIR images revealed mice exposed to LPS and both LPS plus glyphosate showed compromised lung tissue compared to other treatment groups. These results suggest the presence of glyphosate

interacts with the LPS, potentiating the pulmonary inflammatory response to an exposure that has a high probability to occur in an agriculture work environment. Taken together, these results show that female mice, exposed to the combination of LPS and glyphosate for 5 days, exhibit physiological and structural effects that were different from mice exposed to LPS or glyphosate alone.

In addition to the results we obtained from the female mouse exposure, we compared these results to age-matched male mice who received the same exposures. We observed a significantly different inflammatory respiratory response in the female mice compared to male mice. Female mice demonstrated: less severely damaged lung architecture present in histology images from female animals across treatment groups; significantly lower levels of inflammatory markers such as myeloperoxidase expression, ICAM, TLR4, and TLR2 expression; and lower levels of proinflammatory cytokine expression. The effect of estrogen may be an important key explanation into the significant differences we observed between the female and male mice. Estrogen has been shown to dampen the response of many key pathways following exposure to LPS such as inhibiting the TLR4 and TLR2 by NF-κB pathway, (94), and inhibiting the expression of proinflammatory cytokines IL-1, IL-6, and TNF-α (94,95). Estrogen has also been observed to decrease the expression of ICAM and VCAM (145), and inhibit the adhesion of monocytes to endothelial cells following LPS exposure (132). The male mice displayed a strong TLR2 and neutrophilic expression to the 5 day co-exposure (22), not displayed in the female mice. However, the males also displayed an attenuated immune response when cytokines and chemokines were measured at 10 days, although histology revealed leukocyte infiltration persisted in the LPS plus glyphosate treated males (22). Future work to see if the female mice follow a similar attenuation pattern after 10 days of exposure would be enlightening.

It has been observed that females have decreased leukocyte, especially neutrophil, recruitment (128) which is one explanation of the strong neutrophilic response observed in the male exposure study (22) not observed in the females. It has also been observed that states of being where estrogen is decreased, such as menopause and ovariectomized female mice, show increased levels of proinflammatory cytokines such as TNF-α, IL-1, and higher levels of ICAM (146). Taken together, there is significant evidence that the presence of estrogen has

considerable impact on the inflammatory response; and is likely the main reason we are observing pronounced differences between the sexes to this exposure.

Altered lung function, differences in histology, and higher proinflammatory cytokine expression support the major finding from our study that female mice, exposed to an agriculturally relevant concentration of LPS plus glyphosate for 5 days, exhibited respiratory inflammatory effects significantly different compared to each single exposure. However, these inflammatory effects were not as drastic as those observed in male mice. Thus, we utilized multiple image radiography for further structural evaluation of these exposures, which provided another way to image the exposed mouse lungs *in vivo*. While there were some challenges experienced during both the *in vivo* imaging and how to best analyse these results, the research team now has a well-developed protocol for how to conduct image acquisition and analysis in future experiments.

Finally, a more compromised lung architecture, and significantly higher levels of inflammatory mediators observed in the male mice demonstrate that there is a significantly different respiratory inflammatory response between the sexes at 5 days of LPS plus glyphosate exposure. This sex differential response we observed has real world impact. Female agriculture workers may be experiencing inflammatory effects due to agriculture co-exposures, but because these effects are not as severe as those exhibited by their male counterparts, they may not be regarded or treated as seriously. Whether this sex differential response occurs due to the protective effects of estrogen (93), or another factor, has yet to be determined. Did the females never mount as robust of an immune response as the males or; conversely, did the females mount a similarly high inflammatory response before the 5 day measurements and these results had already resolved when our measurements occurred? These queries have yet to be answered and are questions that warrant further investigation.

6.2 Challenges and Limitations

One of the goals of this study was to investigate the inflammatory respiratory response of an agriculture worker, particularly a female agriculture worker. To do this we used a murine model and administered an intranasal exposure. The reasons for this exposure route included

ease of administration and being able to compare results to previously conducted experiments (22). However, an aerosolized exposure would have been closer in accuracy to be able to mimic what is occurring to an agriculture worker. When utilizing an animal model, it is the goal to have the model represent as close as possible the real-world scenario and researchers must come up with creative ways to do so. Ultimately it would be ideal to be able to collect lavage and blood samples from agriculture workers of both sexes and conduct similar assays that were performed in this study and see if the same patterns are observed.

A difficulty we encountered was that differential cell counts (DLC) were not able to be performed due to issues with the protocol used. As the results from our total cell count did not show any differences between treatment groups, having the DLCs may have provided valuable insight as to whether specific cell populations were driving the differences between treatment groups observed in other inflammatory mediators. Based on the myeloperoxidase expression results it is likely there would be higher neutrophil counts for the combined treatment LPS plus glyphosate treatment group.

Another problem encountered was the immunohistochemistry. IHC is a relatively inexpensive and easy assay to perform and way to visualize cell populations, but also possesses several disadvantages (147). It is at the mercy of human error; an experienced and skilled pathologist is necessary to conduct this assay (147). An example of human error occurred during the duration of this project: the wrong secondary antibody was being used for a number of primary antibodies. Once the mistake was identified many slides had been already been used up and some of the paraffin blocks had limited tissues remaining so it was not possible to repeat the IHC staining. Furthermore, IHC is a laborious and difficult way to quantify results, especially considering there are many more efficient ways to quantify cell populations such flow cytometry (148), and less rudimentary ways to visualize cells such as electron microscopy, which is considered the gold standard (149).

6.3 Future Research

It is not clear from the results if the females did not mount as robust an immune response compared to the male mice or if the female response was high before 5 days and

already resolving when our measurements were conducted. Performing additional studies where animals are sacrificed before 5 days (for example 1 or 2 days following exposure) would help to illuminate this query.

The hormone panel analysis was conducted in the female mice only, as we demonstrated a sex differential response to these exposures, in the future it would be of benefit to also conduct a hormone analysis in the male mice too, specifically to look if cortisol, a stress hormone, levels were significantly different between treatment groups in male mice.

The molecule A20 gave us intriguing results in both chapters 3 and 4. In Chapter 3 the glyphosate treated mice expressed higher levels of A20 compared to the LPS plus glyphosate treated animals and had the highest A20 expression compared to all treatment groups. In Chapter 4 the male LPS plus glyphosate treated animals displayed higher levels of A20 compared to the females. As A20 is typically thought to be an inhibitor of the NF-κB pathway, taken together, the results from these two chapters appear contradictory and further research into the sex differential response of A20, along with the potential effect of glyphosate on A20 expression, is necessary.

It has previously been established that castrated C57BL/6 male mice exposed to intratracheal LPS displayed an attenuated response to LPS based on BAL fluid neutrophils and TNF-α levels while ovariectomized female mice did not display a significantly different response to intratracheal LPS compared to sham-operated females (40). Performing a similar study using the same exposures but using castrated and ovariectomized mice would provide further insight into what is the mechanism behind the sex differential inflammatory response we observed.

As our research team has improved the technical procedure needed to perform experiments at the CLS, we are confident we would be approved for more beam time and be able to expand on our initial pilot study, using more mice per treatment group as well as adding male mice to the research study and protocol. Based on what we saw in the MIR analysis from the female mice, it would be of great interest to see how the compromised males lungs exposed to LPS plus glyphosate appears using synchrotron technology.

Chapter 7: References

1. Statistics Canada. Saskatchewan remains the breadbasket of Canada. 2017;(95):9. Available from: www.statcan.gc.ca
2. Ellis H. The lungs. Anaesth Intensive Care Med. 2011;12(11):501–3.
3. Lambrecht BN. Alveolar Macrophage in the Driver's Seat. Immunity. 2006;24(4):366–8.
4. Punchard NA, Whelan CJ, Adcock I. The Journal of Inflammation. J Inflamm. 2004;1:1–4.
5. Rankin JA. Biological Mediators of Acute Inflammation. Adv Crit Care Clin Issues. 2004;15(1):3–17.
6. Tizard IR. Veterinary Immunology. Ninth. Elsevier; 2013.
7. Botting RM, Botting JH. Pathogenesis and Mechanisms of Inflammation and Pain. Clin Drug Investig [Internet]. 2000;19(Supplement 2):1–7. Available from: http://link.springer.com/10.2165/00044011-200019002-00001
8. Xiao TS. Innate immunity and in flammation. 2017;1–3.
9. Medzhitov and Janeway. Immune Recognition. N Engl J Med. 2000;343:338–44.
10. Kolaczkowska E, Kubes P. Neutrophil recruitment and function. Nat Rev Immunol [Internet]. 2013;13(3):159–75. Available from: http://dx.doi.org/10.1038/nri3399
11. Kreisel D, Nava RG, Li W, Zinselmeyer BH, Wang B, Lai J. In vivo two-photon imaging reveals monocyte- dependent neutrophil extravasation during pulmonary in fl ammation. 2010;107(42):18073–8.
12. Zarbock A, Ley K, Mcever RP. Review article Leukocyte ligands for endothelial selectins : specialized glycoconjugates that mediate rolling and signaling under flow. 2019;118(26):6743–52.
13. Kornerup KN, Salmon GP, Pitchford SC, Liu WL, Page CP. Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. J Appl Physiol. 2010;109(3):758–67.
14. Rua R, McGavern DB. Elucidation of monocyte/macrophage dynamics and function by intravital imaging. J Leukoc Biol [Internet]. 2015;98(3):319–32. Available from: http://www.jleukbio.org/cgi/doi/10.1189/jlb.4RI0115-006RR

15. Polf MMJ, Veerdonk F Van De, Do EA, Kesteren-hendrikx EML Van, Rooijen N Van, Dijkstra CD, et al. The role of perivascular and meningeal macrophages in experimental allergic encephalomyelitis. 2002;122:1–8.
16. Gordon S, Plüddemann A. Tissue macrophages : heterogeneity and functions. 2017;1–18.
17. Pandit AA, Gandham RK, Mukhopadhyay CS, Verma R, Sethi RS. Transcriptome analysis reveals the role of the PCP pathway in fipronil and endotoxin- induced lung damage. 2019;1–15.
18. Furst DE, Wallis R, Broder M, Beenhouwer DO. Tumor Necrosis Factor Antagonists : Different Kinetics and / or Mechanisms of Action May Explain Differences in the Risk for Developing Granulomatous Infection. 2006;
19. Ray JLHA. Sex-differences in the inflammatory immune response to multi- walled carbon nanotubes and crystalline silica. Physiol Behav. 2017;176(3):139–48.
20. Blacquière MJ, Hylkema MN, Postma DS, Geerlings M, Timens W, Melgert BN. Airway inflammation and remodeling in two mouse models of asthma: Comparison of males and females. Int Arch Allergy Immunol. 2010;153(2):173–81.
21. Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, et al. Endothelium-derived toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. J Clin Invest. 2003;111(7):1011–20.
22. Pandher U. Pulmonary Inflammation After Exposure To Lps and Glyphosate. 2021.
23. Tang W, Huang S, Du L, Sun W, Yu Z, Zhou Y, et al. Expression of HMGB1 in maternal exposure to fine particulate air pollution induces lung injury in rat offspring assessed with micro-CT. Chem Biol Interact [Internet]. 2018;280(December 2017):64–9. Available from: https://doi.org/10.1016/j.cbi.2017.12.016
24. Carswell EA, Old LJ, Kassel RL, Green S. An endotoxin-induced serum factor that causes necrosis of tumors Immunology : 1975;72(9):3666–70.
25. Chroboczek H, Oppenheim JD, Stone-wolff D, Henriksen-destefano D. CHARACTERIZATION OF HUMAN TUMOR NECROSIS FACTOR PRODUCED BY PERIPHERAL BLOOD MONOCYTES AND ITS SEPARATION FROM LYMPHOTOXIN. 1985;73:69–73.
26. Stamou P, Kontoyiannis DL. Posttranscriptional regulation of TNF mRNA: a paradigm of

signal-dependent mRNA utilization and its relevance to pathology. Curr Dir Autoimmun. 2010;11:61.

27. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloproteinase disintegrin that releases from cells. 1997;385120(February):729–33.
28. Tartaglia LA, Weber RF, Figarit IS, Reynoldst C, Palladino MA, Goeddel D V. The two different receptors for tumor necrosis factor mediate distinct cellular responses. 1991;88(October).
29. Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. Cutting Edge: HMG-1 as a Mediator of Acute Lung Inflammation. J Immunol [Internet]. 2000;165(6):2950–4. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.165.6.2950
30. Tanaka T, Narazaki M, Kishimoto T. IL-6 in Inflammation, Immunity, and Disease. 2014;6(Kishimoto 1989):1–16.
31. Catrysse L, Vereecke L, Beyaert R, van Loo G. A20 in inflammation and autoimmunity. Trends Immunol [Internet]. 2014;35(1):22–31. Available from: http://dx.doi.org/10.1016/j.it.2013.10.005
32. Uchiyama T. Modulation of Immune Response by Bacterial Lipopolysaccharide (LPS): Roles of Macro- phages and T Cells in In Vitro Adjuvant Effect of LPS on Antibody Response to T Cell-Dependent and T Cell- Independent Antigens. 1982;26(3):213–25.
33. Alexander C, Rietschel ET. Invited review: Bacterial lipopolysaccharides and innate immunity. J Endotoxin Res. 2001;7(3):167–202.
34. Qureshi ST, Larivière L, Leveque G, Clermont S, Moore KJ, Gros P, et al. Endotoxin-tolerant mice have mutations in toll-like receptor 4 (Tlr4). J Exp Med. 1999;189(4):615–25.
35. Janardhan KS, McIsaac M, Fowlie J, Shrivastav A, Caldwell S, Sharma RK, et al. Toll like receptor-4 expression in lipopolysaccharide induced lung inflammation. Histol Histopathol. 2006;21(7–9):687–96.
36. Kawai T, Akira S. Toll-like receptor downstream signaling. Arthritis Res Ther. 2005;7(1):12–9.
37. Hoeven JG Van Der, Netea MG, Pickkers P. Gender differences in the innate immune

response and vascular reactivity following the administration of endotoxin to human volunteers*. 2007;35(6).

38. Haegens A, Heeringa P, van Suylen RJ, Steele C, Aratani Y, O'Donoghue RJJ, et al. Myeloperoxidase Deficiency Attenuates Lipopolysaccharide-Induced Acute Lung Inflammation and Subsequent Cytokine and Chemokine Production. J Immunol. 2009;182(12):7990–6.
39. Toews GB. Cytokines and the lung. Eur Respir Journal, Suppl. 2001;18(34):3–17.
40. Card JW, Carey MA, Bradbury JA, DeGraff LM, Morgan DL, Moorman MP, et al. Gender Differences in Murine Airway Responsiveness and Lipopolysaccharide-Induced Inflammation. J Immunol. 2006;177(1):621–30.
41. Jagielo PJ, Thorne PS, Watt JL, Frees KL, Quinn TJ, Schwartz DA. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. Chest. 1996;110(1):263–70.
42. Kirkhorn SR, Garry VF. Agricultural lung diseases. Environ Health Perspect. 2000;
43. Bharadwaj L, Senthilselvan A, Dosman JA, Gusikoski C. Acute changes in lung function in farmers exposed to grain dust. J Agric Saf Health. 1999;5(2):207–14.
44. Brass DM, Savov JD, Gavett SH, Schwartz DA, Subchronic DAS. Subchronic endotoxin inhalation causes persistent airway disease. 2018;0001:755–61.
45. Vernooy JHJ, Dentener MA, Van Suylen RJ, Buurman WA, Wouters EFM. Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. Am J Respir Cell Mol Biol. 2002;26(1):152–9.
46. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-α-activated vascular endothelium under flow. Blood. 2005;106(2):584–92.
47. Andreotti G, Koutros S, Hofmann JN, Sandler DP, Lubin JH, Lynch CF, et al. Glyphosate Use and Cancer Incidence in the Agricultural Health Study. 2018;110:509–16.
48. Szekacs A, Darvas B. Forty Years with Glyphosate. Herbicides - Properties, Synthesis and Control of Weeds. 2012.
49. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally.

Environ Sci Eur. 2016;28(1):1–15.

50. Giesy JP, Dobson S, Solomon KR. Ecotoxicological Risk Assessment for Roundup® Herbicide. In: Ware GW, editor. Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews [Internet]. New York, NY: Springer New York; 2000. p. 35–120. Available from: https://doi.org/10.1007/978-1-4612-1156-3_2
51. Guyton KZ, Loomis D, Grosse Y, El Ghissassi F, Benbrahim-Tallaa L, Guha N, et al. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. Lancet Oncol. 2015;16(5):490–1.
52. Cressey BD. Widely Used Herbicide Linked to Cancer. 2015;
53. Silva V, Montanarella L, Jones A, Fernández-Ugalde O, Mol HGJ, Ritsema CJ, et al. Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural topsoils of the European Union. Sci Total Environ [Internet]. 2018;621:1352–9. Available from: https://doi.org/10.1016/j.scitotenv.2017.10.093
54. Curwin BD, Hein MJ, Sanderson WT, Striley C, Heederik D, Kromhout H, et al. Urinary pesticide concentrations among children, mothers and fathers living in farm and non-farm households in Iowa. Ann Occup Hyg. 2007;51(1):53–65.
55. Henderson AM, Gervais JA, Luukinen B, Buhl K, Stone D. Glyphosate General Fact Sheet. Natl Pestic Inf Center, Oregon State Univ Ext Serv [Internet]. 2010;1–3. Available from: http://npic.orst.edu/factsheets/glyphogen.html
56. Myers JP, Antoniou MN, Blumberg B, Carroll L, Colborn T, Everett LG, et al. Concerns over use of glyphosate-based herbicides and risks associated with exposures: A consensus statement. Environ Heal A Glob Access Sci Source [Internet]. 2016;15(1):1–13. Available from: http://dx.doi.org/10.1186/s12940-016-0117-0
57. Trinidade A, Kumar S, Haji M, Shakeel M, Leong P. The prevalence of oilseed rape hypersensitivity in a mixed cereal farming population. Clin Otolaryngol. 2010;
58. EPA. Epa--R . E . D . Facts. 1993;1–7.
59. Available U, Www AT, Net G, Result DMAY, Numbers IP. Roundup ® Original Complete Directions for Use AVOID CONTACT OF HERBICIDE WITH FOLIAGE , GREEN STEMS , AS SPECIFIED FOR INDIVIDUAL ROUNDUP READY ® CROPS), THIS IS AN END-USE PRODUCT .

THIS COMPANY DOES NOT INTEND AND HAS NOT REGISTERED IT FOR REFORMULATI.

60. Monsanto Canada. Roundup Transorb HC Liquid Herbicide - Safety Data Sheet. 2013;2010:1–72. Available from: http://www.roundup.ca/_uploads/documents/RoundupTransorbHCLiquid-Canada-English.D14.pdf
61. CAREX Canada. Glyphosate - Occupational Exposures - CAREX Canada [Internet]. Available from: https://www.carexcanada.ca/profile/glyphosate-occupational-exposures/
62. Slager RE, Simpson SL, Levan TD, Poole J a, Sandler DP, Hoppin J a. Applicators in the Agricultural Health Study. J Toxicol Env Heal A. 2010;73(20):1382–93.
63. Health Canada. Pesticide Label Search - Health Canada [Internet]. 2018. Available from: http://pr-rp.hc-sc.gc.ca/ls-re/result-eng.php?p_search_label=&searchfield1=ACT&operator1=CONTAIN&criteria1=Clopyralid&logicfield1=AND&searchfield2=NONE&operator2=CONTAIN&criteria2=&logicfield2=AND&searchfield3=NONE&operator3=CONTAIN&criteria3=&logicfield3
64. Shushkova T, Ermakova I, Leontievsky A. Glyphosate bioavailability in soil. Biodegradation. 2010;21(3):403–10.
65. Corrêa EA, Dayan FE, Owens DK, Rimando AM, Duke SO. Glyphosate-Resistant and Conventional Canola (Brassica napus L.) Responses to Glyphosate and Aminomethylphosphonic Acid (AMPA) Treatment. J Agric Food Chem. 2016;64(18):3508–13.
66. Saunders LE, Pezeshki R. Glyphosate in Runoff Waters and in the Root-Zone: A Review. 2015;462–80.
67. World Health Organization. The Who Recommended Classification of Pesticides By Hazard and Guidelines To Classification 2009. World Heal Organ. 2010;1–60.
68. Van Bruggen AHC, He MM, Shin K, Mai V, Jeong KC, Finckh MR, et al. Environmental and health effects of the herbicide glyphosate. Sci Total Environ. 2018;616–617:255–68.
69. Camacho A, Mejía D. The health consequences of aerial spraying illicit crops: The case of Colombia. J Health Econ [Internet]. 2017;54:147–60. Available from:

http://dx.doi.org/10.1016/j.jhealeco.2017.04.005

70. Kumar S, Khodoun M, Kettleson EM, McKnight C, Reponen T, Grinshpun SA, et al. Glyphosate-rich air samples induce IL-33, TSLP and generate IL-13 dependent airway inflammation. Toxicology. 2014;325: 42-51.

71. Pandey A, Dhabade P, Kumarasamy A. Inflammatory Effects of Subacute Exposure of Roundup in Rat Liver and Adipose Tissue. Dose-Response. 2019;17(2):1–11.

72. Spurzem JR, Romberger DJ, Von Essen SG. Agricultural lung disease. Clin Chest Med. 2002;23(4):795–810.

73. Linaker C, Smedley J. Respiratory illness in agricultural workers. Occup Med (Chic Ill). 2002;52(8):451–9.

74. Poole JA. Farming-associated environmental exposures and effect on atopic diseases. Annals of Allergy, Asthma and Immunology. 2012.

75. Radon K, Opravil U, Hartung J, Szadkowski D, Nowak D. Work-related respiratory disorders and farming characteristics among cattle farmers in Northern Germany. Am J Ind Med. 1999;36(4):444–9.

76. Mackiewicz B, Wsi IM, Dutkiewicz J, Wsi IM, Feltovich H, Thorne PS. ORIGINAL ARTICLES. 2014;(February 1998).

77. Ye M, Beach J, Martin JW, Senthilselvan A. Occupational pesticide exposures and respiratory health. Int J Environ Res Public Health. 2013;10(12):6442–71.

78. Hoppin JA, Umbach DM, London SJ, Henneberger PK, Kullman GJ, Alavanja MCR, et al. Pesticides and atopic and nonatopic asthma among farm women in the agricultural health study. Am J Respir Crit Care Med. 2008;177(1):11–8.

79. Zuskin E, Schachter EN, Kanceljak B, Witek TJ, Fein E. Organic dust disease of airways. Int Arch Occup Environ Health. 1993;65(2):135–40.

80. Short SE, Yang YC, Jenkins TM. Sex, gender, genetics, and health. Am J Public Health. 2013;103(SUPPL.1):93–101.

81. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol. 2016;16(10):626–38.

82. Caracta CF. Gender differences in pulmonary disease. Vol. 70, Mount Sinai Journal of

Medicine. 2003. p. 215–24.

83. Cabello N, Mishra V, Sinha U, Diangelo SL, Chroneos ZC, Ekpa NA, et al. Sex Differences in the Respiratory System Sex differences in the expression of lung inflammatory mediators in response to ozone. 2018;1150–63.

84. Schröder J, Kahlke V, Staubach K-H, Zabel P, Stüber F. Gender Differences in Human Sepsis. Arch Surg [Internet]. 1998 Nov 1;133(11):1200–5. Available from: https://dx.doi.org/10.1001/archsurg.133.11.1200

85. Everhardt Queen A, Moerdyk-Schauwecker M, McKee LM, Leamy LJ, Huet YM. Differential expression of inflammatory cytokines and stress genes in male and female mice in response to a lipopolysaccharide challenge. PLoS One. 2016;11(4):1–13.

86. McClelland EE, Smith JM. Gender specific differences in the immune response to infection. Arch Immunol Ther Exp (Warsz). 2011;59(3):203–13.

87. Chen Y, Horne SL, Dosman JA. Increased susceptibility to lung dysfunction in female smokers. Am Rev Respir Dis. 1991;143(6):1224.

88. Schachter EN, Zuskin E, Moshier EL, Godbold J, Mustajbegovic J, Pucarin-cvetkovic J, et al. Gender and respiratory findings in workers occupationally exposed to organic aerosols : A meta analysis of 12 cross-sectional studies. 2009;16.

89. Fuentes N, Cabello N, Nicoleau M, Chroneos ZC, Silveyra P. Modulation of the lung inflammatory response to ozone by the estrous cycle. 2019;7.

90. Pignataro FS, Bonini M, Forgione A, Melandri S, Usmani OS. Asthma and gender: The female lung. Pharmacol Res [Internet]. 2017;119:384–90. Available from: http://dx.doi.org/10.1016/j.phrs.2017.02.017

91. Center for Disease Control and Prevention. 2018. Most recent asthma data.

92. Oguzulgen IK, Turktas H, Erbas D. Airway inflammation in premenstrual asthma. J Asthma. 2002;39(6):517–22.

93. Speyer CL, Rancilio NJ, McClintock SD, Crawford JD, Gao H, Sarma JV, et al. Regulatory effects of estrogen on acute lung inflammation in mice. Am J Physiol - Cell Physiol. 2005;288(4 57-4).

94. Deshpande R, Khalili H, Pergolizzi RG, Michael SD, Chang MDY. Estradiol down-regulates

LPS-induced cytokine production and NFkB activation in murine macrophages. Am J Reprod Immunol. 1997;38(1):46–54.

95. Demyanets S, Pfaffenberger S, Kaun C, Rega G, Speidl WS, Kastl SP, et al. The estrogen metabolite 17β-dihydroequilenin counteracts interleukin-1α induced expression of inflammatory mediators in human endothelial cells in vitro via NF-κB pathway. Thromb Haemost. 2006;95(1):107–16.
96. Gleicher N, Barad DH. Gender as risk factor for autoimmune diseases. J Autoimmun. 2007;28(1):1–6.
97. Angum F, Khan T, Kaler J, Siddiqui L, Hussain A. The Prevalence of Autoimmune Disorders in Women: A Narrative Review. Cureus. 2020;12(5).
98. Migeon BR. The role of X inactivation and cellular mosaicism in women's health and sex-specific diseases. Jama. 2006;295(12):1428–33.
99. Spolarics Z. The X-files of inflammation: Cellular mosaicism of X-linked polymorphic genes and the female advantage in the host response to injury and infection. Shock. 2007;27(6):597–604.
100. Townsend EA, Miller VM, Prakash YS. Sex differences and sex steroids in lung health and disease. Vol. 33, Endocrine Reviews. 2012. 1–47 p.
101. Jain R, Ray JM, Pan JH, Brody SL. Sex hormone-dependent regulation of cilia beat frequency in airway epithelium. Am J Respir Cell Mol Biol. 2012;46(4):446–53.
102. Raghavan D, Jain R. Increasing awareness of sex differences in airway diseases. Respirology. 2016;21(3):449–59.
103. Thurlbeck WM. Postnatal human lung growth. Thorax. 1982;37(8):564–71.
104. Cai KC, van Mil S, Murray E, Mallet JF, Matar C, Ismail N. Age and sex differences in immune response following LPS treatment in mice. Brain Behav Immun [Internet]. 2016;58:327–37. Available from: http://dx.doi.org/10.1016/j.bbi.2016.08.002
105. Mabley JG, Horváth EM, Murthy KGK, Zsengellér Z, Vaslin A, Benko R, et al. Gender differences in the endotoxin-induced inflammatory and vascular responses: Potential role of poly(ADP-ribose) polymerase activation. J Pharmacol Exp Ther. 2005;315(2):812–20.

106. Kuo SM. Gender difference in bacteria endotoxin-induced inflammatory and anorexic responses. PLoS One. 2016;11(9):1–12.
107. Wichmann MW, Ayala A, Chaudry IH. Male sex steroids are responsible for depressing macrophage immune function after trauma-hemorrhage. Am J Physiol - Cell Physiol. 1997;273(4 42-4):1335–40.
108. Grimaldi CM, Hill L, Xu X, Peeva E, Diamond B. Hormonal modulation of B cell development and repertoire selection. Mol Immunol. 2005;42(7):811–20.
109. Orbach H, Shoenfeld Y. Hyperprolactinemia and autoimmune diseases. Autoimmun Rev. 2007;6(8):537–42.
110. Bouman A, Schipper M, Heineman MJ, Faas MM. Gender difference in the non-specific and specific immune response in humans. Am J Reprod Immunol. 2004;52(1):19–26.
111. Darnall BD, Suarez EC. Sex and gender in psychoneuroimmunology research: Past, present and future. Brain Behav Immun [Internet]. 2009;23(5):595–604. Available from: http://dx.doi.org/10.1016/j.bbi.2009.02.019
112. EL Temple S, Pham K, Glendenning P, Phillips M, Waterer GW. Endotoxin induced TNF and IL-10 mRNA production is higher in male than female donors: Correlation with elevated expression of TLR4. Cell Immunol. 2008;251(2):69–71.
113. Roberts BJ, Dragon JA, Moussawi M, Huber SA. Sex-specific signaling through Toll-Like Receptors 2 and 4 contributes to survival outcome of Coxsackievirus B3 infection in C57Bl/6 mice. Biol Sex Differ. 2012;3(1):1–13.
114. Castro JM de A, Russo M. Use and limitations of noninvasive and invasive methods for studying pulmonary function. Drug Discov Today Dis Model [Internet]. 2019;29–30:3–9. Available from: https://doi.org/10.1016/j.ddmod.2019.07.001
115. Hamelmann E, Schwarze K, Takeda A, Oshiba A, Larsen C, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography [1] (multiple letters). Am J Respir Crit Care Med. 1998;158(1):340–1.
116. DeLorme MP, Moss OR. Pulmonary function assessment by whole-body plethysmography in restrained versus unrestrained mice. J Pharmacol Toxicol Methods. 2002;47(1):1–10.

117. Aulakh GK, Mann A, Belev G, Wiebe S, Kuebler WM, Singh B, et al. Multiple image x-radiography for functional lung imaging. Phys Med Biol. 2018;63(1).
118. Wernick MN, Wirjadi O, Chapman D, Zhong Z, Galatsanos NP, Yang Y, et al. Multiple-image radiography. Phys Med Biol. 2003;48(23):3875–95.
119. Kaur M. Understanding the Structural and Functional Correlates of Acute Lung Inflammation in Two Murine Models. 2022;33(1):1–12.
120. Marriott I, Bost KL, Huet-Hudson YM. Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: A possible mechanism for gender-based differences in endotoxic shock susceptibility. J Reprod Immunol. 2006;71(1):12–27.
121. Pandher U, Kirychuk S, Schneberger D, Thompson B, Aulakh G, Sethi RS, et al. Pulmonary inflammatory response from co-exposure to LPS and glyphosate. Environ Toxicol Pharmacol [Internet]. 2021;86(March):103651. Available from: https://doi.org/10.1016/j.etap.2021.103651
122. Natarajan S, Kim J, Remick DG. Chronic pulmonary lps tolerance induces selective immunosuppression while maintaining the neutrophilic response. Shock. 2010;33(2):162–9.
123. Huxtable. Systemic inflammation impairs respiratory chemoreflexes and plasticity. Bone [Internet]. 2014;23(1):1–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf
124. Sandstrom T, Bjermer L, Rylander R. Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. Eur Respir J. 1992;5(8):992–6.
125. Rose CE, Lannigan JA, Kim P, Lee JJ, Fu SM, Sung SSJ. Murine lung eosinophil activation and chemokine production in allergic airway inflammation. Cell Mol Immunol [Internet]. 2010;7(5):361–74. Available from: http://dx.doi.org/10.1038/cmi.2010.31
126. Angele MK, Pratschke S, Hubbard WJ, Chaudry IH. Gender differences in sepsis: Cardiovascular and immunological aspects. Virulence. 2014;5(1):12–9.
127. Howie SEM. Go girls! Efficient female innate immunity. Blood. 2011;118(22):5718–9.

128. Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. Blood. 2011;118(22):5918–27.
129. Pan Z, Chang C. Gender and the regulation of longevity: Implications for autoimmunity. Autoimmun Rev [Internet]. 2012;11(6–7):A393–403. Available from: http://dx.doi.org/10.1016/j.autrev.2011.12.004
130. Aulakh GK. Neutrophils in the lung: “the first responders.” Cell Tissue Res. 2018;371(3):577–88.
131. Beck-Schimmer B, Madjdjpour C, Kneller S, Ziegler U, Pasch T, Wüthrich RP, et al. Role of alveolar epithelial ICAM-1 in lipopolysaccharide-induced lung inflammation. Eur Respir J. 2002;19(6):1142–50.
132. Gao H, Liang M, Bergdahl A, Hamrén A, Lindholm MW, Dahlman-Wright K, et al. Estrogen attenuates vascular expression of inflammation associated genes and adhesion of monocytes to endothelial cells. Inflamm Res. 2006;55(8):349–53.
133. Aomatsu M, Kato T, Kasahara E, Kitagawa S. Gender difference in tumor necrosis factor-α production in human neutrophils stimulated by lipopolysaccharide and interferon-γ. Biochem Biophys Res Commun [Internet]. 2013;441(1):220–5. Available from: http://dx.doi.org/10.1016/j.bbrc.2013.10.042
134. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biol Reprod. 2008;78(3):432–7.
135. Girón-González JA, Moral FJ, Elvira J, García-Gil D, Guerrero F, Gavilán I, et al. Consistent production of a higher T(H)1:T(H)2 cytokine ratio by stimulated T cells in men compared with women. Eur J Endocrinol. 2000;143(1):31–6.
136. Schuijs MJ, Willart MA, Vergote K, Gras D, Ege MJ, Madeira FB, et al. Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells Published by : American Association for the Advancement of Science Linked references are available on JSTOR for this article : digitize , preserve and extend access to Science Farm dust dust and endotoxin protect Farm and endotoxin protect against allergy allergy

through induc against through induction in cells in lung lung epithelial epithelial cells Aflergic is such characterized by eosinophil responses asthma. 2023;

137. Miller L, Hunt JS. Regulation of TNF-alpha production in activated mouse macrophages by progesterone. J Immunol [Internet]. 1998;160(10):5098–104. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9590261

138. Klein SL. The effects of hormones on sex differences in infection: From genes to behavior. Neurosci Biobehav Rev. 2000;24(6):627–38.

139. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: When a chromosome makes the difference. Nat Rev Immunol [Internet]. 2010;10(8):594–604. Available from: http://dx.doi.org/10.1038/nri2815

140. Zuk M. The sicker sex. PLoS Pathog. 2009;5(1):1–3.

141. Zhong Z, Thomlinson W, Chapman D, Sayers D. Implementation of diffraction-enhanced imaging experiments: at the NSLS and APS. Nucl Instruments Methods Phys Res Sect A Accel Spectrometers, Detect Assoc Equip. 2000;450(2):556–67.

142. Gao W, Li L, Wang Y, Zhang S, Adcock IM, Barnes PJ, et al. Bronchial epithelial cells: The key effector cells in the pathogenesis of chronic obstructive pulmonary disease? Respirology. 2015;20(5):722–9.

143. Glauser MP, Zanetti G, Baumgartner JD, Cohen J. Septic shock: pathogenesis. Lancet. 1991;338(8769):732–6.

144. Asti C, Ruggieri V, Porzio S, Chiusaroli R, Melillo G, Caselli GF. Lipopolysaccharide-induced lung injury in mice. I. Concomitant evaluation of inflammatory cells and haemorrhagic lung damage. Pulm Pharmacol Ther. 2000;13(2):61–9.

145. Caulin-Glaser T, Farrell WJ, Pfau SE, Zaret B, Bunger K, Setaro JF, et al. Modulation of circulating cellular adhesion molecules in postmenopausal women with coronary artery disease. J Am Coll Cardiol [Internet]. 1998;31(7):1555–60. Available from: http://dx.doi.org/10.1016/S0735-1097(98)00145-4

146. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Chronic tumor necrosis factor-α inhibition enhances NO modulation of vascular function in estrogen-deficient rats. Hypertension. 2005;46(1):76–81.

147. Hawes D, Shi S-R, Dabbs DJ, Taylor CR, Cote RJ. Immunohistochemistry as a laboratory test. Immunohistochemistry. 2020;(January):48–70.
148. Drescher H, Weiskirchen S, Weiskirchen R. Flow cytometry: A blessing and a curse. Biomedicines. 2021;9(11):1–12.
149. Dar AM, Kapil S, Goyal SM. Comparison of immunohistochemistry, electron microscopy, and direct fluorescent antibody test for the detection of bovine coronavirus. J Vet Diagnostic Investig. 1998;10(2):152–7.