PULMONARY INFLAMMATION AFTER EXPOSURE TO LPS AND GLYPHOSATE

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ABSTRACT

Background: Agricultural workers experience chronic respiratory effects due to regular exposure to their work-place environments. The adaptation in respiratory inflammatory response is well recognized in workers after chronic agricultural exposures; however, decline in worker's lung function continues at a higher rate than any other industry exposed worker. Agricultural work-place environments are complex and include a multitude of molecules. Two of the most common agricultural exposures are endotoxin (lipopolysaccharide, LPS) and glyphosate. LPS is well studied, whereas the lung inflammatory capability of glyphosate is poorly understood. Further, the lung inflammatory effects to combined exposure to LPS and glyphosate have not been studied. We hypothesized that exposure to the combination of LPS and glyphosate would induce additive or synergistic lung inflammatory response as compared to individual exposures. The primary aim was to study the lung inflammation after exposure to glyphosate alone and in a combination to LPS for single and repeated exposures.

Objectives: Utilize a mouse model to: i) characterize the pulmonary inflammation after single and repeated exposure to glyphosate; and ii) evaluate the differences in pulmonary inflammation after single and repeated exposure to combined LPS and glyphosate, glyphosate alone and LPS alone.

Methods: Male mice of C57BL/6 strain were intranasally treated with saline, LPS (0.5 μg/ml), glyphosate (1 μg/ml), or a combination of LPS (0.5 μg/ml) and glyphosate (1 μg/ml). Exposures were conducted for one day, or daily for five days, and 10 days, excluding weekends. Bronchoalveolar lavage (BAL) fluid was assessed for cellular and cytokine changes and lungs were processed for histology, mRNA, and protein analysis.

Results: Repeated exposure to glyphosate (five-days and 10-days) showed infiltration of inflammatory cells in lungs with significantly higher neutrophil counts, and eosinophil marker. Increases in IL-4, IL-5 and IL-13 observed after 10-days of glyphosate exposure compared to one-day or five-days exposure. Expression of ICAM-1, VCAM-1 and vWF increased in lungs after glyphosate exposure. Exposure to a combination of LPS and glyphosate synergistically increased

lung inflammation markers as compared to individual exposures to glyphosate or LPS. Short-term (five-days) repeated exposure to the combination of LPS and glyphosate resulted in robust accumulation of inflammatory cells in the perivascular, peribronchiolar and alveolar regions of the lungs. The inflammatory changes were characterized such as significantly higher total leukocytes counts comprised predominantly of neutrophils; higher expression of ICAM-1; 70-fold greater expression of TLR-2, higher levels of proinflammatory mediators (TNF-α, KC, IL-6) and myeloperoxidase (MPO); and intense immune-staining of CD45+ B and CD3+ T lymphocytes in the perivascular region of the lungs in comparison to the same length of exposure to LPS alone and glyphosate alone. Longer-term exposure (10-days) to a combination of LPS and glyphosate resulted in generally reduced expression of inflammatory markers; however, accumulation of inflammatory cells including CD45+ B and CD3+ T lymphocytes specifically around the perivascular regions, persisted. Longer-term exposure to the combination of LPS and glyphosate resulted in IL-4 increases that remained higher in comparison to the individual exposure groups. MPO and IL-6, although lower than the five-day levels, remained higher as compared to the other exposure groups.

Conclusions: Repeated exposure to glyphosate at exposure levels relevant for agricultural workers induces lung inflammation. Moreover, repeated exposure to the same dose of glyphosate in combination to LPS induces synergistic effect on lung inflammation as induced by glyphosate alone or LPS alone. Longer-term exposure to the combination of glyphosate and LPS induces reduction in inflammatory response with the persistence in accumulation of inflammatory cells in lungs, suggesting an adaptive immune response. Repeated exposure to the combination of agents in a mouse could be used as a animal model to study chronic inflammatory adaptation response in lungs that may help to explain the respiratory effects caused by complex agricultural exposures.

Keywords: Glyphosate, LPS, Combination, Repeated exposure, Lung inflammation

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Thank you!

DEDICATION

To

My Loving Family

With the Grace of GOD

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LIST OF ABBREVIATIONS

%	Percentage
A20	Tumor necrosis factor alpha-induced protein 3 (TNFAIP3)
A549	Human alveolar epithelial cells
AHR	Airway Hyperresponsiveness
ANOVA	Analysis of variance
B	Bronchus
B cells	B lymphocytes
BAL	Bronchoalveolar lavage fluid
BV	Blood vessels
CCHSA	Canadian Centre for Health and Safety in Agriculture
CD	Cluster of differentiation
cDNA	Complementary DNA
cm^3	Cubic centimeter
CO_2	Carbon dioxide
COPD	Chronic obstructive lung disease
Ct	Threshold cycle
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide

E. coli	*Escherichia. coli*
ELISA	Enzyme linked immunosorbent assay
EPO	Eosinophil peroxidase
EU	Endotoxin Units
FBS	Fetal bovine serum
FEF	Forced Expiratory Flow
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
g	Gram
Gly	Glyphosate
H&E	Hematoxylin and eosin
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution/saline
Hsp72	Heat shock protein 72
ICAM-1	Intercellular adhesion molecule-1
IFN-y	Interferon-gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin

KC	Keratinocyte chemoattractant
Kg	Kilogram
l/min	Litre per minute
LBP	Lipopolysaccharide binding proteins
LPS	Lipopolysaccharides
LPS+Gly	Combined LPS and glyphosate
M	Molar
m^3	Cubic meter
MCP	Monocyte chemoattractant protein
mg	Milligram
Min.	Minutes
MIP	Macrophage inflammatory protein
mm	Millimeter
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MYD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
ng	Nanogram
NH_3	Ammonia

nm	Nanometer
^{o}C	Degree Celsius
OR	Odds ratio
PA	Pulmonary artery
PBS	Phosphate buffered saline
PEF	Peak expiratory flow
PFA	Paraformaldehyde
PIMMs	Pulmonary intravascular macrophages
PKC	Protein kinase C
PMN	Neutrophils
ppm	Parts per million
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
TLR-2	Toll-like receptor-2

TLR-4	Toll-like receptor-4
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	Toll/IL-1 receptor-domain-containing adaptor inducing IFN-β
VCAM-1	Vascular adhesion molecule-1
vWF	Von willebrand factor
WBP	Whole-body plethysmography
µ	Micron

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Exposures in agricultural work environments are a risk for respiratory diseases such as work-related asthma. Agricultural workers, in general, reported asthma related symptoms such as a cough, phlegm, shortness of breath, and chest tightness and showed progressive decline in lung function (1–5). Depending upon the type of agricultural exposures, differential respiratory responses were observed in agricultural workers such that animal confinement workers show greater decline in lung function than grain workers and both groups of farmers show greater decline in lung function than non-agricultural workers (2,4,6–8).

Individuals having no history of exposure to the agricultural environment show strong pulmonary inflammatory response (higher cell counts and proinflammatory cytokines such as interleukin (IL)-6, IL-8 in sputum, blood or nasal lavage) on initial exposure to an agricultural environment, and the response is greater than the response of workers regularly exposed to agricultural environments (9–12). The agricultural work environment is composed of multiple contaminants capable of inducing pulmonary inflammation and inflammatory effects in agricultural workers (13,14).

Glyphosate and endotoxin are two common contaminants in the agricultural work environment. Glyphosate is a common active ingredient in agricultural herbicides (15–18). Glyphosate treatments in in-vitro experiments has been shown to cause toxic effects on various human organ cells (19–21). Mice intranasally exposed to glyphosate for seven days showed increases in airway inflammatory markers including eosinophils (lungs), neutrophils (Bronchoalveolar lavage fluid; BAL), IL-5 (BAL), IL-13 (serum), IL-33 (serum), and IL-10 (serum) cytokines and lung pathology (22). The inflammatory potential of glyphosate when it is inhaled repeatedly, such as in the case of respiratory exposure levels and patterns of agricultural workers, have not been studied.

Endotoxin (Lipopolysaccharide, LPS) is an ubiquitous agricultural contaminant, however, it accounts for only a fraction of the inflammatory response from agricultural exposures (23–26). The agricultural environment is a complex mixture of contaminants in which the inflammatory potential from the combination of exposures is not fully understood.

Co-exposure to endotoxin and glyphosate is common in the agricultural environment (22,27,28); however, it is not known if the inflammatory response is altered when these agents are co-presented to the respiratory system, versus, if they are presented as a single agent. Unravelling the inflammatory potential from co-exposure to common agricultural agents is important for understanding the respiratory response experienced by exposed workers.

The aim of this thesis was to characterize pulmonary inflammation after exposure to agricultural relevant doses of glyphosate, alone, and in combination with LPS after single and repetitive exposure periods. This work demonstrated, for the first time, the lung inflammatory potential of a single exposure to glyphosate. Further, this work demonstrated the differential lung inflammatory response after exposure to the combination of LPS and glyphosate as compared to glyphosate or LPS alone.

This chapter aims to comprehensively review the literature on; a) epidemiological evidence on respiratory outcomes in agricultural workers commonly exposed to glyphosate and endotoxin; b) glyphosate exposure and pulmonary inflammation; c) LPS exposure and pulmonary inflammation; and d) co-exposure to LPS and glyphosate.

1.2 Respiratory health effects in agricultural workers

As early as 1555, occupational exposures in agriculture were recognized as a risk for respiratory diseases in workers (29). Work-related asthma is well recognized in agricultural workers (5,27,30–35). Work-related asthma is a common occupational lung disease and can result in significant morbidity in affected individuals (36). Work-related asthma accounts for 16 % (95 % CI, 10 - 22 %) of cases of adult asthma and contributes to approximately one in six adult asthma cases (36–39).

Long-term agricultural exposures have been associated with chronic respiratory symptoms such as cough, phlegm, and wheezing (1–3,6–8,40). Furthermore, prior exposure to the agricultural environment appears to play a role in the inflammatory response. Individuals with no prior exposure to the agricultural environment (i.e., naïve to agricultural exposures) showed a greater increase of leukocytes and proinflammatory cytokines (IL-6 and IL-8) in sputum, blood or nasal lavage upon exposure to the agricultural environment as compared to individuals with a history of agricultural exposures (6,7,11,41). Workers regularly exposed to the agricultural work environment show an attenuation of inflammatory response over time (11,13,42). However, lung function continues to decline over time with the greatest decline in those exposed to a more complex environment, such that swine workers have greater longitudinal decline in lung function as compared to grain farmers, and both have greater lung function decline than those not exposed to an agricultural work environment (2,4,6,7,12,27,30,43). Currently, neither is there evidence to explain the respiratory inflammatory effects induced from complex agricultural respiratory exposures nor to help explain how inflammation and lung pathology alter with repeated agricultural exposures, specifically when glyphosate is combined with endotoxin.

1.3 Immunopathogenesis of work-related respiratory outcomes: agricultural workplace exposures

Asthma is considered a chronic inflammatory disease having multiple phenotypes and immunological mechanisms (44). Work-related asthma is a common type of adult-onset asthma. Work-related asthma is the most common work-related respiratory disease in many countries (39). Work-related asthma comprises occupational asthma and work-exacerbated asthma (39). Occupational asthma refers to new onset asthma caused by work exposures. Work-exacerbated asthma is preexisting asthma that is made worse by working conditions. Occupational asthma can further be distinguished by whether it appears after a latency period of exposure necessary for an individual to acquire sensitization to an agent, or without the latency period. Single exposure to a very high concentration of an irritant in the workplace can induce a non-immunologic asthma without the latency period (i.e., irritant-induced asthma)

(45). Approximately 15 to 25 % of new agricultural workers experience work-related asthma with elements of both occupational asthma and work-exacerbated asthma (5,33,34,46–48), while other respiratory conditions remain common in experienced worker. The immunopathogenesis of respiratory health outcomes in relation to agricultural exposures is not understood.

The agricultural environment is heterogenous and includes airborne contaminants such as organic dust, allergens, pesticides, microbes, and components like endotoxin and other biologically active molecules (14). Agricultural agents which can sensitize or irritate the airway and induce inflammatory effects can be either high molecular weight or low molecular weight agents. Allergenic agents such as pollens, grasses, and proteins derived from wheat etc. can induce allergic asthma phenotype, involving airway inflammation with eosinophils, T helper (Th) 2 cytokines (IL-4, IL-5 and IL-13) and IgE mediated sensitization mechanism (49–51). In contrast, a predominant neutrophilic response with the release of proinflammatory cytokines (tumour necrosis factor (TNF)-α, IL-6 and IL-8) has been shown after agricultural environment exposures in particular grain dust or swine dust exposures (9,10,12,42,52–55).

Pesticides are low molecular weight agents to which agricultural workers are commonly exposed. Glyphosate is the most common active ingredient in herbicides used for weed control across the globe (27,56). Epidemiological studies have associated the use of glyphosate with exacerbation of existing asthma in agricultural workers (27,32,57). The pathogenesis of respiratory diseases caused by low molecular weight agents such as glyphosate is not known (58). Most of the low molecular weight agents cause sensitization through non-IgE mediated mechanisms (58–60). Therefore, it is possible that respiratory exposure to glyphosate may induce non-IgE mediated respiratory effects.

Endotoxin is ubiquitous in the agricultural environment and there is evidence that endotoxin exposure can induce occupational asthma and work-exacerbated asthma (48,59,60). Endotoxin is a high molecular weight agent and is a potent inflammatory agent. Endotoxin exposure in early life has been associated with protection against the development of asthma (49,61). Endotoxin exposure has been associated with respiratory symptoms and longitudinal

lung function decline in agricultural workers (62–68). The underlying pathology from endotoxin exposure is different from that observed in “classic” allergic/atopic asthma and does not include sensitization and eosinophil involvement (69). An endotoxin exposure mediated acute inflammatory response involves various cytokines including TNF-α, IL-6, and IL-8, and the subsequent massive recruitment and activation of neutrophils in the lower and upper airways (70–73).

Endotoxin is well defined for its airway inflammatory effects. There remains very limited data on the airway inflammatory effects of glyphosate exposure. In agricultural workplaces exposure to a combination of endotoxin and glyphosate would be common; however, the airway inflammatory effects of the combined exposures to these agricultural agents are not known. It is not known if the inflammatory system has a differential response when these common agricultural respiratory agents are presented individually versus in combination.

1.4 Glyphosate exposure in agriculture and pulmonary effects

Glyphosate exposure is common in agricultural work environments and the inflammatory and toxic effects on lungs are not clear.

1.4.1 Glyphosate and herbicide

Glyphosate is the most common active ingredient in herbicides which are highly applied on crops worldwide (15,18). About 826 million kgs of glyphosate was used worldwide in 2014, and > 90% of it applied in the agriculture sector (15). Glyphosate is a N-(phosphonomethyl) glycine as per International Union of Pure and Applied Chemistry nomenclature, and its empirical formula is $C_3H_8NO_5P$. The molecular weight of glyphosate is 169.07 g/mol. Glyphosate is a broad-spectrum herbicide and kills weeds by inhibiting action of the EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase) enzyme involved in the shikimate pathway (15). Glyphosate is applied in different formulations along with other ingredients including an adjuvant. For example, RoundUp is the world’s most widely used commercial herbicide and it includes the isopropylamine salt of glyphosate and the polyethoxyethylene amine (POEA) adjuvant (15,16). RoundUp is used as a pre- and post-emergence herbicide and as a desiccator in crop farming

(74,75). Recent studies have shown that other components within the herbicides are more important in health outcomes than glyphosate (20,76,77); however, little is known about the respiratory inflammatory effects of agricultural workplace exposure to glyphosate.

1.4.2 Glyphosate, environment, and exposure

Glyphosate is ubiquitous in agricultural environments (78) and has been detected in soil (79,80), dust (81,82), air (22), plants (83), foods (84) and animals (85,86). Glyphosate has been shown to dissipate slowly and to be present in soil even 28 days after application (80).

Glyphosate inhalation exposure is most common during its application on crops (47,87). Kumar et al., 2014 detected glyphosate in air samples collected from crop farms at a concentration of 22.59 ng/m^3 (22). Glyphosate has been shown to have an affinity for organic matter, and glyphosate content has been shown to be highest in the smaller, inhalable, particle sizes less than 10 µm (21). It has been estimated that more than 56,000 workers are exposed to glyphosate in Canadian agricultural workplaces (87). Glyphosate has been detected in urine and blood samples of agricultural workers who were involved with the use of glyphosate-based herbicides (17,88,89).

Glyphosate exposure is also likely in agricultural workplaces such as grain handling operations, the animal feed industry and animal farming as these workplaces involve working with grains that could carry residues of glyphosate (87). The residues of glyphosate have been detected on the grains of crops sprayed with glyphosate (83,84) transferred for processing to various crop farming-related secondary operations (81,82). Secondary operations such as the animal feed industry reported the presence of glyphosate residues (85).

1.4.3 Glyphosate exposure and respiratory symptoms in agricultural workers

Glyphosate use by agricultural workers has been associated with allergic and non-allergic wheeze (47), atopic and non-atopic asthma (34) and rhinitis (90). Glyphosate use has been linked with the exacerbation of symptoms of asthma already existing in pesticide

applicators (33). Although agricultural workplace exposures to glyphosate are common, the airway immunopathogenesis of glyphosate exposure is not known.

1.4.4 Glyphosate exposure and pulmonary inflammation: in-vivo studies

Glyphosate exposure (via oral or intraperitoneal route) in animal models has been shown to induce inflammation in the kidney, intestine, spleen, and liver (91–96); however, data are lacking on the ability of glyphosate to induce inflammation in the lungs. There is only a single animal model study on glyphosate exposure and the evaluation of inflammatory markers in lungs (22). Kumar et al., 2014 challenged mice intranasally with 1 µg, 100 µg, and 100 ng of glyphosate for seven days and results showed no dose dependent effect on airway inflammatory markers and lung pathology of exposed mice (22). Glyphosate exposure for 7-days (at 1µg and 100 µg) increased the neutrophils and IL-5 in BAL, eosinophils in lungs and IL-13, IL-33, TSLP, IL-10 except IL-4 in the blood of exposed mice (22). These cytokines, such as IL-5, and IL-13 are mainly derived from T helper type 2 (Th2) cells; IL-33 and TSLP are mainly sourced from epithelial cells and are involved in the recruitment of eosinophils into allergic lungs (97–99). The specific cellular source of cytokines in lungs after glyphosate treatment is unknown. Invitro experiment on glyphosate treatment to lung cells are needed to demonstrate whether glyphosate possesses the ability to directly stimulate the lung cells to release cytokines. Moreover, the lung histology of seven days glyphosate exposed mice (1µg, 100 µg, 100 ng) showed damage to airway epithelium and leukocyte infiltration in parenchymal, peribronchiolar and perivascular regions (22). Similarly, lungs from animal exposed to glyphosate for 21 days (3 times per week for 3 weeks) showed lung histological changes, and release of asthma-related cytokines without any effect on IL-4 (22). Interestingly, glyphosate exposure (1 µg) in IL-13 deficient mice significantly reduced cellular infiltration and IL-5 production in both 7-days and 21-days exposure groups as compared to similarly exposed wild type mice (22). These results suggested the central role of IL-13 in mediating the Th2 cytokine response independent of IL-4 in lungs of glyphosate-exposed mice.

An increase in neutrophils and eosinophils was observed in lungs of mice after glyphosate exposure (22). The leukocyte migrates from circulation into tissue in response to

inflammatory stimuli. The recruitment of inflammatory cells is a multi-step process and is facilitated by adhesion molecules. Leukocyte ligands bind to adhesion molecules on blood vessel endothelium and results in leukocytes rolling, firm adhesion and eventual transmigration into tissue. Upregulation of adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1 and von Willebrand factor (vWF) is known to be important for migration of neutrophils and eosinophils into inflamed lungs (100,101). ICAM-1 and VCAM-1 belong to a family of immunoglobulin-like molecules. The vWF adhesion molecule is stored in the Weibel-Palade bodies of platelets and endothelial cells that form the lining of the blood vessels (159). This protein is exocytosed by endothelial cells constitutively. vWF is involved in binding to various leukocyte ligands, including integrin and P-selectin. ICAM-1, and VCAM-1 are constitutively expressed on endothelial cells and bind to leukocytes via integrin ligands CD11b/CD18 (Mac-1) and CD11a/CD18 (LFA-1) and integrin ligand VLA-4 (α4β1), respectively (102). Antibody blocking of ICAM-1 and VCAM-1 expression has been shown to reduce neutrophils and eosinophil migration into the lungs (103,104). Glyphosate treatment induces migration of neutrophils and eosinophils into lungs of mice; however, there are no data on the expression of adhesion molecules in lungs after glyphosate exposure. Examining the expression of adhesion molecules in lungs could assist in unravelling mechanism of neutrophilic or eosinophilic response after glyphosate exposure. Therefore, we hypothesized that glyphosate treatment at agriculturally relevant levels utilized in a previous study (22) would upregulate the expression of ICAM-1, VCAM-1 and vWF in lungs which would result in recruitment of inflammatory cells into the lungs.

In addition, no data exist on lung inflammation after a single exposure to agriculturally relevant levels of glyphosate. As glyphosate is a low molecular weight agent, unlike LPS, the lung inflammatory potential after a single versus multiple exposures was of interest. This research was designed to explore if a single exposure to a low dose of glyphosate can induce an innate immune response in the lungs. The pulmonary inflammation data from the single glyphosate exposure were compared to repeated exposures to glyphosate to further our understanding about the immune response generated following repeated inhalation exposures to glyphosate.

1.4.5 Glyphosate treatment and cytotoxic effects in lungs: in-vitro studies

The genotoxic, neurologic, cardiovascular and carcinogenic effects of glyphosate and glyphosate-based herbicides have been extensively reviewed (105–107). Glyphosate treatment has been shown to cause cytotoxic effects on various human organ cells (19,105,107); however, data are limited on the effects on human lung cells. Li and colleagues treated human alveolar epithelial cells (A549) for 72 hours with different doses of glyphosate (between 15 mM to 50 mM) dissolved in DMEM media with fetal bovine serum and assessed cytotoxicity using a Cell titer Glu Luminescent Cell Viability assay (108). Glyphosate treatment at 50 mM significantly reduced (about 17 %) viability of A549 cells compared to the control cells (108). In addition, based on linear regression modelling, authors estimated a 136.5 mM concentration of glyphosate as the half maximal (50%) inhibitory concentration (IC50) for A549 cells (108).

RoundUp, a glyphosate containing herbicide formulation has also been studied for its cytotoxic effects (20). The RoundUp herbicide formulation contains the isopropylamine salt of glyphosate, and the adjuvant polyoxyethylene amine (POEA) along with other ingredients. RoundUp, but not isopropylamine salt of glyphosate, showed cytotoxic effects in A549 cells (76). Studies by Hao and colleagues showed that a two hour treatment to isopropylamine salt of glyphosate at 100 µg/mL (0.59 mM) did not show significant toxicity in A549 cells, whereas the adjuvant polyoxyethylene amine (POEA) at 35 µg/mL and RoundUp herbicide at 100 µg/mL induced significant cytotoxicity through DNA damage and mitochondria-associated apoptosis in A549 cells (76,77,109). It is important to note that A549 cells toxicity results by Li et al., 2013 were based on pure glyphosate (108), whereas Hao and colleagues studied the isopropylamine salt of glyphosate present in RoundUp (76,77,109,110). The results indicate that the toxicity effect by the commercial herbicide formulation may be mainly attributed to other ingredients in the formulation, such as adjuvants, rather than glyphosate (111,112).

Overall, the lung inflammatory potential after single and repetitive exposure to glyphosate is not well known, nor is the impact of glyphosate treatment on cytotoxicity of human lung cells.

1.5 Endotoxin exposure in agriculture and pulmonary effects

1.5.1 Endotoxin and agriculture

Agricultural exposure to endotoxin has been associated with respiratory effects in workers and the inflammatory potential and exposure levels of endotoxin (LPS) are well-studied (63,64,113,114).

1.5.2 Endotoxin exposure and association with respiratory effects in agricultural workers

Endotoxin is a primary agent in agricultural organic dust that is associated with respiratory reactions experienced by workers (12,62,63,115,116). Endotoxins are lipopolysaccharides (LPS), found in the outer membrane of most gram-negative bacteria and released into the environment mainly after the lysis of bacteria (117). The term LPS commonly refers to purified endotoxin. A classic LPS molecule is comprised of lipid A, core oligosaccharide, and O antigen polysaccharide; lipid A is mainly responsible for stimulating the immune response (118). LPS purified from *E. coli* gram-negative bacteria is a potent inflammatory agent (118).

Airborne endotoxin is highly associated with adverse respiratory outcomes in exposed agricultural workers (12,62–64,68,115,119). Levels of airborne endotoxin are highly variable in the agricultural environment (66,117,120,121). Airborne endotoxin levels depend upon many factors such as season, ventilation, geography, climate, area of sampling, and type of farming (63,122,123). Endotoxin has been reported in the range of 110 - 1400 EU/m^3 in grain farming environments (66,114,124–126) and 400 - 6600 EU/m^3 in swine farming environments (123,124). Endotoxin levels in extracts have been studied in the range of 0.9 - 6 μg/ml for grain dust extract (24,26,127,128) and 0.325 - 0.875 μg/ml in swine dust extract (13,129). Agricultural workers exposed to airborne endotoxin exhibit respiratory symptoms, such as chronic cough, chronic phlegm, and shortness of breath (23,24,26,62–64,115,130). Various studies have shown significant dose-response relationships between airborne endotoxin levels and lung function decline in grain workers (25) and animal workers (67,131).

1.5.3 LPS activated TLR-4 signaling pathway

Endotoxin (LPS) binds to the TLR-4 receptor and activates the TLR-4 signaling pathway leading to cellular and molecular inflammatory changes in the lungs (132–134). In LPS-induced lung inflammation, TLR-4 expression on the vascular endothelium of lungs has been shown as a direct role for recruitment of neutrophils into lungs (135–137). Historically, TLR-4 was discovered as a receptor for LPS by identifying endotoxin hyporesponsive strains of mice, C3H/HeJ and C57BL/10ScCr, due to point mutation (pro712 to his712) in the TLR-4 gene (113,134). TLR-4 in both humans and mice shows high sequence similarity of about 70% at the nucleotide level and about 80% at the protein level (132,138). The TLR-4 signaling pathway is conserved in both humans and mice (113).

The cell surface binding of LPS and TLR-4 initiates a cascade of signaling events in cytoplasm and releases inflammatory mediators (113). The cascade includes a series of phosphorylation events of various accessory molecules and subsequently allows the nuclear translocation of nuclear factor-κB (NF-κB) transcription factor for induction of inflammatory gene transcription. There are a few reports on LPS binding with the TLR-2 receptor and the subsequent activation of the NF-κB transcription factor (139).

1.5.4 LPS exposure and neutrophilic inflammation and cytokine release

Exposure to LPS has been shown to induce both dose and time dependent effects on airway inflammatory markers and lung pathology (73,140,141). A single exposure to a low dose of LPS (1 ng – 10 μg; intranasal exposure) in a mouse model showed an influx of neutrophils, increases of tissue myeloperoxidase (MPO), and release of proinflammatory cytokines including TNF-α, IL-6, KC in lungs (142–145). Proinflammatory cytokines can be produced by monocytes and macrophages, inflammatory cells, endothelial cells and epithelial cells (146). KC and TNF-α are potent neutrophilic chemoattractant while IL-6 is involved in both pro-inflammatory and anti-inflammatory activities that range from recruitment of mononuclear cells to regeneration of tissue (71,147–150). The pathological manifestations of LPS exposure were neutrophil

infiltration in perivascular, peribronchiolar and alveolar regions, thickening of alveolar septa, edema and other signs of pulmonary inflammation (142,143).

Long-term exposure to LPS has been shown to induce chronic lung inflammatory changes such as infiltration of macrophages, CD4+ T lymphocytes, CD8+ T lymphocytes and B lymphocytes, and increase of mucus producing goblet cells due to metaplasia (72,151,152). The lung inflammatory changes persisted after a recovery period (151). The inflammatory changes induced by LPS exposure are implicated in the pathogenesis of various chronic respiratory diseases of humans, such as asthma (97).

1.5.5 LPS exposure and adhesion molecule expression: ICAM-1, VCAM-1, and vWF

LPS exposure induces upregulation in the ICAM-1, VCAM-1, and vWF expression on endothelial cells (153–156). Expression of ICAM-1 and VCAM-1 on pulmonary endothelial cells has been linked with the migration of neutrophils and eosinophils in LPS induced lung inflammation (157,158). Blocking of expression of ICAM-1 and VCAM-1 has been shown to inhibit migration of neutrophils and eosinophils in lungs (103,104,159). In addition, LPS exposure for acute lung injury increases the expression of vWF, P-selectin, migration of neutrophils, and release of MPO in lungs (160,161).

The pulmonary activation of adhesion molecules including ICAM-1, VCAM-1 and vWF has been implicated in the pathogenesis of asthma in humans and in animal models (101,154,162).

1.6 Pulmonary effects from co-exposure to LPS and glyphosate

Respiratory exposure to LPS and glyphosate, individually, have been shown to induce lung innate immune responses, as discussed previously; however, the inflammatory effects when glyphosate is exposed in combination with LPS has not been studied.

1.6.1 Evidence of co-exposure to endotoxin and glyphosate

The regular activities in agricultural workplaces result in the aerosolization of dust particles into the breathing zone of workers leading to exposure to contaminated aerosols (163). Both endotoxin and glyphosate are present in inhalable components of agricultural aerosols (15,16,22,81,82,88,89). Both endotoxin and glyphosate have been shown to have an affinity in the inhalable fraction of dusts (62,63,164). Exposure levels to endotoxin and glyphosate were shown by Kumar et al., 2014 at an average airborne concentration of 4.87 EU/m^3 and 22.59 ng/m^3 respectively (22).

1.6.2 Pulmonary inflammation from exposure to LPS alone versus co-exposures in agricultural environment

Exposure to agricultural environments has proven to be a much stronger inflammatory stimulus than exposure to LPS alone, in both human and animal model exposure studies (11,23,25,26). Human participants exposed in an animal barn setting had higher IL-6, IL-8 and total cell numbers in sputum as compared to exposure to LPS alone (11). Even when subjects were exposed to LPS alone at dose levels higher than those found in animals barns, the inflammatory response was weaker than that of subjects exposed to the animal barn environment (11). The results suggest LPS alone may not induce as intense an inflammatory response as induced by agricultural exposures. It is possible, and likely, that the combination of agents in agricultural exposures contributes to the intensity of pulmonary inflammation.

Intranasal exposure to extracts of agricultural dust has been shown to induce a stronger pulmonary inflammation as induced by LPS exposure alone (25,128,165,166). Agricultural dusts are a complex exposure and include components of gram positive bacteria, gram negative bacteria and fungi (167,168). Mice exposed to grain dust extract for five hours have shown a strong neutrophilic response and greater release of proinflammatory cytokines such as TNF-α in comparison to mice exposed to LPS alone (128,166). TLR-4 signaling appears to play an important role in the inflammatory response to grain dust and swine dust extract as experiments in TLR-4 knock out mice showed a reduction in neutrophil counts and TNF-α

concentration as compared to exposed wild type mice (128,169). It is likely that multiple components within dust are contributing to an overall stronger neutrophilic response than LPS exposure alone. It is also possible that multiple components within dust potentiate the inflammatory effects induced by a single agent like LPS. The delineation of the immune response after single agent exposures versus after exposure to a combination of agents is important to further our understanding of the inflammatory response from agricultural co-exposures.

Agricultural co-exposures, including dust exposure, have induced intense pulmonary inflammation in comparison to LPS exposure alone (11,128,166). Individual exposure to LPS or glyphosate at agriculturally relevant doses has been shown to induce lung inflammation (22,128). A stronger inflammatory response has been shown from real agricultural environmental exposures compared to the same level of LPS when given alone (11,128,166). Further, when LPS is presented at levels higher than that found in agriculture, the inflammatory response has still been found to be weaker than that induced from real agricultural exposures (11). This finding leads to the question of whether the combination of agents in the agricultural environment could induce additive or synergistic inflammatory effects. Therefore, we hypothesized that exposure to a combination of LPS and glyphosate would induce additive or synergistic effects on lung inflammation compared to LPS or glyphosate alone exposures.

1.6.3 Repeated exposure to LPS alone versus co-exposures in an agricultural environment

The reduction in inflammatory response has been seen after repetitive exposure to LPS alone or agricultural exposures (11,128–130). Repetitive exposure to the farming environment has been shown to result in lower levels of IL-6, IL-8, and total cells in sputum and nasal lavage as compared to levels of individuals exposed for the first time to a farm environment (11,42). Reduction in BAL total cells and neutrophils and lung chronic histological changes including increases in mucus containing goblet cells and activation of bronchus associated lymphoid tissue have been shown after repeated swine barn exposure in mice for 20-days as compared to 1-day and 5-days exposures (130). Repeated exposure to swine dust extract in mice for two weeks resulted in significantly lower levels of proinflammatory mediators including TNF-α, IL-6,

KC, and MIP-2, with mononuclear cellular aggregates composed of CD3+ T-cells, CD45+ B cells, and mononuclear phagocytes as compared to the 1-day and one week exposed mice (129). A 4-day pretreatment of mice with LPS prior to grain dust extract exposure resulted in lower neutrophils counts and TNF-α levels as compared to saline pretreated mice and grain dust extract exposure (128), suggesting that LPS pretreatment modulated the inflammatory response to a grain dust exposure. Overall, these studies suggest an adaptive immune response. The mechanism of adaptation in the immune response after repeated exposure is not known.

We hypothesized that repeated exposure to a combination of LPS and glyphosate would result in a reduction in airway inflammatory markers and changes in lung histology as compared to single day combined exposures.

1.7 Summary of the literature review

The differential respiratory responses have been observed in agricultural workers such that the decline in lung function among animal workers is greater than in the grain workers and the lung function decline in both groups is greater than non-agricultural workers. Adaptation in the immune response is observed in farmers after chronic agricultural exposures; however, long-term agricultural exposure often results in chronic respiratory symptoms and longitudinal decline in the lung function of workers. The lung inflammatory effects resulting from these exposures have not been characterized.

Glyphosate and endotoxin are two common agricultural exposures. Glyphosate is the most common active ingredient in agricultural herbicides and glyphosate inhalation is common during spraying on crops. The residues of glyphosate have been detected on crops and in soil, air, dust, and grains. Glyphosate exposure directly is likely in crop farming when glyphosate is used to spray grain crops. As well, secondary exposure through grains is a part of animal feeding and grain processing. An animal model study on glyphosate respiratory exposure in mice found an increase in airway inflammatory markers after seven days and 21-days (3 times a week for 3-weeks). Glyphosate treatment showed toxic effects in human cells of various

tissues, but data are limited on human lung cells. The pulmonary inflammatory and toxic effects of glyphosate exposure are not clear.

LPS is ubiquitous in the agricultural environment and exposure to LPS is highly associated with respiratory symptoms in grain and animal workers. The lung inflammatory response from LPS exposure is well studied through the TLR-4 signalling pathway. Single and repeated exposure to LPS have shown neutrophil migration, cytokine release and upregulation of ICAM-1, VCAM-1 and vWF adhesion markers in lungs.

Notably, considering the complexity of the agricultural environment, it is reasonable to assume that an agricultural worker will be exposed to multiple contaminants at a given time. The air samples collected from glyphosate sprayed crop farms showed both endotoxin and glyphosate at levels of 4.87 EU/m^3 and 22.59 ng/m^3 respectively (22). It has been demonstrated in both human and animal studies that LPS alone induces a weaker lung inflammatory response compared to complex agricultural environment exposures. These complex agricultural exposures likely include a combination of endotoxin and glyphosate; however, the lung inflammatory impact of exposure to this combination of LPS and glyphosate, as a single dose and after repeated exposure, has not been studied.

1.8 Gaps and rationale

The primary aim of this dissertation was to study the pulmonary inflammation after single and repetitive exposure to glyphosate alone and in combination, with LPS at an agriculturally relevant dose.

The ability of glyphosate to stimulate the lung cells after a single treatment was not known and toxicity data on human lung cells effects from glyphosate were limited. Prior to an animal experiment, in-vitro experiments were planned and conducted using human alveolar epithelial cells (A549 cells) to study the inflammatory and toxic effects of low doses of glyphosate alone, LPS alone, and a combination of LPS and glyphosate. The aim was to rule out the possibility that very low doses of agents alone or in combination would have toxic cellular effects. Cell viability was utilized to assess the toxicity to low doses of these agents (170). The

A549 cells were chosen as these cells are derived from the alveolar epithelium which is exposed to various agents upon their inhalation and these cells have been used by other in-vitro studies on glyphosate cytotoxicity (76,77,108,109). For A549 cellular treatments, serum free DMEM media was used as serum proteins may buffers the toxic effects of the agents due to the protein's ability to bind to agents and subsequently reduces their availability to cells (111,171). Cell viability and measures of IL-8 and IL-6 were used to confirm toxicity and stimulatory effects (172).

The lung inflammatory potential of glyphosate is not well known. In addition, no data exist on lung effects after a single exposure to glyphosate. This research investigated if a single exposure to a low agricultural environment dose of glyphosate can induce sufficient lung cell stimulation to generate an innate immune response in lungs. The pulmonary inflammation data from the single glyphosate exposure were compared to repeated exposures to glyphosate to further our understanding of the immune response generated following inhalation exposures to glyphosate. Further, the research sought to understand the expression of ICAM-1, VCAM-1 and vWF adhesion markers in lungs after glyphosate exposures. Expression of adhesion molecules could assist in unravelling differential inflammatory responses (i.e., eosinophilic, neutrophilic or mixed responses) after glyphosate exposure. Therefore, we hypothesized that glyphosate treatment would upregulate the pulmonary expression of ICAM-1, and VCAM-1 adhesion markers which would be important for recruitment of inflammatory cells into the lungs. Male C57BL/6 mice were intranasally treated with glyphosate at 1 µg for one day or daily for five days and 10-days, excluding weekend days. Control exposure mice were treated with Hank's Balanced Salt Solution (HBSS or saline) as it was used to dissolve glyphosate before treatments in the mice. Experimental design and dose of glyphosate were kept similar to a previous study on glyphosate exposure by Kumar and colleagues (22). Importantly, this research examined different exposure periods than those previously studied (22) and chose different patterns of exposure to more closely mimic the work-shift schedule of agricultural workers (14,130). At the end of exposure periods, respiratory parameters were measured by using whole body plethysmography chambers. Analysis of blood, BAL fluid and lung tissue from the glyphosate treated group of mice were compared with the HBSS treated control group. Cytokines

important to innate and adaptive immune response and shown to be important by others in response to glyphosate and LPS treatments were analyzed. The expression of adhesion markers ICAM-1, VCAM-1 and vWF in lungs of glyphosate-exposed mice were analyzed through immunohistochemistry by using previously used monoclonal antibodies against these markers (173,174). It is unknown whether glyphosate affect the expression of genes related to TLR signaling pathway. Therefore, broad-spectrum expression profiling of genes related to the TLR pathway was undertaken. Receptors of interest were further confirmed with primer based real-time PCR for specific receptors and subsequently used to confirm specific gene expression changes after glyphosate exposure.

The second gap addressed in this research was to test the lung immune response on exposure to the combination of LPS and glyphosate as compared to LPS or glyphosate alone at agriculturally relevant doses, and to evaluate the inflammatory effects after repeated exposure. Male C57BL/6 mice were intranasally treated with combined or individual LPS and glyphosate for one day or daily for five days and 10-days. The experimental design of the experiment with mice, including its glyphosate dose, was kept similar to the glyphosate alone study. The dose of LPS (0.5 µg) was calculated using Guyton's formula: the relevant airborne endotoxin levels commonly found in agricultural environments (50 µg/m^3); the minute volume of a mouse (average weight 20 g); and an 8-hour period (166,175). The dose of 0.5 µg LPS matches to endotoxin levels in grain dust extract utilized in previous studies (24,26,127,128). The doses of both LPS and glyphosate were kept constant for the individual and combined LPS and glyphosate exposure groups. As these experiments were the first to study the inflammatory potential of combined LPS and glyphosate, the aim was to broadly examine the spectrum of inflammatory changes in blood, BAL fluid and lung tissue of treated mice. The monoclonal antibodies against CD45+ B and CD3 lymphocyte markers were selected as these were previously used for immunohistochemical staining of lung tissue (129).

Chapter 2

HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

a) Single and repeated exposure to glyphosate increases pulmonary inflammation.

b) Glyphosate exposure would upregulate the expression of ICAM-1, VCAM-1 and vWF adhesion molecules in lungs.

c) Exposure to a combination of LPS and glyphosate would induce additive or synergistic effect on pulmonary inflammation as compared to individual LPS or glyphosate exposures.

d) Repeated exposure to a combination of LPS and glyphosate would result in a reduction in airway inflammatory markers and changes in lung histology as compared to a single day combined LPS and glyphosate exposure.

2.2 Objectives

a) To utilize a mouse model to characterize the pulmonary inflammation after single and repeated exposure to glyphosate.

b) To utilize a mouse model to evaluate the differences in pulmonary inflammation after single and repeated exposure to combined LPS and glyphosate, glyphosate alone and LPS alone.

CHAPTER 3

LUNG INFLAMMATION AFTER SINGLE AND REPEATED EXPOSURE TO GLYPHOSATE (MANUSCRIPT I)

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3.1 Abstract

Glyphosate is an active ingredient in herbicides used worldwide in agriculture. Exposure to glyphosate has been associated with respiratory dysfunctions in agricultural workers; however, the ability of glyphosate to induce inflammation in the lung is not well studied. There is no data on pulmonary expression of endothelial adhesion molecules after glyphosate exposure which regulates the recruitment of leukocytes. Therefore, we exposed C57BL/6 mice intranasally to saline or glyphosate (1 μg/40 μl) for one day or once daily for five days and 10-days. Lung tissue of exposed mice were used for histology, immunostaining of adhesion molecules ICAM-1, VCAM-1 and vWF and for quantification of eosinophil peroxidase, a marker for eosinophils. Bronchoalveolar lavage (BAL) fluid was analyzed for leukocytes counts and cytokine changes. Single exposure to glyphosate did not show increase in total and differential leukocyte counts and cytokine release in the BAL fluid as compared to control exposure. Repeated glyphosate exposure for five days and 10-days showed an increase of neutrophils in BAL fluid and eosinophil marker in lungs. Leukocyte infiltration in the lungs was further confirmed through lung histology. Th2 cytokines including IL-4, IL-5 and IL-13 were increased in BAL fluid after 10-days of glyphosate exposure. Lung sections from all glyphosate groups showed higher immunostaining for ICAM-1, VCAM-1, and vWF adhesion molecules. Expression of innate immune receptor TLR-4 and TLR-2 was increased in the lungs after repeated glyphosate. We conclude that repeated exposure to glyphosate induces migration of eosinophils and neutrophils and release of Th2 cytokines. This study provides first evidence for the role of ICAM-1, VCAM-1 and vWF in the lungs of glyphosate-treated animals. Overall, our study suggest that glyphosate may modulate the inflammatory response to other agricultural contaminants, at least after repeated exposures.

Keywords: Glyphosate; Repeated exposure; Lung inflammation; Adhesion markers

3.2 Background

Glyphosate [N-(phosphonomethyl) glycine] is the most common active ingredient in herbicides. Glyphosate-based herbicides are extensively used worldwide in agriculture (15). Glyphosate has been detected in urine samples of agricultural workers and their family members, indicating exposure to glyphosate (17,82,89).

Glyphosate use has been associated with increased risk of rhinitis and allergic and non-allergic wheeze among pesticide applicators (34,35,47,90,176). Moreover, glyphosate use is linked with exacerbation of existing asthma in workers (33). Glyphosate has been detected at a level of 17.33 µg in air from farms sprayed with glyphosate (22). Although there is evidence of glyphosate exposure as a risk factor for human respiratory problems, the ability of glyphosate to induce inflammation in lungs is not well studied.

Recently, Kumar and colleagues challenged female C57BL/6 mice intranasally with different doses of glyphosate (100 ng, 1 µg or 100 µg) for seven days and did not observe dose dependent effects of glyphosate on markers of airway inflammation and lung pathology (22). Glyphosate exposure for seven days (1 µg or 100 µg) induced an increase of neutrophils in the bronchoalveolar lavage (BAL) fluid, eosinophils in lungs, and IL-5 (BAL fluid), IL-13, IL-10, IL-33 and TSLP cytokines in blood samples of challenged mice (22). Glyphosate exposures in IL-13 deficient mice (for 7-days or 3-times per week for total 3 weeks; 1 µg) showed reduction in leukocyte infiltration, and IL-5 release, suggesting central role of IL-13 for lung inflammatory changes (22). The leukocyte recruitment is facilitated through an increase in expression of endothelial adhesion molecules due to the release of cytokines. The pulmonary endothelial cells showed constitutive expression of various adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and von Willebrand factor (vWF) (100). However, there are no data on the expression of adhesion molecules in the lungs of glyphosate-exposed animals. We hypothesized that glyphosate exposure would upregulate the ICAM-1, VCAM-1 and vWF expression in lungs which would results in the recruitment of inflammatory cells in lungs.

The present study was designed to characterize the lung inflammation induced with exposure to an agriculturally relevant dose of glyphosate (1 µg) for different periods (1-day, 5-days and 10-days) by using mouse model. Our data showed that repetitive exposure to glyphosate increases the inflammatory markers and expression of adhesion molecules in the lungs.

3.3 Materials and Methods

3.3.1 Mice exposures

The experimental protocols were approved by the Animal Ethics Research Board of the University of Saskatchewan (Protocol # 20160106). Male C57BL/6 mice (Charles River Laboratories, Montreal, QC Canada), 6-8 weeks old, were maintained at the Laboratory Animal Services Unit of the University of Saskatchewan. Mice were fed ad libitum and were acclimatized for one week after arrival.

Mice were divided into glyphosate and control treatment groups (n = 5 per group). The 1 µg dose of glyphosate treatment was selected based on glyphosate levels found in the agricultural environment and has been utilized in other studies (22). The stock solution of glyphosate (0.8 M; analytical grade PESTANAL standard, Sigma, St. Louis, MO USA) was prepared in Hank's Balanced Salt Solution (HBSS). It was vortexed for 10 minutes and syringe filtered (0.22 µm; Fisher Scientific). Mice received 40 µL of either glyphosate (1 µg/40 µl) or saline intranasally for one day or daily for five days or 10-days. Mice were lightly anesthetized using isoflurane before treatments. There were no differences in the weight of mice in the control and the glyphosate-treated groups. Four hours after the last treatment, mice were euthanized humanely by CO_2 inhalation, and BAL fluid and lung samples were collected.

3.3.2 Bronchoalveolar lavage collection and leukocyte counts

BAL fluid was collected by washing the airways three times with 0.5 ml ice-cold HBSS. The collected BAL fluid was centrifuged at 1000 g for 10 minutes at 4° C, and supernatants were stored at -80° C for cytokine analysis. Cells from the BAL fluid were resuspended in HBSS and

kept on ice until used for leukocyte counts. The total and differential leukocyte counts in BAL fluid were performed using a hemocytometer and cytospin stained with Protocol Hema 3 kit (ThermoFisher Scientific, Waltham, MA USA), respectively.

3.3.3 Cytokine levels

Multiple cytokines in BAL fluid were measured using a Custom Mouse Procartaplex Multiplex Immunoassay (ThermoFisher Scientific, Waltham, MA USA), according to manufacturer's instructions for magnetic bead-based ELISA. Plates were read using a Bioplex 200 system (Bio-Rad, Mississauga, ON Canada) and Bioplex Manager Software (Bio-Rad, Mississauga, ON Canada).

3.3.4 Lung tissue collection and processing

Following BAL fluid collection, the right lung was tied off at the primary bronchus, and the left lung was fixed in-situ through intratracheal instillation of 200 µl of 4% paraformaldehyde (PFA). The right lung was removed and snap-frozen in liquid nitrogen and stored at - 80^0 C for eosinophil peroxidase (EPO) and RNA analysis. The fixative-instilled left lung was further submerged in 4% PFA for 16 hours at 4^0 C. Lung tissue was then washed through ascending grades of alcohol before embedding in paraffin. Lung sections of 5 µm thickness were cut from paraffin-embedded tissues. Hematoxylin and eosin (H&E) staining, and immunohistochemistry were performed on these lung sections.

3.3.5 Eosinophil peroxidase quantification

Lung tissues were homogenized using 2 mm Zirconia beads (BioSpec, Bartlesville, OK USA) in tubes containing RIPA lysis buffer supplemented with 1X Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA USA) in a Mini- Beadbeater-24 homogenizer (BioSpec, Bartlesville, OK USA) for two, 1-minute rounds. The tubes were cooled on ice in between rounds of homogenization. The total protein concentration of lung homogenates was determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. EPO was quantified using a

Mouse Eosinophil Peroxidase DuoSet ELISA (LifeSpan Biosciences, Seattle, WA USA). Plates were read using a BioTek Synergy HT plate reader (BioTek, Winooski, VT USA) at 450 nm.

3.3.6 Histology and scoring for lung inflammation

Lung sections from all the mice were stained with H&E stain. Two stained lung sections from each mouse were reviewed and scored for lung inflammation. Each of the sections on the slide was reviewed at different magnifications (x20, x40, x100). Each section's scoring was performed by multiple reviewers blinded to the exposure groups, and scores were averaged. Cellular infiltration in alveolar, perivascular, and peribronchiolar compartments of lungs were scored. Each parameter was given a score based on the intensity and was statistically analyzed (0: absent, 1: mild, 2: moderate, 3: severe).

3.3.7 Immunohistochemistry and analysis

Lung sections from all mice were stained with antibodies against ICAM-1, VCAM-1, and vWF markers. Briefly, lung sections were immersed in a series of xylene baths for deparaffinization and different alcohol grades for rehydration. Endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in methanol for 20 minutes. Antigen unmasking and blocking were done for 30 minutes with 2 mg/ml pepsin and 1% bovine serum albumin, respectively. The lung sections were incubated overnight at 4^0 C with the following primary antibodies: ICAM-1 (dilution 1: 100; rabbit monoclonal anti-mouse ICAM-1, ab79707, Abcam Inc., ON Canada), VCAM-1 (dilution 1:100; rabbit monoclonal anti-mouse VCAM-1, ab134047, Abcam Inc., ON Canada), and vWF (dilution 1:200; rabbit monoclonal anti-mouse vWF, ThermoFisher Scientific, Waltham, MA USA). Following overnight incubation, the secondary goat anti-rabbit antibody (dilution 1:200; ThermoFisher Scientific, Waltham, MA USA) was added onto tissue sections. Slides were incubated for one hour at room temperature in a humidified chamber. The color was developed using a peroxidase kit (Vector laboratories, Burlington, ON Canada) according to manufacturer’s instructions and counterstained with methyl green (Vector laboratories, Burlington, ON Canada). In the end, slides were dehydrated through a series of ethanol concentrations and were fixed with xylene before mounting.

Controls with the omission of the primary antibody or secondary antibody were run at the same time.

The expression of ICAM-1, VCAM-1, and vWF was reviewed in five random fields of the lung sections from each mouse (N = 3). Expression of ICAM-1 was scored in the vasculature and bronchial epithelium. Each parameter staining was given a score based on staining intensity by a reviewer blinded to exposure groups (0: no or occasional staining, 1: weak staining, 2: moderate staining, 3: intense staining).

3.3.8 RNA isolation and real-time PCR

Lung homogenates were prepared using 2 mm Zirconia beads (BioSpec, Bartlesville, OK USA) in tubes containing RLT lysis buffer (Qiagen, Chatsworth, CA USA) in a Mini-Beadbeater-24 homogenizer (BioSpec, Bartlesville, OK USA). RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA USA) according to the manufacturer's instructions. Purified mRNA was quantified 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) with 0.5 µg mRNA. PCR was conducted in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA) using the following protocol: 25^0 C for five minutes, 46^0 C for 20 minutes, and 95^0 C for one minute.

Real-time PCR was performed using probes for mouse ICAM-1 (Mm00516023_m1), TLR-4 (Mm00445273_m1), and TLR-2 (Mm00442346_m1). Each reaction was carried out in duplicate using ribosomal RNA (Life Technologies, Grand Island, NY USA) as an endogenous control. PCR was conducted in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA). PCR reactions were carried out as follows: 50° C for two minutes, 95° C for 10 minutes followed by 40 cycles at 95° C for 15 seconds and 60° C for one minute. Relative quantification was estimated from each target gene's cycle threshold obtained from real-time PCR data followed by analysis with the ΔΔCt method.

3.3.9 Data analysis and statistics

Data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Error bars represent mean +/- standard deviation (SD). For values outside the assay limit of detection, either the LLOD/2 (lowest limit of detection) or a minimum value below the lowest attained value was designated. Statistical significance was determined using one-way ANOVA with a follow-up Tukey test for multiple comparisons. If the assumption of equal variance was not met, the data were either log-transformed, followed by one-way ANOVA and multiple comparison tests, or a non-parametric Kruskal-Wallace test was conducted. A p-value < 0.05 was considered significant for differences between groups. For graphing of data, "a" indicates a significant difference compared with the control group; "1" indicates a significant difference compared with the 1-day exposure group; "2" indicates a significant difference compared with the 5-days exposure group.

3.4 Results

3.4.1 Leukocyte counts in bronchoalveolar lavage fluid

After 1-day of glyphosate exposure, total and differential leukocyte counts in BAL fluid were not significantly different as compared to 1-day control exposure (Figure 3.1A, B). However, for 5-days glyphosate exposure group, total leukocyte counts were significantly higher as compared to 5-days control exposure group. Counts were further higher after 10-days glyphosate exposure and were significantly higher as compared to 1-day glyphosate exposure.

In differential leukocyte counts neutrophil counts were significantly higher in both 5-days and 10-days glyphosate exposure groups compared with respective time-matched control exposure groups (Figure 3.1C, D). Macrophage and lymphocyte counts were not significantly different between any of the glyphosate exposure groups.

3.4.2 Cytokine levels in bronchoalveolar lavage fluid

After 1-day or 5-days of glyphosate exposure, none of the cytokine levels were significantly different as compared to the respective time-matched control exposure (Figure 3.2A-K). However, for 10-days glyphosate exposure group, IL-5 and IL-13 levels were

significantly higher as compared to 10-days control exposure group. Furthermore, IL-5 level in 10-days glyphosate exposure group was significantly higher than 1-day glyphosate exposure group whereas IL-4 level in 10-days glyphosate exposure group was significantly higher from both 1-day and 5-days glyphosate exposure groups.

3.4.3 Eosinophil peroxidase in lungs

EPO is released by activated eosinophils and acts as a surrogate marker for the presence of eosinophils. In all glyphosate exposure groups i.e., 1-day, 5-days and 10-days, EPO levels in lungs were significantly higher as compared to the respective time-matched control exposure (Figure 3.3).

3.4.4 Lung histology

Lungs of control mice showed normal architecture after 1-day (Figure 3.4A), 5-days (Figure 3.4B), and 10-days (Figure 3.4C) of exposure. After 1-day of glyphosate exposure, there was little infiltration of leukocytes, occasional sloughing, and increased thickness of bronchial epithelium in the lungs (Figure 3.4D, G). In both 5-days (Figure 3.4E, H) and 10-days (Figure 3.4F, I) glyphosate exposure groups, lung sections had greater infiltration of leukocytes including alveolar, perivascular, and peribronchiolar region infiltration. In addition, both group of lung sections, 5-days, and 10-days glyphosate exposure groups, showed cellular binding to the vascular endothelium, occasional sloughing, and increased thickness of the bronchial epithelium.

Semi-quantification data revealed that perivascular leukocyte infiltration in lung sections of 1-day glyphosate exposure group was significantly higher compared to the 1-day control exposure group (Figure 3.4J). In both 5-days and 10-days glyphosate group lung sections, there was significantly higher leukocyte infiltration in the perivascular, peribronchiolar (Figure 3.4K), and alveolar (Figure 3.4L) regions as compared to the respective control exposure.

3.4.5 ICAM-1, VCAM-1, and vWF staining

Lungs of control mice in 1-day, 5-days, and 10-days exposure groups showed positive endothelial staining in large blood vessels for ICAM-1 (Figure 3.5A-C), VCAM-1 (Figure 3.6A-C), and vWF (Figure 3.7A-C). Staining for all these adhesion molecules was minimal in the bronchial epithelium and alveolar septa regions of the lungs of control mice.

After 1-day (Figure 3.5D), 5-days (Figure 3.5E), or 10-days (Figure 3.5F) of glyphosate exposure, there was increased ICAM-1 immunostaining for bronchial epithelium, alveolar septa, and endothelium of large blood vessels in lungs. Semi-quantification data showed that lung sections from 1-day, 5-day and 10-days glyphosate exposure groups had significantly higher ICAM-1 staining in the blood vessel endothelium as compared to the respective control lung section staining (Figure 3.5G). ICAM-1 staining in bronchial epithelium of 5-days and 10-days glyphosate exposure groups was significantly higher compared to control group staining (Figure 3.5H).

Immunostaining for VCAM-1 (Figure 3.6D-F) and vWF (Figure 3.7D-I) was also increased in the bronchial epithelium, alveolar septa, and large blood vessel endothelium in lung sections of 1-day, 5-days, and 10-days glyphosate groups.

3.4.6 ICAM-1, TLR-4, and TLR-2

After 1-day of glyphosate exposure, there was no significant difference in expression of ICAM-1 (Figure 3.8A), TLR-4 (Figure 3.8B), and TLR-2 (Figure 3.8C) in the lungs as compared to the 1-day control exposure. However, for 5-days glyphosate exposure group, expression of ICAM-1, TLR-4 and TLR-2 was significantly higher as compared to 5-days control exposure. In addition, expression of all these molecules in 5-days glyphosate groups (ICAM-1, TLR-4 and TLR-2), were significantly higher as compared to the 1-day glyphosate exposure group. After 10-days of glyphosate exposure, only TLR-4 expression was significantly higher than 10-days control exposure.

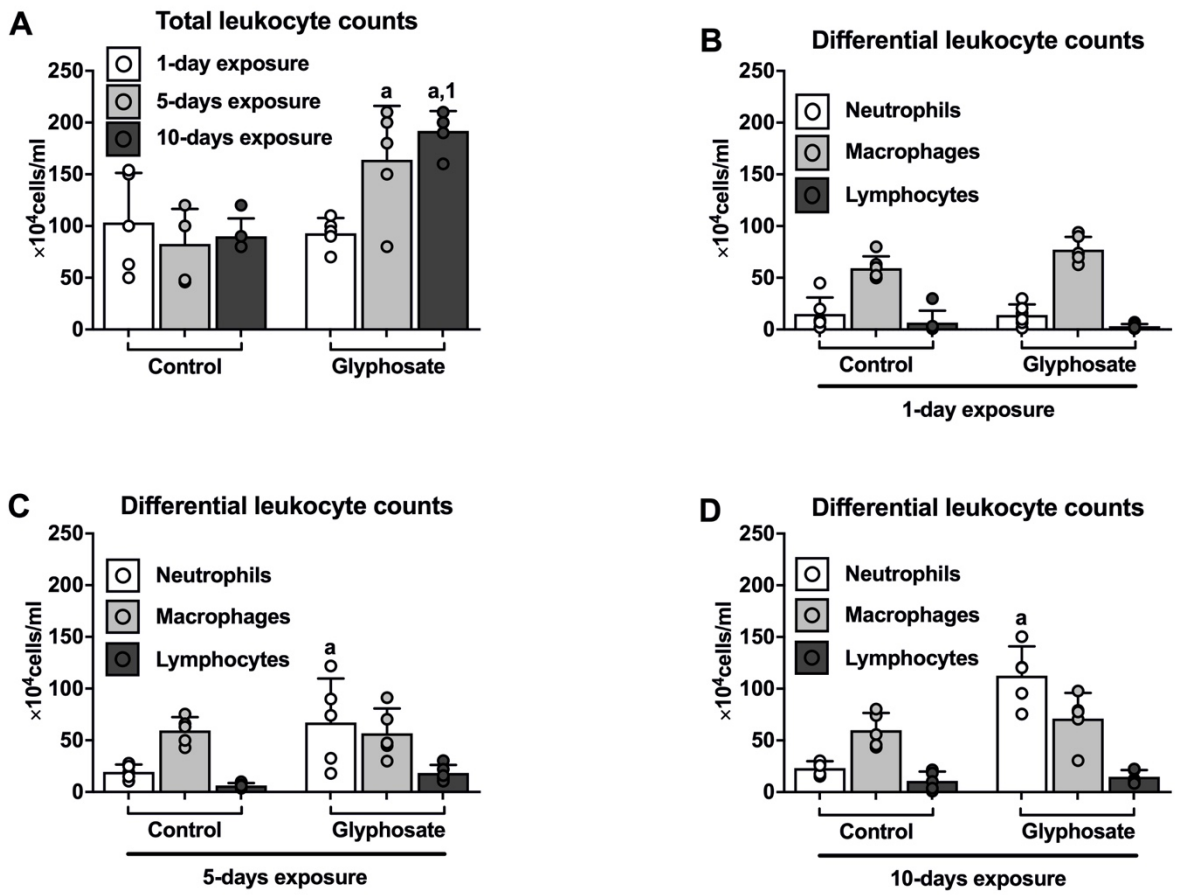

Figure 3.1: Leukocyte counts in bronchoalveolar lavage fluid. Total leukocyte counts (A) and differential leukocyte counts (B-D) were measured in BAL fluid of mice after exposure to control or glyphosate for 1-day, 5-days and 10-days. Total leukocyte counts were measured using hemocytometer and differential leukocyte counts were measured on cytospins stained with Protocol Hema 3 kit. Data presented as mean ± SD (N = 5 mice per group). Significance ($p < 0.05$) is denoted as such: “a” indicates a significant difference compared with the control group; “1” indicates a significant difference compared with 1-day exposure group.

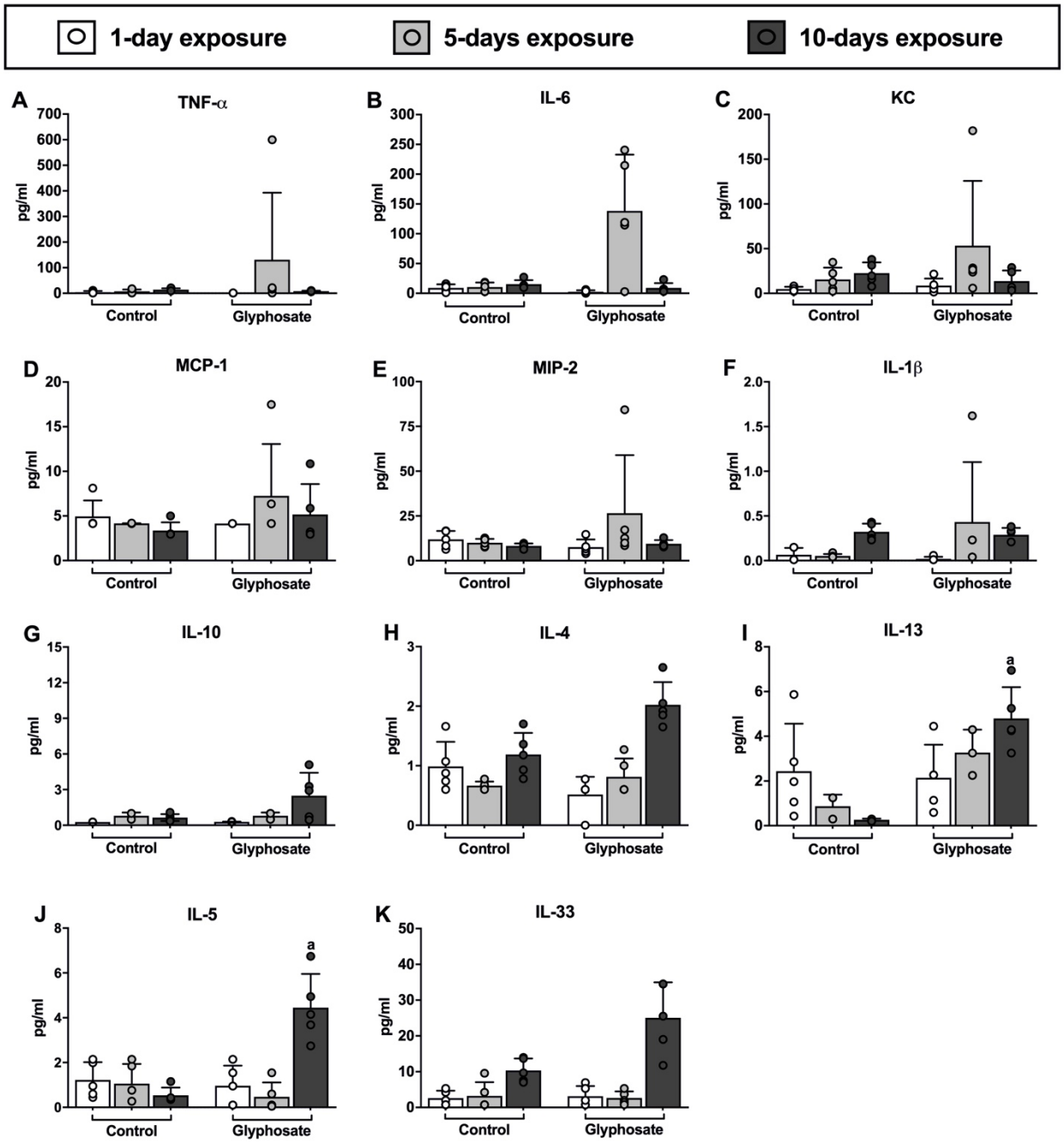

Figure 3.2: Cytokine levels in bronchoalveolar lavage fluid. Cytokines were measured in supernatant of BAL fluid collected from mice after exposure to control or glyphosate for 1-day, 5-days, and 10-days (A-K). Proteins levels were assessed using a Custom Mouse Procartaplex Multiplex Immunoassay. Data presented as mean ± SD (N = 5 mice per group). “a” indicates a significant difference ($p < 0.05$) compared with the control group.

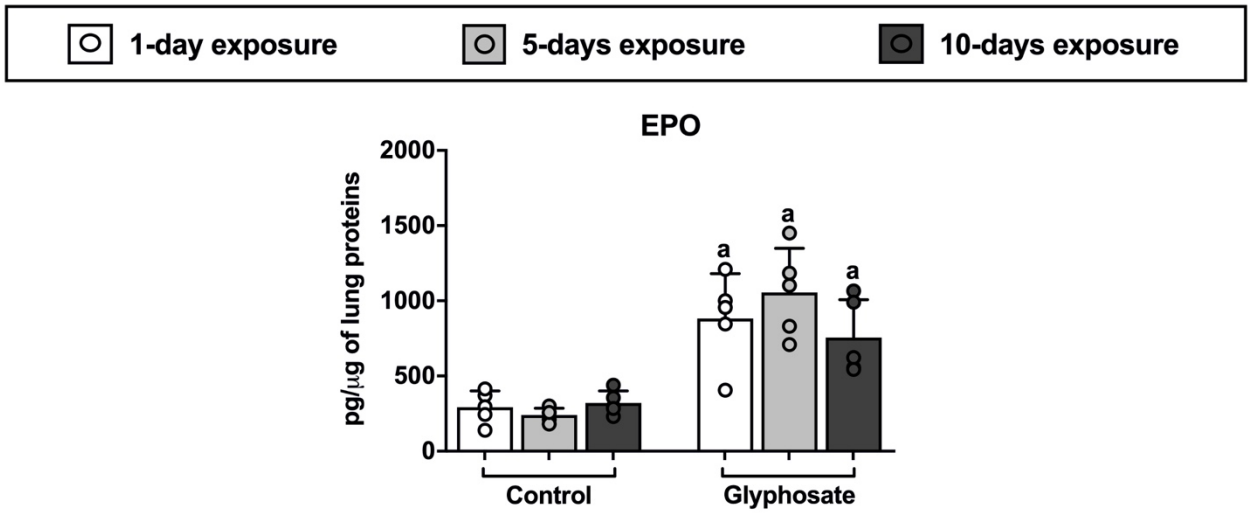

Figure 3.3: Eosinophil peroxidase levels in lungs. Eosinophil peroxidase (EPO) levels were measured in lungs of mice after exposure to control or glyphosate for 1-day, 5-days, and 10-days. Protein levels were measured using Mouse Eosinophil Peroxidase ELISA. Data presented as mean ± SD (N = 5 mice per group). “a” indicates a significant difference ($p < 0.05$) compared with the control group.

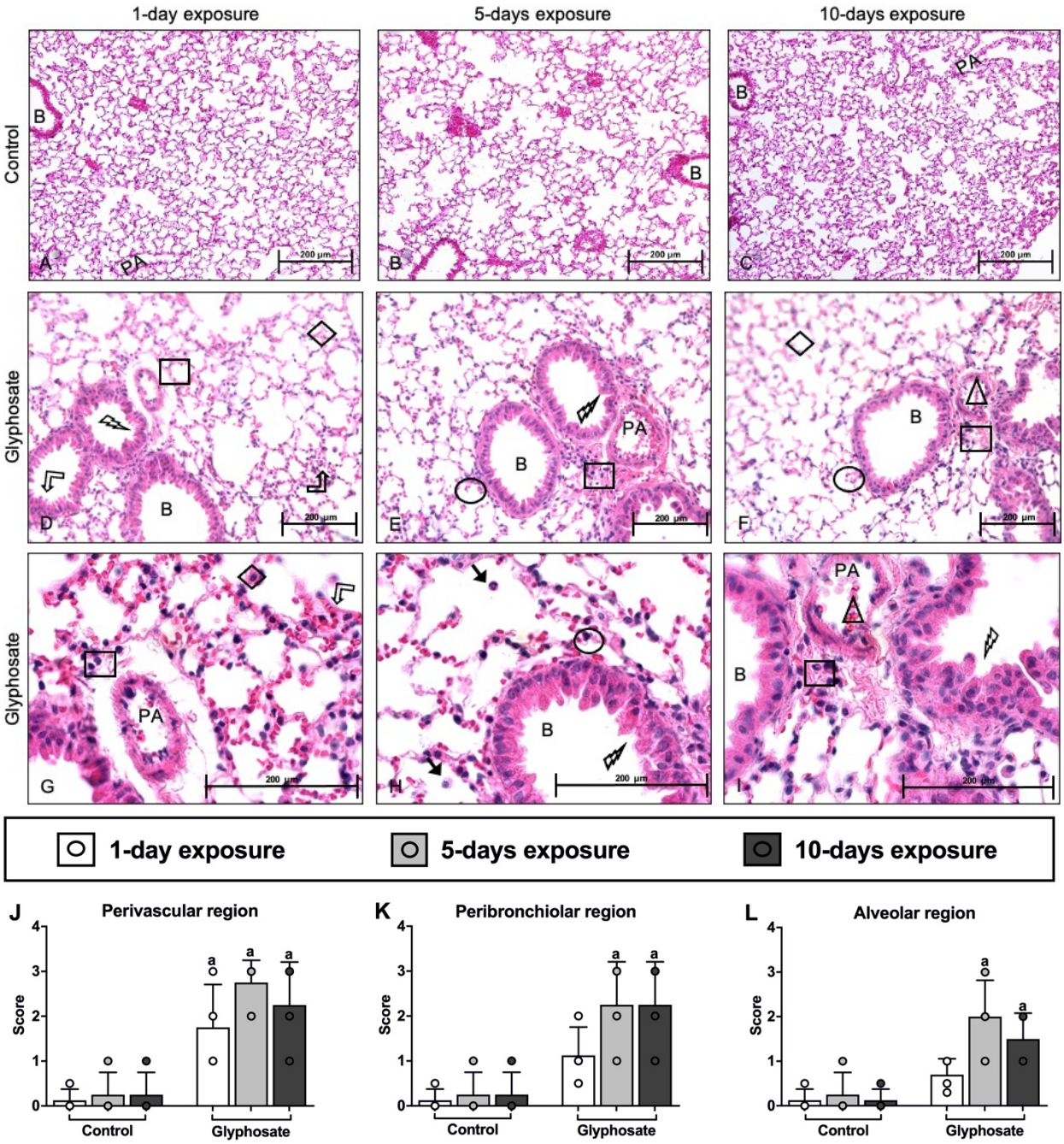

Figure 3.4: Hematoxylin and eosin-staining of lung sections of mice. Hematoxylin and eosin-stained lung sections of mice after exposure for 1-day, 5-days and 10-days to control (A-C) or glyphosate (D-I) and scoring of lung sections for inflammation (J-L). Group representative images showing perivascular infiltration (square), peribronchiolar infiltration (circle), alveolar infiltration (diamond), perivascular space increase (double arrow), blood vessel congestion (triangle), bronchial epithelium thickness increase (bent up arrow) and sloughing of bronchial

epithelial surface (lightning bolt). Data presented as mean ± SD (J-L, N = 5 mice per group; 5 fields per section). “a” indicates a significant difference ($p < 0.05$) compared with the control group. Magnification: ×200 (A-C); ×400 (D-F); ×1000 (G-I). Scale bar: 200 µm (A-I). PA: Pulmonary artery; B: Bronchus.

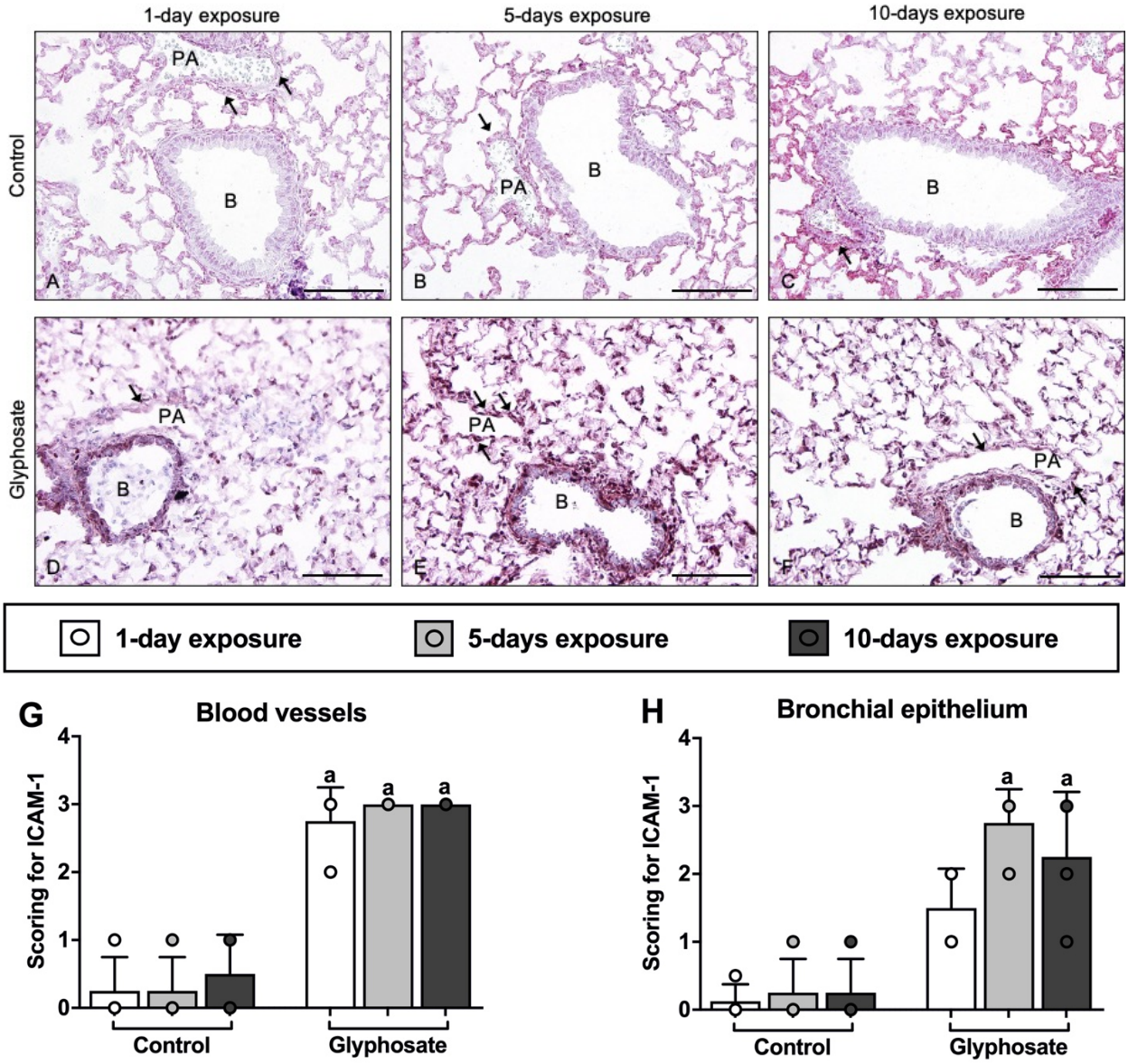

Figure 3.5: Expression and quantification of ICAM-1 in lung sections of mice. Immunohistochemical expression of ICAM-1 in lung sections of mice after 1-day, 5-days and 10-days of exposure to control (A-C) or glyphosate (D-F) and its scoring (G-H). Arrow indicates ICAM-1 positive staining in group representative images. Data presented as mean ± SD (N = 3 mice per group; 5 random fields per section). "a" indicates a significant difference ($p < 0.05$) compared with the control group. Magnification: ×400 (A-F). Scale bar: 50 µm (A-F). PA: Pulmonary artery; B: Bronchus.

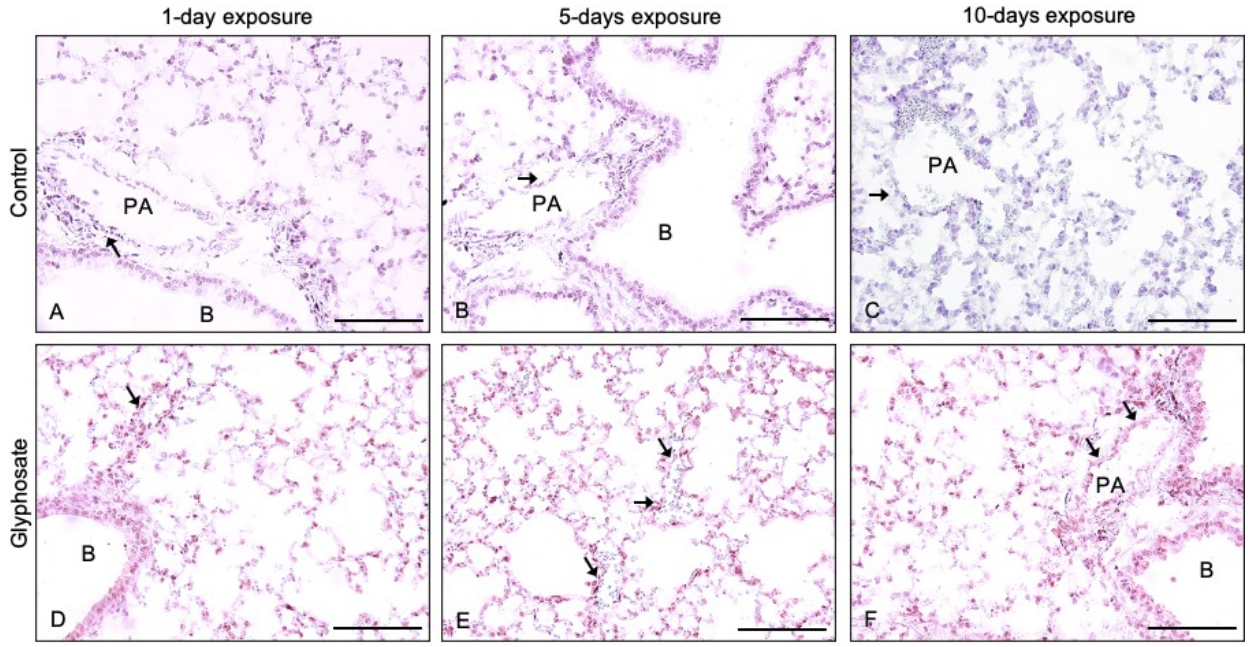

Figure 3.6: Expression of VCAM-1 in lung sections of mice. Immunohistochemical expression of VCAM-1 in lung sections of mice after 1-day, 5-days, and 10-days of exposure to control (A-C) or glyphosate (D-F). Arrow indicates VCAM-1 positive staining in group representative images. Magnification: ×400 (A-F). Scale bar: 50 µm (A-F). PA: Pulmonary artery; B: Bronchus.

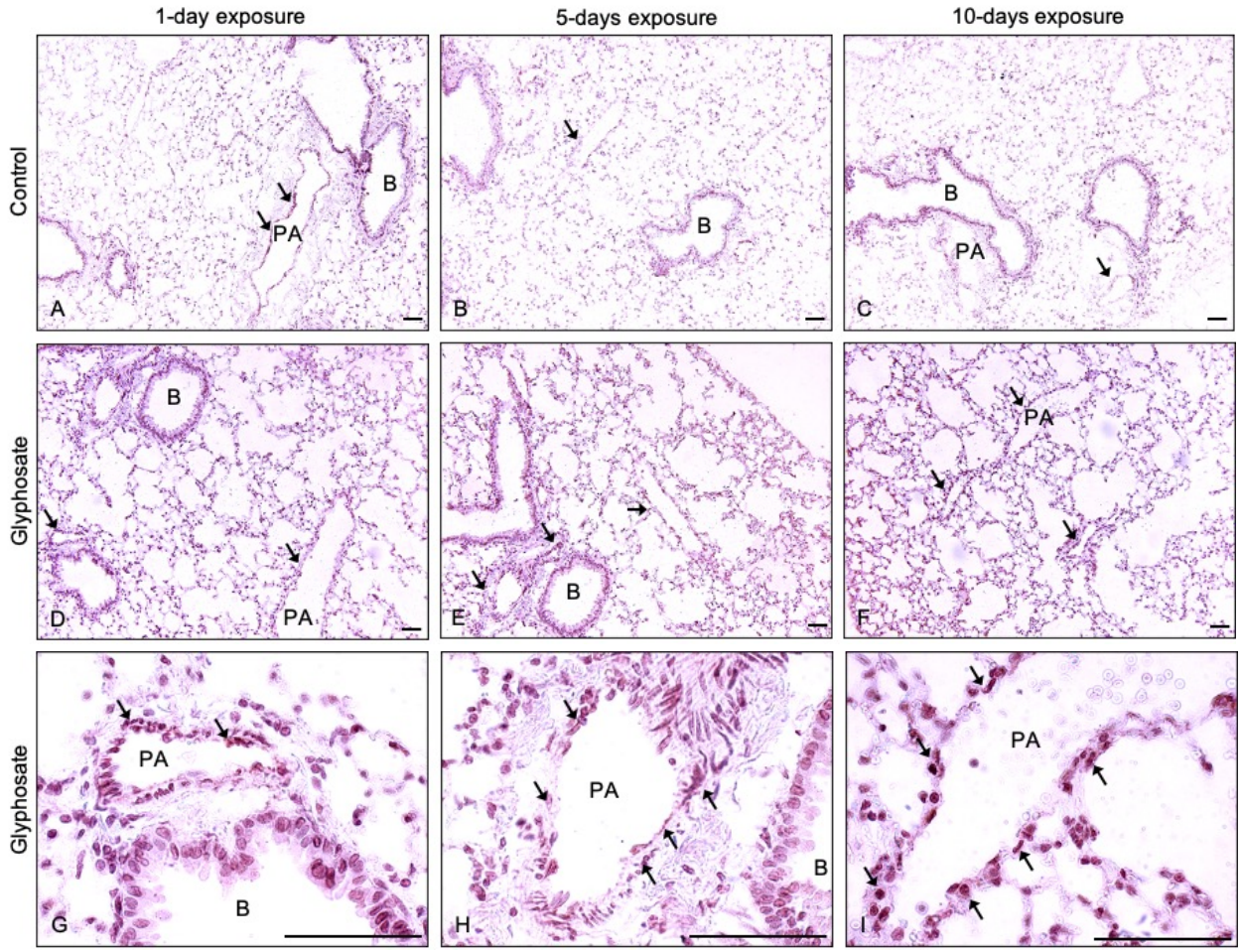

Figure 3.7: Expression of vWF in lung sections of mice. Immunohistochemical expression of vWF in lung sections of mice exposed for 1-day, 5-days, and 10-days to control (A-C) or glyphosate (D-I). Arrow indicates vWF positive staining in group representative images. Magnification: ×400 (A-F). Scale bar: 50 µm (A-F). PA: Pulmonary artery; B: Bronchus.

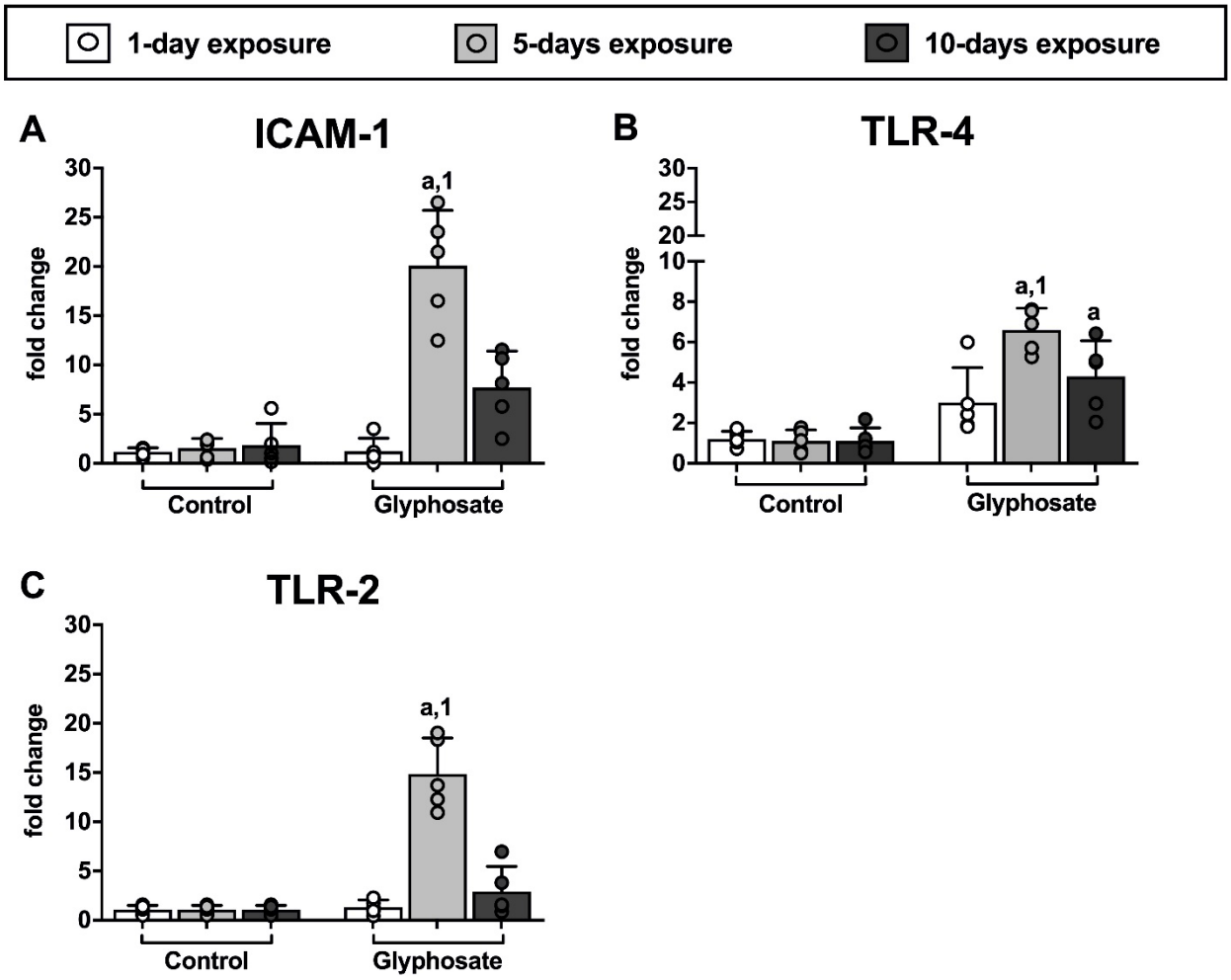

Figure 3.8: Real time PCR for expression of ICAM-1, TLR-4, and TLR-2 in lungs. Fold change expression of ICAM-1 (A), TLR-4 (B), and TLR-2 (C) in lungs of mice after exposure to control or glyphosate for 1-day, 5-days, and 10-days. Data presented as mean ± SD (N = 5 mice per group). Significance ($p < 0.05$) is denoted as such: “a” indicates a significant difference compared with the control group; “1” indicates a significant difference compared with 1-day exposure group.

3.5 Discussion

This is the first study where mice were treated with glyphosate for single and multiple days to study its effect on lung inflammation. The data reported in this paper show that multiple exposure to glyphosate induced migration of eosinophils and neutrophils and release of IL-4, IL-5 and IL-13 cytokines. Immunostaining for ICAM-1, VCAM-1, and vWF was increased in the alveolar septa and endothelium of large blood vessels in all glyphosate treated lungs, suggesting their role in leukocyte migration after glyphosate exposure. These data add to the evidence on the ability of glyphosate to induce lung inflammation and therefore it may play a role in human respiratory problems.

Lung inflammation is characterized by the recruitment of inflammatory cells such as neutrophils and eosinophils in response to an inflammatory agent. Glyphosate herbicides are used worldwide in agriculture and glyphosate exposure has been associated with respiratory dysfunction in agricultural workers (15,47). Glyphosate's ability to induce inflammation in the lungs is not well understood. Therefore, we used mice to evaluate lung inflammation after single (1-day) and repeated exposure (5-days and 10-days) to glyphosate. The pattern of exposure to glyphosate in mice more closely mimicked the work-shift schedule of agricultural workers (130). The dose of glyphosate was based on the levels of glyphosate detected in the crop farming environment and it was used in the previous study (22). Our results showed that repeated exposure to glyphosate compared to single exposure induced greater cellular infiltration in lungs at agricultural relevant dose. The induction of inflammation was further underscored by counts of neutrophils in BAL fluid and levels of eosinophil marker in the lungs after repetitive exposure to glyphosate. The data from an earlier study (22) showed similar findings after seven days of glyphosate exposure. While Kumar and colleagues examined lung effects at only one time-point (seven days) (22), we did so at 1-day, 5-days, and 10-days of glyphosate exposure. The lung inflammation caused by chronic exposure to glyphosate may be associated with lung function impairments observed in pesticide applicators as high prevalence of chronic respiratory symptoms and decline in lung function were reported (27,177,178).

The recruitment of inflammatory cells is a multi-step process and is facilitated through the release of cytokines and upregulation of endothelial adhesion molecules. Our results show that single exposure to glyphosate did not increase cytokines in lungs, suggesting that it may not induce strong stimulation in lung cells. However, IL-5 and IL-13 cytokines were significantly increased after repeated exposure to glyphosate for 10-days. These cytokine results are similar to the findings of others reported after seven days of glyphosate exposure (22). IL-13 deficient mice exposed to glyphosate for seven days and 21-days (3 exposures per week for 3 weeks) showed less cellular infiltration and diminished production of IL-5 in the lungs (22). These results suggest that IL-13 signaling in the lungs may be critical in observed inflammation after glyphosate exposure. Both IL-5 and IL-13 have important roles in lung inflammation associated with asthma or chronic obstructive pulmonary disease (179–181). IL-5 is involved in the recruitment of eosinophils in the asthmatic lung; Interestingly, we did notice an increase in eosinophils in the lung tissues from glyphosate-treated mice.

Kumar and colleagues showed no effect on IL-4 and an increase of IL-33, and IL-10 after seven days of glyphosate exposure (22). Our use of the same dose of glyphosate and longer exposure periods showed an increase of IL-4 in 10-days group and no differences in IL-33 and IL-10; however, glyphosate-treated lungs showed damage to lung epithelium which is one of the potential sources of these cytokines (182,183). These differences in cytokine results could be possibly explained by three factors. Firstly, it may be that we missed the time point for their observed cytokine increase in the five days gap between our exposure periods. Secondly, pattern of glyphosate exposure may be important for cytokine changes in lungs after glyphosate exposure. Thirdly, the two studies used different sexes of mice to test glyphosate exposure. There is a known difference in inflammatory response between the different sexes (184,185). A sex-related response may be an important factor for differences in cytokine responses after glyphosate exposure and needs further investigation.

The activation of pulmonary endothelium by cytokines leads to the expression of adhesion molecules which are important for the tissue recruitment of inflammatory cells such as neutrophils and eosinophils (100). There is no data on the expression of adhesion molecules

in lungs of animals treated with glyphosate. Our study provides first the immunohistochemical data showing increased pulmonary expression of ICAM-1, VCAM-1 and vWF proteins in glyphosate treated mice. It is well known that ICAM-1 and VCAM-1 engage selectins to slow-down rolling neutrophils and vWF secreted from Weibel-Palade bodies in endothelial cells facilitates recruitment of platelets (186,187) and is a marker of inflammation (188–190). The higher levels of cytokines such as IL-13 and IL-5 likely caused the observed increase in the expression of adhesion molecules in the lungs of animals treated with glyphosate. Nevertheless, the increased endothelial expression of ICAM-1, VCAM-1 and vWF provides molecular evidence of vascular inflammation in the lungs of glyphosate exposed mice.

For agricultural crop production workers, glyphosate is often co-exposed with other well-known lung inflammation stimulants, most notably endotoxin (LPS) as a component of organic dust. Grain dust inhalation has been shown to induce neutrophilic inflammation in exposed individuals as well as in animal models, and endotoxin (LPS) in grain dust is often associated with respiratory dysfunction among grain farmers (4,64,128). Because bacterial molecules activate cells upon binding with toll like receptors (TLR) (191), we examined the expression of TLR4 and TLR2 in lungs from mice in our experiments and found an increase in their expression following repeated exposures to glyphosate. While it is not known whether glyphosate directly activates TLR-mediated cell activation, the increased expression of TLRs will enhance sensitivity of the lung to bacterial challenges. Activation of the TLR receptors is critical for inflammatory signaling resulting in the release of cytokines and upregulation of endothelial adhesion molecules, which are necessary for leukocyte migration (192). These results suggest the need to study glyphosate exposure by using TLR receptor knock-out mice which would provide a better understanding about the mechanistic role of TLRs in glyphosate induced lung inflammation. Taken together, these results suggest that repeated glyphosate exposure may modulate the lung inflammation induced by bacterial molecules in agricultural dust.

3.6 Conclusions

Repeated exposure to glyphosate induced a migration of eosinophils, neutrophils and release of IL-4, IL-5 and IL-13. It was concomitantly associated with increased pulmonary

expression of ICAM, VCAM-1, and vWF adhesion molecules and TLR-4 and TLR-2 receptors. Exposure to glyphosate could be a risk factor for respiratory dysfunction observed among agricultural workers. Future studies are needed to investigate the chronic effects of glyphosate on markers of airway inflammation and lung pathology.

CHAPTER 4

PULMONARY INFLAMMATORY RESPONSE FROM CO-EXPOSURE TO LPS AND GLYPHOSATE (MANUSCRIPT II)*

*Chapter 4 has been published as a full-length research article in *Environmental Toxicology and Pharmacology* journal (193). This article is licensed under a Creative Commons Attribution 4.0 International License. Elsevier allows the authors to include their articles in full or in part in a thesis or dissertation.

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Author's contributions: Mr. Upkardeep Pandher conceptualized and designed the study, conducted the experiments, analyzed the samples, interpreted the data, and prepared and revised the manuscript; Dr. Shelley Kirychuk, Mr. Upkardeep Pandher's Ph.D. supervisor, contributed to the study concept, design, data management and interpretation, reviewed and revised the manuscript; Dr. David Schneberger contributed to study methodology, sample preparation, laboratory analysis, results interpretation, reviewed and revised the manuscript; Ms. Brooke Thompson contributed to the study methodology, reviewed and revised the manuscript; Drs. Gurpreet Aulakh, RS Sethi, and Baljit Singh contributed to the study methodology, results interpretation, reviewed and revised the manuscript. All authors read and approved the final manuscript.

4.1 Abstract

Agricultural workplace exposures are a risk for respiratory outcomes in workers and exposures are complex in nature. Endotoxin and glyphosate are two common agents in agricultural exposures. Endotoxin is a bacterial molecule whereas glyphosate is a main active ingredient in agricultural herbicides. While endotoxin (lipopolysaccharide, LPS) is a potent inflammatory agent it explains only a portion of the respiratory inflammatory response. The inflammatory potential when LPS is presented with another common agricultural respiratory agent, glyphosate, is not known. It was hypothesized that exposure to a combination of LPS and glyphosate would induce additive or synergistic effects on lung inflammation than individual agents' exposure. Mice were assigned to four treatment groups: control (saline), LPS alone (0.5 μg/40 μl), glyphosate alone (1 μg/40 μl), and combined LPS (0.5 μg/40 μl) and glyphosate (1 μg/40 μl). Treatments were for one, five or 10 days. Leukocyte counts and cytokine levels were assessed in bronchoalveolar lavage fluid. Lung tissue was used for levels of myeloperoxidase, a neutrophil marker, expression of ICAM-1 adhesion marker, and TLR-4 and TLR-2 innate receptors and for immunostaining against CD45+ B and CD3+ T lymphocytes. Five days of repeated exposure to the combination of LPS and glyphosate resulted in higher neutrophil counts, myeloperoxidase, TNF-α, IL-6, KC levels, and ICAM-1 and TLR-2 expression compared to the same length of treatment to LPS or glyphosate alone. After 10-days of exposure, airway inflammatory responses decreased; however, leukocyte infiltration persisted along with increases in IL-4. Neutrophil counts and myeloperoxidase levels were still higher. Immunostaining for CD45+ B and CD3+ T lymphocytes was intense, specifically around the perivascular region of lung sections in both 5-days and 10-days combined LPS and glyphosate exposure groups. Glyphosate exposure modified LPS induced lung inflammatory responses with synergistic effect on neutrophilic infiltration. TLR-2 may be important in the modulated lung inflammatory response after co-exposure to LPS and glyphosate.

Keywords: Glyphosate; LPS; Combined exposure; Lung inflammation

4.2 Background

Agricultural exposures have been associated with various respiratory outcomes and diseases (14). Long-term agricultural exposures have been associated with chronic respiratory symptoms and conditions in workers (1–3,7,8,194,195). The worker respiratory response is greater as the complexity of the exposure increases, such that animal confinement workers have a greater response than grain workers, and both are greater than blue collar exposed workers (2,4,7,12,27,30,43,194). An endotoxin, (lipopolysaccharide, LPS), an ubiquitous component of agricultural organic dust, has been shown to have a critical role in worker respiratory response (62,63,196) and pulmonary inflammation (24,166,169,197). Endotoxin exposure mediated acute inflammatory response has been shown to involve various cytokines including interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor (TNF)-α, and the subsequent recruitment and activation of neutrophils in the lower and upper airways (70–73), mainly through the TLR-4 signaling pathway (132–134). However, endotoxin (LPS) explains only a fraction of the responses. LPS exposure alone, at concentrations higher than that found in agricultural dusts, has been shown to generate less of an inflammatory response than exposure to agricultural dusts (11,25,26,169). It is possible that exposure to a combination of molecules, common in agriculture, may differentially alter pulmonary inflammatory responses. In agricultural workplaces endotoxin is encountered in organic dust exposures which contain many other molecules (7,12,81,172,198,199); common among them is glyphosate. Glyphosate is the most common active ingredient used in agricultural herbicides, which are extensively applied to crops worldwide (15,16,87). Glyphosate residue has been shown to be present in agricultural organic dusts (22,164,200), and to have an affinity for organic matter, particularly the inhalable particle sizes (164), making it important to worker inhalation effects. Agricultural worker exposures that include exposure to organic dust and herbicides containing glyphosate have been associated with respiratory conditions including allergic and non-allergic wheeze, asthma, and rhinitis (34,47,90). The lung inflammatory potential of inhaled glyphosate is not known. A single animal study has shown the potential for glyphosate to induce type 2 airway inflammation (22). More importantly, the inflammatory effects when glyphosate is exposed in combination with LPS have not been studied. Unravelling the inflammatory potential from co-

exposure to common agricultural agents is important to better understand the respiratory responses experienced by exposed workers. We hypothesized that exposure to a combination of LPS and glyphosate would induce additive or synergistic effects on lung inflammation compared to individual LPS or glyphosate exposures.

4.3 Materials and methods

4.3.1 Study protocol

Male C57BL/6 mice (Charles River Laboratories, Senneville, QC Canada), seven weeks of age were assigned to four experimental groups (5 mice/group): control, LPS alone (LPS), glyphosate alone (glyphosate), or a combination of LPS and glyphosate (LPS+glyphosate). Mice were group-housed in the Laboratory Animal Services Unit at the University of Saskatchewan and fed with commercial rodent chow and water *ad libitum*. All animals were acclimatized to whole-body plethysmography (WBP) chambers (Buxco FinePointe Whole Body Plethysmography 4-site system; Data Sciences International, New Brighton, MN USA) daily for two hours per day for one week before the start of treatments. The Animal Research Ethics Board of the University of Saskatchewan approved all the experimental protocols (Protocol# 20160106) and procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 which complies with EU Directive 2010/63/EU.

The dose of LPS (0.5 µg) was calculated using Guyton's formula: the relevant airborne endotoxin levels commonly found in agricultural environments (50 µg/m^3); the minute volume of a mouse (average weight 20 g); and an 8-hour period (166,175). The dose of glyphosate (1 µg) was selected based on the level of glyphosate in agricultural dust samples (22).

The control group received saline (Hank's Balanced Salt Solution (HBSS), without calcium, pH 7.4; (Life Technologies, Grand Island, NY USA). The LPS alone group received 0.5 µg/40 µl LPS (stock: 1 mg/ml, *E.coli* serotype 0111:B4; Sigma, St. Louis, MO USA) dissolved in HBSS. The glyphosate alone group received 1 µg/40 µl of glyphosate (stock: 0.85 M, analytical grade PESTANAL standard; Sigma, St. Louis, MO USA) dissolved in HBSS. The combined LPS and glyphosate received the mixture of LPS (0.5 µg/40 µl) and glyphosate (1 µg/40 µl) in HBSS.

Before treatment, each solution was vortexed for 10 minutes and then filter sterilized (size: 0.22 µm; Millipore, Burlington, MA USA).

Mice were treated intranasally with 40 µL of treatment solution under light isoflurane anesthesia. Exposures were performed for one day, or daily for five days, or daily for 10-days. For the 10-day exposure, no exposure occurred on weekends to mimic agricultural worker exposure patterns. Post intranasal exposure, mice were placed back into their respective cages.

4.3.2 Whole-body plethysmography

On the last day of treatment, four hours post-treatment, respiratory measures were undertaken using mouse WBP chambers as per the manufacturer's instructions (Buxco small animal whole body plethysmography 4-site system, Data Sciences International, New Brighton, MN USA). Briefly, mice were placed in calibrated chambers and respiratory measures (PenH, peak inspiratory volume, peak expiratory volume, volume per minute, breath frequency) were taken every two seconds for 20 minutes, and results were averaged for each mouse. Data were collected using FinePointe software (Data Sciences International, New Brighton, MN USA).

Post-WBP measurement mice were sacrificed by CO_2 inhalation after isoflurane anesthesia and bronchoalveolar lavage (BAL) fluid and lung tissue were collected.

4.3.3 Bronchoalveolar lavage fluid collection, processing and analysis

Bronchoalveolar lavage fluid was collected by washing the lungs three times with 0.5 ml ice-cold HBSS. BAL fluid was then centrifuged at 1000 x g for 10 minutes at 4^0 C. Supernatants were stored at -80^0 C for further analysis. Cells from BAL fluid was resuspended in HBSS and kept on ice until used for leukocyte counts. Total leukocyte counts in BAL fluid were measured using a hemocytometer and expressed as an absolute cell number. For differential leukocyte counts, approximately 50,000 cells were cytospun onto a glass slide using a cytocentrifuge for three minutes. Adhered cells were stained with a Protocol Hema 3 kit (ThermoFisher Scientific, Waltham, MA USA) and coverslips mounted using Surgipath MM24 Mounting Media (Leica

Biosystems, Richmond, IL). A total of 200 cells were counted based on morphology and expressed as an absolute cell number.

The total protein concentration in BAL fluid supernatant was quantified using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. Plates were read using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT USA) at 562 nm.

TNF-α, keratinocyte chemoattractant (KC), monocyte chemoattractant protein -1 (MCP-1), macrophage inflammatory protein -2 (MIP-2), IL-1β, IL-10, IL-13, IL-33, IL-4, IL-5, and IL-6, were measured in BAL fluid using a Custom Mouse Procartaplex Multiplex Immunoassay (ThermoFisher Scientific, Waltham, MA USA) according to the manufacturer's instructions for magnetic bead-based ELISA. Samples on the plate were read using a BioPlex 200 system (Bio-Rad, Mississauga, ON Canada) and BioPlex Manager Software (Bio-Rad, Mississauga, ON Canada).

4.3.4 Lung tissue collection and processing

After BAL fluid collection, the right lung was tied off at the primary bronchus, and the left lung was inflated with 200 µl of 4% paraformaldehyde (PFA). The right lung was removed, flash-frozen in liquid nitrogen, and stored at -80^0 C to be used for myeloperoxidase (MPO) and RNA analyse. The inflated left lung was removed afterward and stored in PFA for 16 hours at 4^0 C for histopathology and immunohistochemistry.

4.3.5 Histopathology

The fixed lung tissues were processed in an Intelsint RVG/1 Histology Vacuum tissue processor (Intelsint; Turin, Italy) and followed by mounting in paraffin blocks in Tissue Tek II tissue embedder (Sakura Finetek; Nagano, Japan). Lung sections of 5 µm thickness were cut from paraffin embedded tissue on an American Optical Rotary Microtome (Model 820, American Optical, Buffalo, NY USA). Tissue sections on slides were stained with hematoxylin

and eosin and mounted using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL USA) before histopathological analysis.

4.3.6 Immunohistochemistry

Lung sections on slides were stained with CD45R/B220 and CD3 antibodies. Briefly, lung sections were deparaffinized and rehydrated through a series of xylene and graded alcohol baths. Endogenous peroxidase in tissue was quenched by incubating with 0.5% hydrogen peroxide in methanol for 20 minutes. Antigen unmasking was done with pepsin (2 mg/ml of pepsin in 0.01 N HCL) for 30 minutes. Non-specific binding was avoided by blocking with 1% bovine serum albumin for 30 minutes. Lung sections were incubated with the following unconjugated monoclonal primary antibodies overnight at 4^0 C: rat anti-CD45R/B220 (Pan-B cell marker, dilution 1:400, clone RA3-6B2; ThermoFisher Scientific, Waltham, MA USA), and rat anti-CD3 (Pan-T cell marker, dilution 1:100, clone 17A2; ThermoFisher Scientific, Waltham, MA USA). The next day, anti-rat secondary antibody (dilution 1:200; ThermoFisher Scientific, Waltham, MA USA) conjugated to horseradish peroxidase was allowed to bind with primary antibody for one hour at room temperature. The colored reaction was developed using a commercial kit (Vector Laboratories, Burlington, ON Canada). Counterstaining was done with methyl green nuclear stain (Vector Laboratories, Burlington, ON Canada). Stained lung sections were mounted using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL USA). Positive control (Von Willebrand Factor antibody; dilution 1:200; ThermoFisher Scientific, Waltham, MA USA), isotype control, and controls with the omission of primary antibody or secondary antibody were similarly run at the same time.

4.3.7 Myeloperoxidase levels

Lung tissue homogenates were prepared using 2 mm Zirconia beads (BioSpec Products, Bartlesville, OK USA) in tubes containing RIPA lysis buffer with 1X Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA USA) in a Mini-Beadbeater-24 homogenizer (BioSpec Products, Bartlesville, OK USA), for two, 1-minute rounds, with cooling on ice in between rounds. Total protein concentration in lung homogenates was

quantified with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA). MPO was quantified using a Mouse Myeloperoxidase DuoSet ELISA (R&D Systems, Minneapolis, MN USA). Plates were read using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT USA) at 450 nm.

4.3.8 RNA purification, cDNA synthesis, and Real-time PCR

RNA was purified from lung homogenates using the RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA USA) according to manufacturer instructions. Purified mRNA was quantified using a Take3 plate and BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT USA). cDNA was generated using the iScript Reverse Transcription Supermix (BioRad Laboratories, Hercules, CA USA) with 0.5 μg mRNA as per the manufacturer's instructions.

Real time-PCR was performed with probes for mouse ICAM-1 (Mm00516023_m1), TLR-4 (Mm00445273_m1), and TLR-2 (Mm00442346_m1) (Life Technologies, Grand Island, NY USA) using a CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA USA). Ribosomal RNA (Life Technologies, Grand Island, NY USA) was used as an endogenous control. PCR was conducted using a CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA USA). The reaction mix was incubated for two minutes at 50^0 C, 10 minutes at 95^0 C, followed by 40 cycles at 95^0 C for 15 seconds, followed by 60^0 C for one minute. Reactions were carried out in duplicate. The cycle threshold of each target gene obtained from real-time PCR data was analyzed with the ΔΔCt method to determine its relative quantification.

4.3.9 Statistical Analyses

GraphPad Prism (Graph-Pad Software, San Diego, CA USA) was used for statistical analysis and graph preparation. Data are presented as mean ± standard deviation (SD). For cytokine levels below the software predicted estimates, a value lower than the lowest extrapolated value was selected for statistical analysis of each cytokine. All data were tested for equal variance assumption using the Brown-Forsythe test. Cytokine data were log-transformed if normality was judicious for analysis. Statistical analysis of data was performed using one-way

ANOVA with a follow-up Tukey's posthoc test for multiple comparisons. Non-parametric data were analyzed by Kruskal-Wallis. In all tests, a p-value ≤ 0.05 was considered significant.

For graphing of data, "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group; "1" indicates a significant difference compared with the 1-day exposure group; "2" indicates a significant difference compared with the 5-days exposure group.

4.4 Results

4.4.1 Whole-body plethysmography

There were no significant differences in PenH between any of the 1-day or 10-days exposure groups. However, after 5-days of exposure (Figure 4.1), PenH was significantly higher in the combined LPS+glyphosate group and the LPS group as compared to the control group, while the PenH in the combined LPS+glyphosate group was also higher than the glyphosate only group. After 10-days of exposure, the PenH in both the combined LPS+glyphosate group and the LPS alone group was back down to the same level as after one day of exposure, and similar to the measures of all the other exposures. The PenH in the control and glyphosate exposed groups remained similar between all of the exposure periods (1, 5 and 10-days). The results suggest that the respiratory response in the group exposed to the combination of LPS+glyphosate is similar in manner to that of the LPS group.

4.4.2 Total and differential leukocyte counts in bronchoalveolar lavage fluid

After the one day of exposure, total leukocyte counts (Figure 4.2A) in BAL fluid were not significantly different between any of the exposure groups. For both the control and glyphosate groups, total leukocyte counts did not change between the 1, 5, and 10-days exposures. However, for both the combined LPS+glyphosate group and the LPS alone group, total leukocyte counts were significantly higher after five days of exposure and remained at this level

after 10-days of exposure. Furthermore, total leukocyte counts in the combined LPS+glyphosate group were significantly higher than the LPS only group after both five and 10-days of exposure.

In the differential leukocyte counts the major response was neutrophilic for all groups. The neutrophilic response was very robust in the combined LPS+glyphosate group at five days and remained at this robust level after 10-days of exposure (Figure 4.2C, D). The neutrophilic response in the group exposed to the combination of LPS+glyphosate was significantly greater than all other treatments (LPS alone, glyphosate alone, and control) after both five days and 10-days of exposure. For the macrophages and lymphocytes, the LPS+glyphosate exposed group was not significantly different from the LPS alone group after both five and 10-days of exposure, however, the macrophage and lymphocyte levels in the combined LPS+glyphosate group did continue to be significantly different from the glyphosate alone groups.

4.4.3 Lung vascular permeability

There were no significant differences in BAL proteins between the groups after the one day of exposure (Figure 4.3); however, after five days of exposure BAL fluid proteins were significantly higher in the group exposed to the combination of LPS+glyphosate as compared to all other 5-days groups. The levels after 10-days of exposure remained similar to the 5-days exposure levels for all groups. As such, after 10-days of exposure to the combination of LPS+glyphosate, BAL fluid proteins were significantly higher as compared to all other 10-day groups.

4.4.4 Cytokines in bronchoalveolar lavage fluid

After one day of exposure to the combination of LPS+glyphosate, IL-6 (Figure 4.4B) and MIP-2 (Figure 4.4E) were significantly higher as compared to all other exposures. IL-1β (Figure 4.4F) was significantly higher after 1-day exposure for both combined LPS+glyphosate and LPS alone exposure.

After five days of exposure IL-6 was significantly higher than the one-day results for all treatments as compared to the control. Furthermore, the IL-6 level for the combined

LPS+glyphosate exposure was significantly higher as compared to all other exposures, suggesting an additive effect. In addition, TNF-α (Figure 4.4A), and KC (Figure 4.4C) were also significantly higher with exposure to the combination of LPS+glyphosate as compared to all other 5-days exposure groups, also suggesting an additive effect. After five days of exposure, levels of MIP-2 (Figure 4.4E), IL-1β (Figure 4.4F), IL-10 (Figure 4.4G), and IL-5 (Figure 4.4J) were not different between LPS alone and LPS+glyphosate exposure groups. IL-4 (Figure 4.4H), MCP-1 (Figure 4.4D), IL-13 (Figure 4.4I), and IL-33 (Figure 4.4K) were not significantly different between groups.

After 10-days of exposure, IL-6 (Figure 4.4B) was significantly higher with exposure to the combination of LPS+glyphosate as compared to all other 10-days exposure groups. IL-4 (Figure 4.4H) was significantly higher after exposure to the combination of LPS+glyphosate as compared to the LPS alone and the control groups, but not different from the glyphosate alone group.

After 10-days of exposure, TNF-α, IL-6, KC, IL-1β and MIP-2 were significantly lower in the combined LPS+glyphosate exposure group as compared to the 5-days combined LPS+glyphosate exposures. After 10-days of exposure, IL-4 was significantly higher in both the combined LPS+glyphosate exposure group and the glyphosate alone group as compared to their respective 5-days exposures, but not in the LPS alone group. Moreover, at 10-days, the IL-4 level in the combined LPS+glyphosate exposure group was significantly higher than that of both the LPS alone and glyphosate alone exposure groups.

4.4.5 Histopathology

Lungs of control mice showed normal architecture after 1-day (Figure 4.5A), 5-days (Figure 4.5B), and 10-days (Figure 4.5C) exposure.

After one day of exposure there was little cellular infiltration in the lungs of any of the exposure groups (combined LPS+glyphosate (Figure 4.5J), LPS alone (Figure 4.5D) or glyphosate alone (Figure 4.5G).

After five and 10-days of exposure there was robust accumulation of inflammatory cells in the perivascular, peribronchiolar, and alveolar regions of the lungs, with more intense accumulation in the lungs of mice exposed to the combination of LPS+glyphosate for both five days (Figure 4.5K) and 10-days (Figure 4.5I) as compared to the 5-days and 10-days exposures with LPS alone (Figure 4.5E, F) and glyphosate alone (Figure 4.5H, I).

4.4.6 Myeloperoxidase in lungs

After one day of exposure, MPO was significantly higher in the combined LPS+glyphosate exposure group as compared to only the 1-day control exposure (Figure 4.6). After five days of exposure, MPO was significantly higher in the lungs of the combined LPS+glyphosate exposure group as compared to all other 5-days exposure groups (LPS, glyphosate alone, and control). After 10-days of exposure MPO levels in the LPS+glyphosate exposure group were similar to the five days levels and remained significantly higher than all other exposure groups.

4.4.7 B and T lymphocyte expression in lungs

Lung sections were stained with CD45+ (pan-B lymphocyte murine marker; Figure 4.7) or CD3+ (pan-T lymphocyte murine marker; Figure 4.8) antibodies. The B lymphocyte staining was absent in lung sections from control mice for 1-day (Figure 4.7A), 5-days (Figure 4.7B), and 10-days (Figure 4.7C) exposures. After five days of exposure, there was intense expression of B lymphocytes, specifically centered around the perivascular regions in the lung sections of the mice exposed to the combination of LPS+glyphosate for both five days (Figure 4.7K) and 10-days (Figure 4.7I). After 5-days and 10-days of exposure to LPS alone (Figure 4.7E, F) and glyphosate alone (Figure 4.7H, I), B lymphocyte expression was weak around the perivascular region of stained lung sections.

T lymphocytes were not detected in lung sections of control mice for 1-day (Figure 4.8A), 5-days (Figure 4.8B), or 10-days (Figure 4.8C) exposure. After five days (Figure 4.8K) and 10-days (Figure 4.8I) of exposure to the combination of LPS+glyphosate, there was intense T lymphocyte expression around the perivascular regions of lung sections. The 10-days LPS alone

exposed mice showed weak T lymphocyte expression around the perivascular regions of lung sections (Figure 4.8F), whereas the glyphosate exposed mice did not show T lymphocyte expression in lung sections after either 5-days (Figure 4.8H) or 10-days (Figure 4.8I) of exposure.

4.4.8 ICAM-1, TLR-4 and TLR-2 expression in lungs

ICAM-1 expression (Figure 4.9A) was not significantly different between any of the 1-day exposed groups. After five days of exposure ICAM-1 expression was significantly higher in the group exposed to the combination of LPS+glyphosate as compared to all other 5-day exposure groups. After 10-days of exposure, ICAM-1 expression was significantly lower in both the LPS+glyphosate group and the glyphosate alone group as compared to their respective 5-day exposure groups. Furthermore, after 10-days of exposure, ICAM-1 expression in the group exposed to the combination of LPS+glyphosate remained significantly higher than the glyphosate alone group and the control group, but not the LPS group.

After five days of exposure, TLR-4 expression (Figure 4.9B) was significantly higher in the combined LPS+glyphosate exposure group as compared to the glyphosate alone group. After 10-days of exposure, the TLR-4 expression in the combined LPS+glyphosate exposure group remained similar to that of the 5-days exposure and remained significantly higher than the glyphosate alone group.

TLR-2 expression (Figure 4.9C) was not significantly different between any of the 1-day exposure groups. After five days of exposure, TLR-2 expression increased significantly in all treatment groups (except control), as compared to the 1-day exposures. Moreover, TLR-2 expression in the group exposed to the combined LPS+glyphosate was significantly higher (~70 fold) as compared to all other five days exposure groups. After 10-days of exposure, the TLR-2 expression in the group exposed to the combined LPS+glyphosate was significantly lower than its respective 5-days exposure.

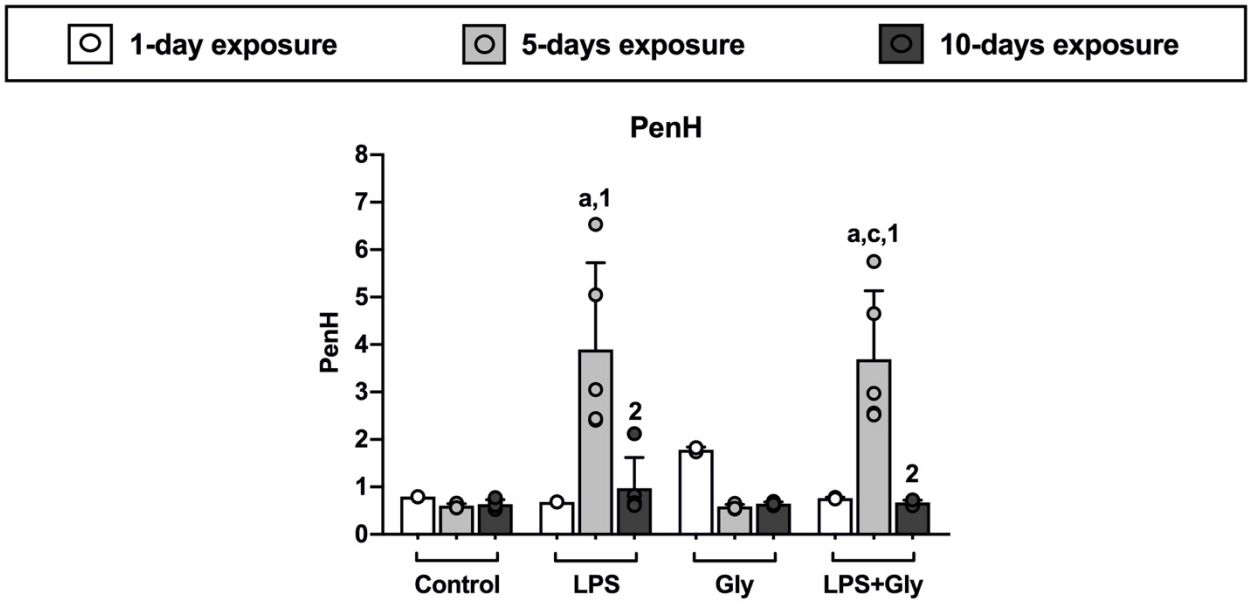

Figure 4.1: Mice PenH data. Mice were exposed to control, LPS, glyphosate (Gly), or combined LPS and glyphosate (LPS+Gly) for 1-day, 5-days, and 10-days. Enhanced pause (PenH) data was averaged for 20 minutes and are presented as mean ± SD. Due to technical difficulties with the WBP equipment during 1-day exposures the N = 2 mice for each of the treatments; N = 5 mice for all the 5-day and 10-day exposure groups. Significance ($p < 0.05$) is denoted as such: “a” indicates a significant difference compared with the control group; “c” indicates a significant difference compared with the glyphosate group; “1” indicates a significant difference compared with 1-day exposure group; “2” indicates a significant difference compared with 5-day exposure group.

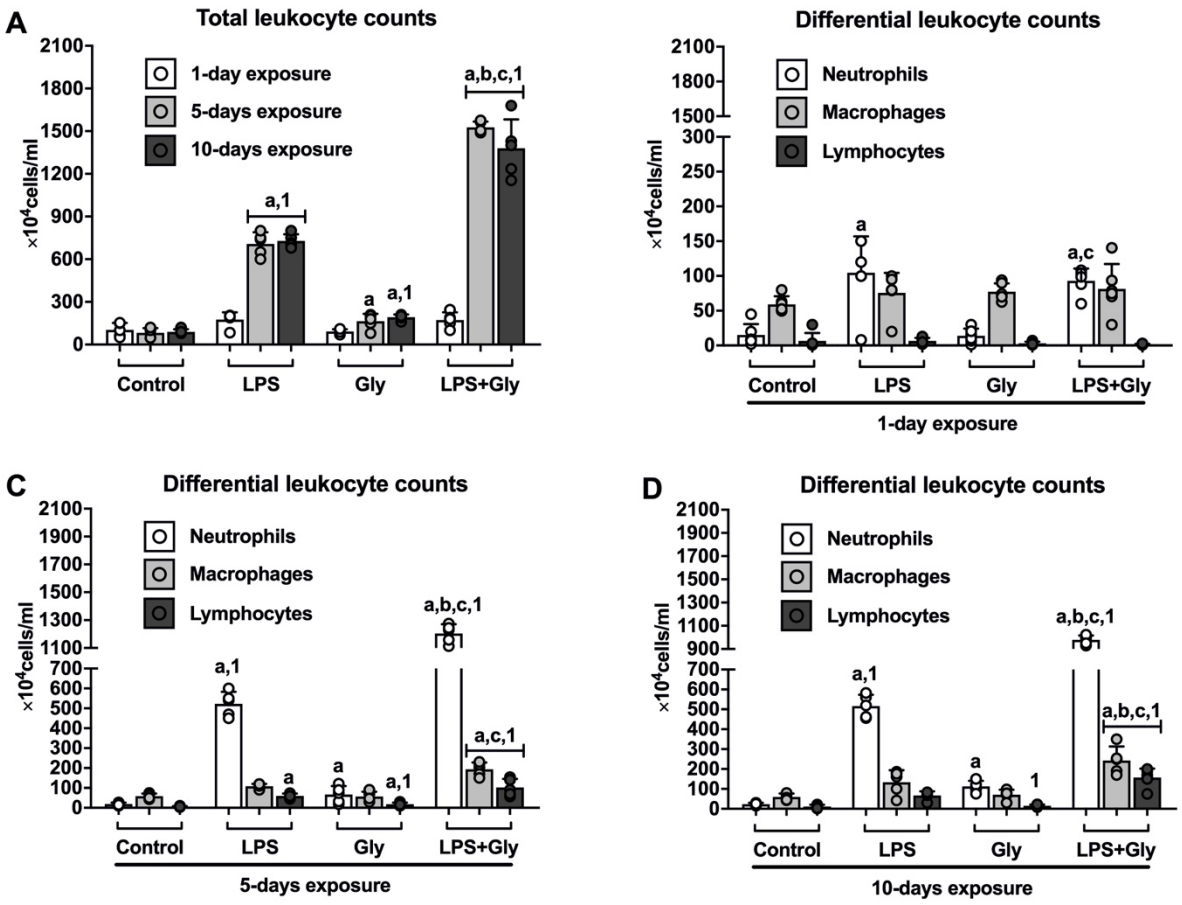

Figure 4.2: Leukocyte counts in bronchoalveolar lavage fluid. Total leukocyte counts (A) and differential leukocyte counts (B-D) in bronchoalveolar lavage (BAL) fluid of mice after exposure to control, LPS, glyphosate (Gly), or combined LPS and glyphosate (LPS+Gly) for 1-day, 5-days, and 10-days. Total leukocyte counts were measured using a hemocytometer, and differential leukocyte counts were performed on Protocol Hema 3 kit stained cytospins. Data presented as mean ± SD (N = 5 mice per group). Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group; "1" indicates a significant difference compared with 1-day exposure group.

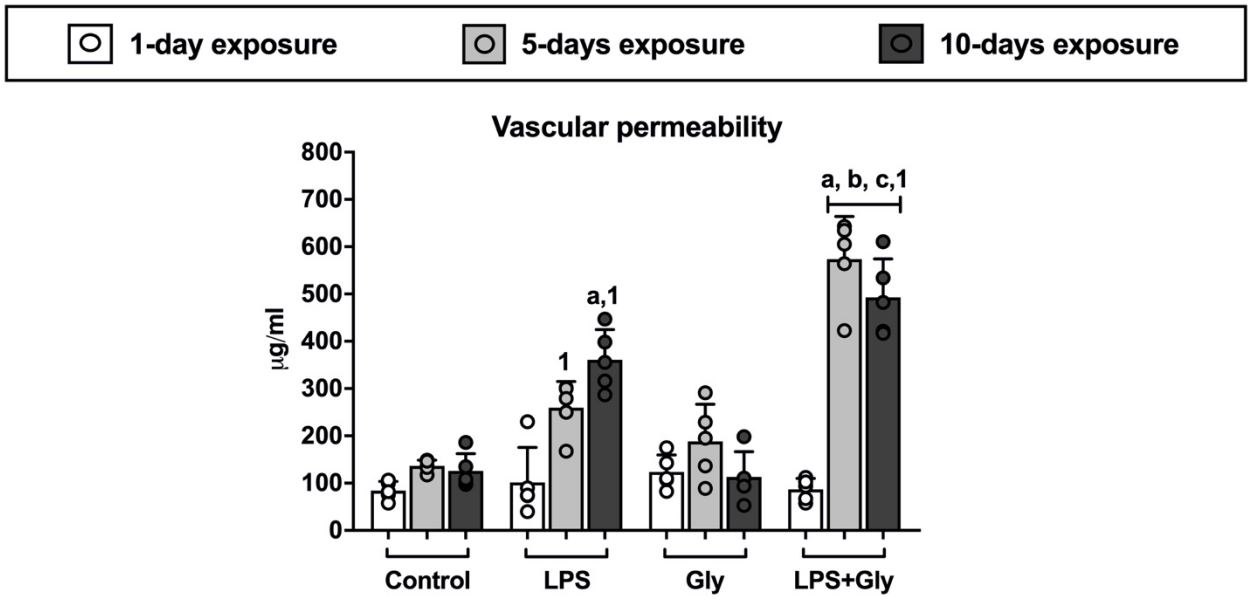

Figure 4.3: Total protein levels in bronchoalveolar lavage fluid. Proteins were measured in BAL fluid after mice exposure to control, LPS, glyphosate (Gly), or combined LPS and glyphosate (LPS+Gly) for 1-day, 5-days, and 10-days. Protein concentration was estimated using a Pierce BCA Protein Assay Kit. Data presented as mean ± SD (N = 5 mice per group. Significance ($p < 0.05$) is denoted as such: “a” indicates a significant difference compared with the control group; “b” indicates a significant difference compared with the LPS group; “c” indicates a significant difference compared with the glyphosate group; “1” indicates a significant difference compared with 1-day exposure group.

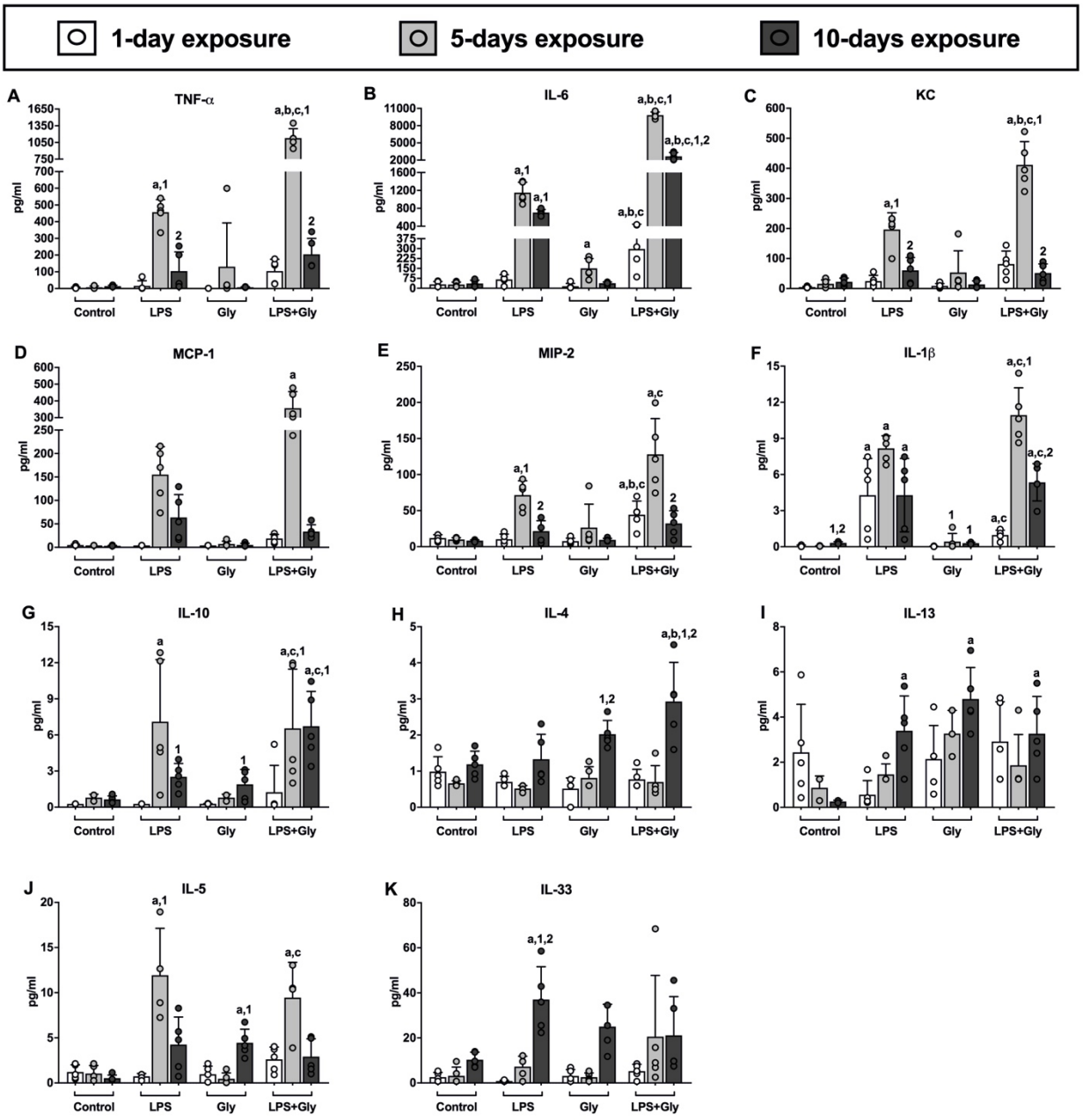

Figure 4.4: Cytokine levels in bronchoalveolar lavage fluid. Protein levels detected in supernatant of BAL fluid after mice exposure to control, LPS, glyphosate (Gly), or combined LPs and glyphosate (LPS+Gly) for 1-day, 5-days, and 10-days. Cytokines such as TNF-α (A), IL-6 (B), KC (C), MCP-1 (D), MIP-2 (E), IL-1β (F), IL-10 (G), IL-4 (H), IL-13 (I), IL-5 (J) and IL-33 (K) were measured using Custom Mouse Procartaplex Multiplex Immunoassay. Data presented as mean ± SD (N = 5 mice per group). Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group;

“1” indicates a significant difference compared with 1-day exposure group; “2” indicates a significant difference compared with 5-day exposure group.

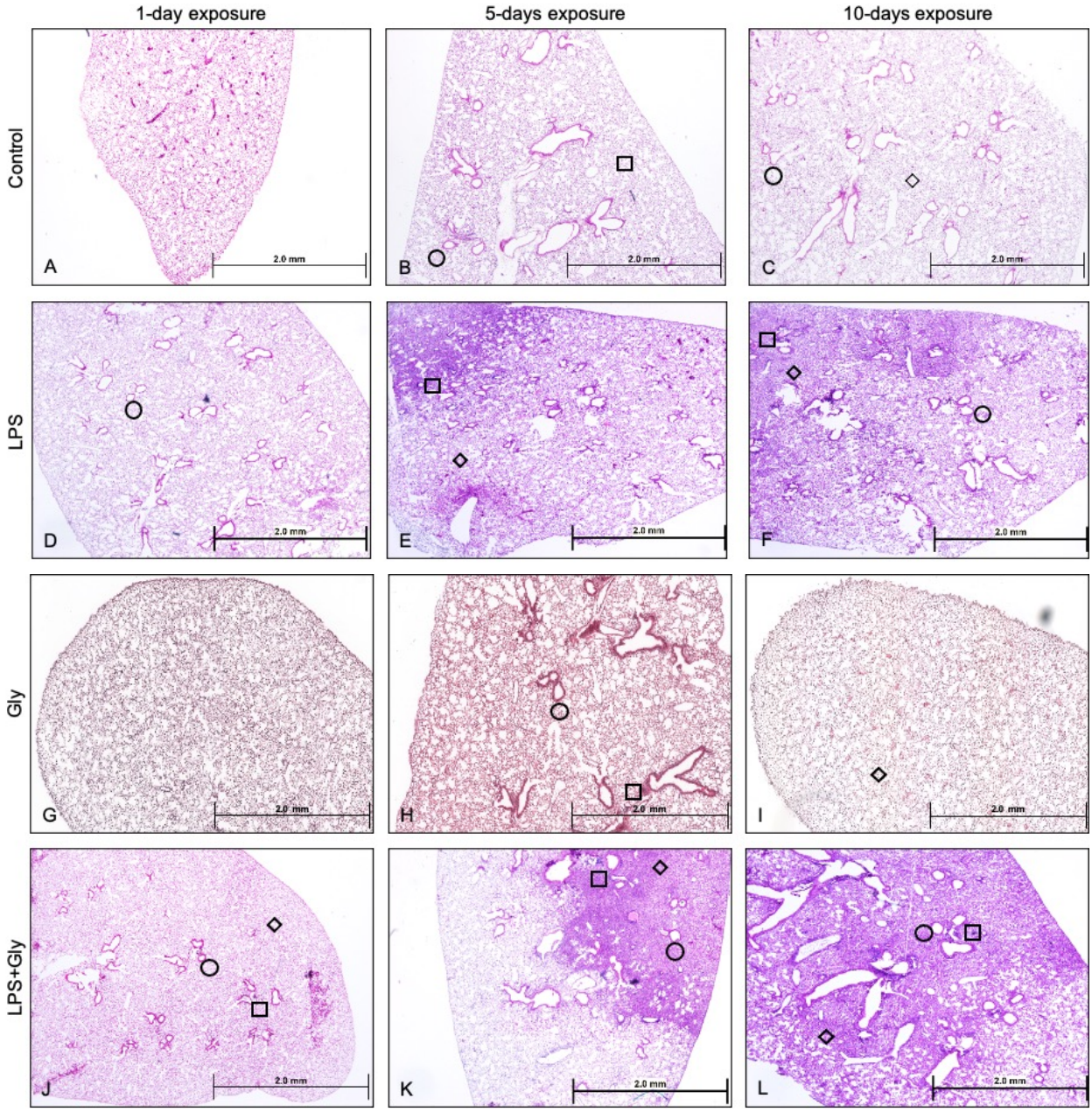

Figure 4.5: Hematoxylin and eosin-staining of lung sections from treated mice. Mice were treated for 1-day, 5-days, and 10-days with control (A-C), LPS (D-F), glyphosate (Gly; G-I) or combined LPS and glyphosate (LPS+Gly; J-L). Group representative images showing whole lungs having infiltration of leukocytes in perivascular (square), peribronchiolar (circle) and alveolar (diamond) regions. Magnification: ×40 (A-L). Scale bar: 2 mm (A-L)

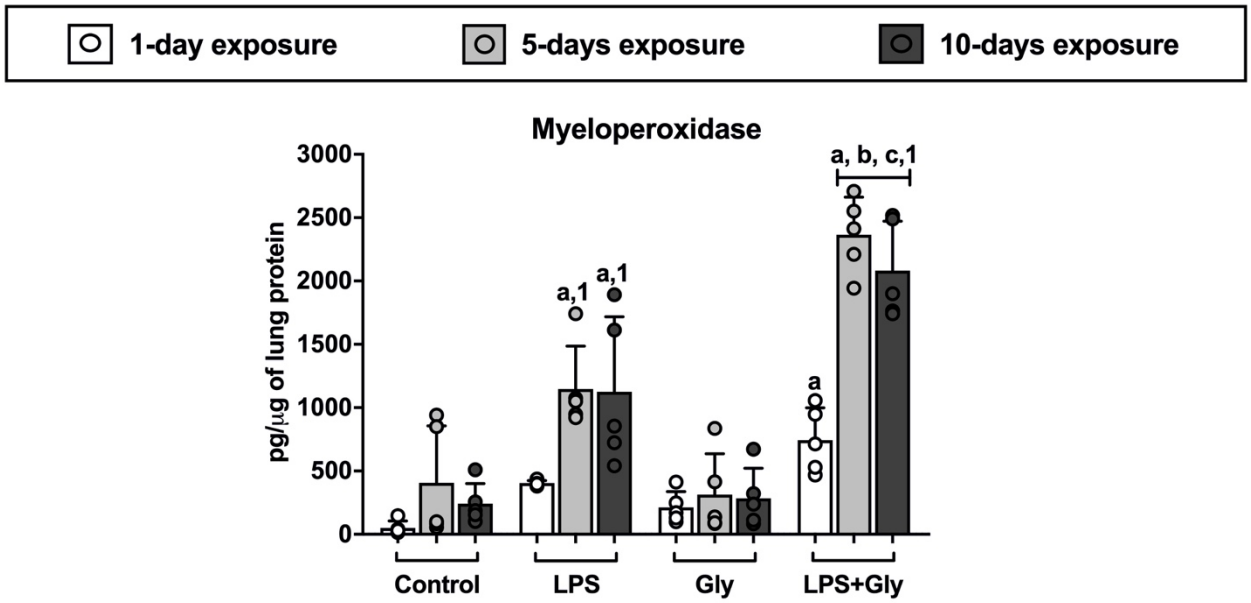

Figure 4.6: Myeloperoxidase levels in mice lung tissue. Myeloperoxidase measured in lung tissues of mice after 1-day, 5-days, and 10-days exposure to control, LPS, glyphosate (Gly), or combined LPS and glyphosate (LPS+Gly). Myeloperoxidase was detected in lung homogenates using a Mouse Myeloperoxidase DuoSet ELISA. Data presented as mean ± SD (N = 5 mice per group). Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group; "1" indicates a significant difference compared with 1-day exposure group.

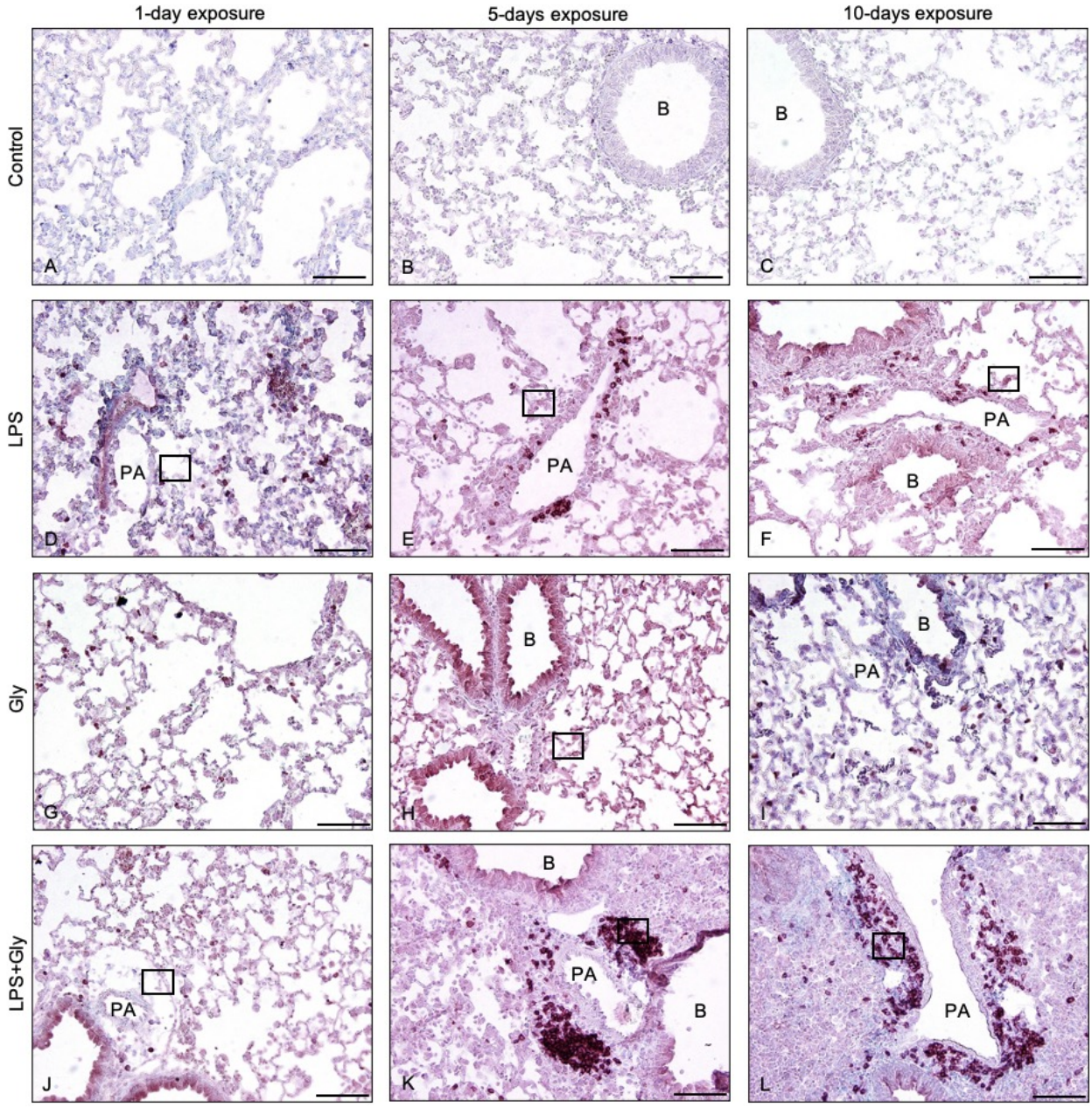

Figure 4.7: Immunohistochemical staining on lung tissue using B lymphocyte antibody. Expression of B lymphocytes in mice lung sections after 1-day, 5-days and 10-days exposure to control (A-C), LPS (D-F), glyphosate (Gly; G-I), or combined LPs and glyphosate (LPS+Gly; J-L). Staining was performed with CD45R/B220 antibody, a pan-B lymphocyte marker. Group representative images showing perivascular region by square. Magnification: ×400 (A-L). Scale bar: 50 µm (A-L). PA: Pulmonary artery; B: Bronchus

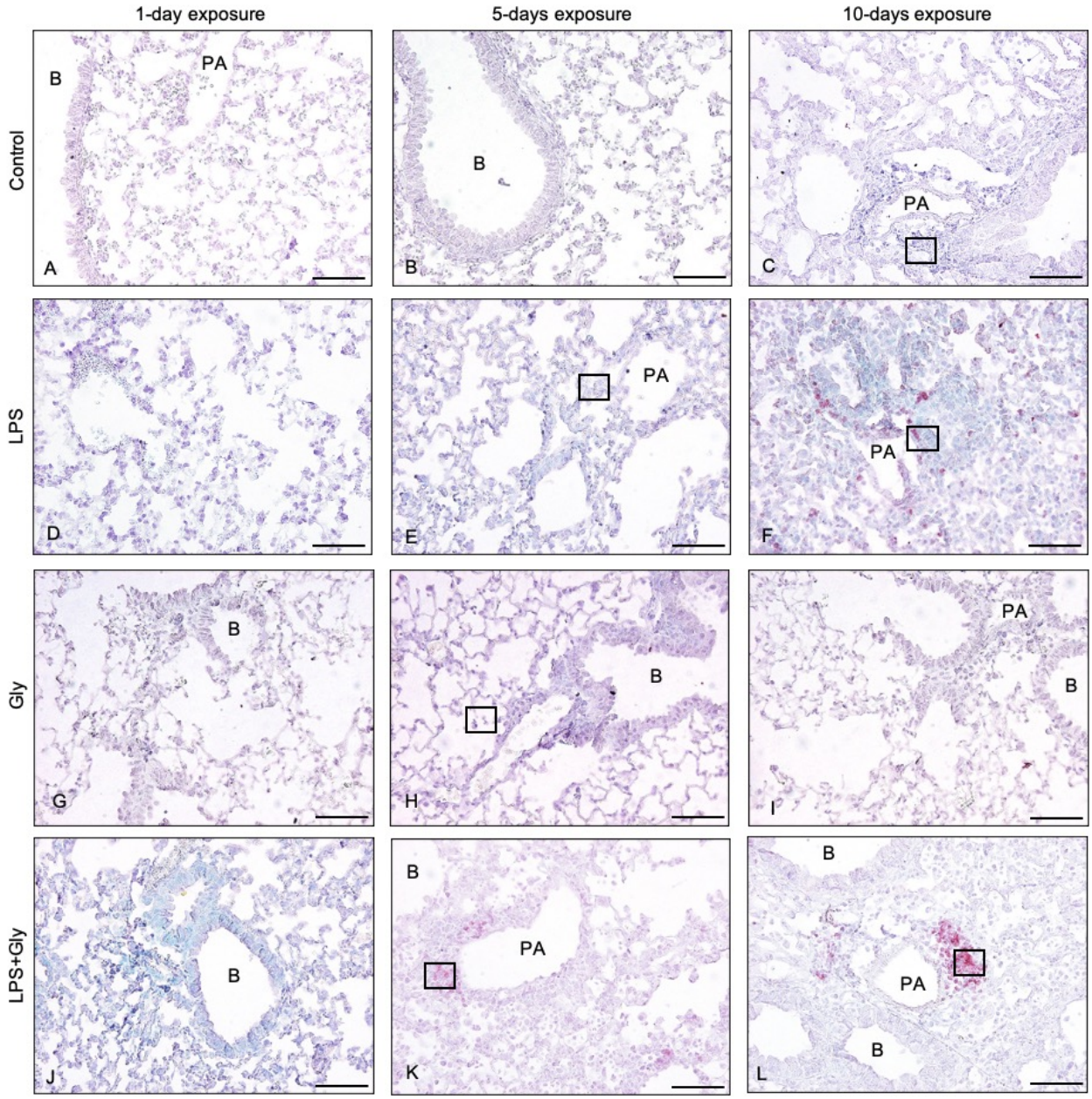

Figure 4.8: Immunohistochemical staining on lung tissue using T lymphocyte antibody. Expression of T lymphocytes in mice lung sections exposed for 1-day, 5-days, and 10-days to control (A-C), LPS (D-F), glyphosate (Gly; G-I) or combined LPS and glyphosate (LPS+Gly; J-L). Staining was performed with CD-3 antibody, a pan-T lymphocyte marker. Group representative images showing perivascular region by square. Magnification: ×400 (A-L). Scale bar: 50 µm (A-L). PA: Pulmonary artery; B: Bronchus

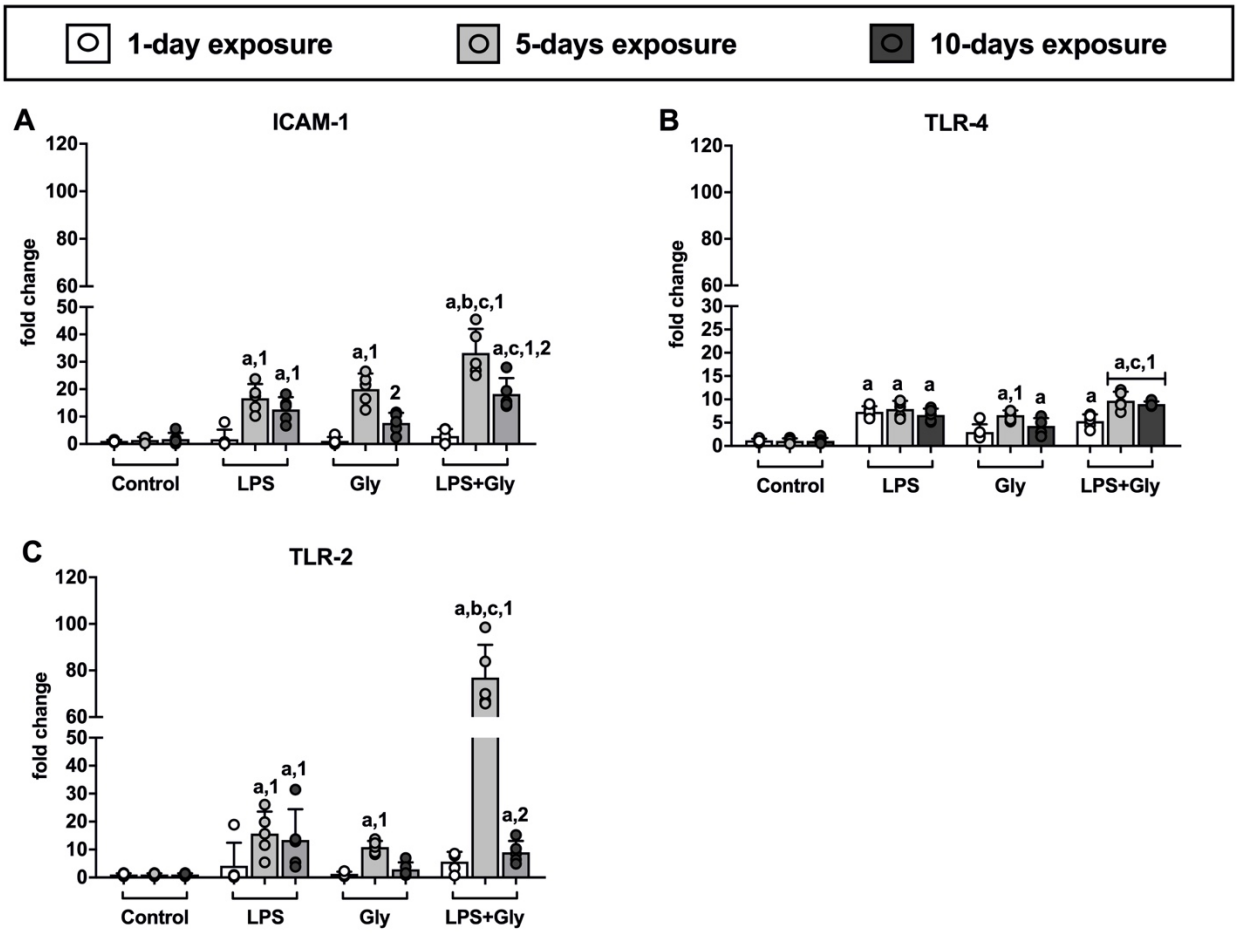

Figure 4.9: Real time PCR for expression of ICAM-1, TLR-4 and TLR-2 in mice lung tissue. Fold change of ICAM-1 (A), TLR-4 (B), and TLR-2 (C) in mice lungs after 1-day, 5-days, and 10-days exposure to control, LPS, glyphosate (Gly), or combined LPs and glyphosate (LPS+Gly). Data presented as mean ± SD (N = 5 mice per group). Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group; "1" indicates a significant difference compared with 1-day exposure group; "2" indicates a significant difference compared with 5-day exposure group.

4.5 Discussion

We are the first to present data showing that exposure to a combination of LPS and glyphosate, at exposure levels relevant for agricultural workers, enhanced lung inflammation as compared to individual exposures to LPS or glyphosate. Short-term (5-days) repeated exposure to the combination of LPS and glyphosate resulted in enhanced inflammatory response, robust accumulation of inflammatory cells in the perivascular, peribronchiolar, and alveolar regions of the lungs, and increases in respiratory response. Inflammatory changes after short-term exposure to the combination of LPS and glyphosate included higher total leukocytes comprised of a robust influx of neutrophils; significantly higher expression of ICAM-1; 70-folds greater expression of TLR-2; higher release of proinflammatory mediators (TNF-α, KC, IL-6); greater MPO expression; and intense immune-staining of B and T lymphocytes in the perivascular region of the lungs in comparison to the same length of exposure to LPS alone and glyphosate alone. Longer-term exposures (10-days) to a combination of LPS and glyphosate resulted in generally reduced expression of inflammation; however, accumulation of inflammatory cells including CD45+ B and CD3+ T lymphocytes specifically around the perivascular regions, persisted. Longer-term exposure to the combination of LPS and glyphosate resulted in IL-4 increases that remained higher in comparison to the other exposure groups. MPO and IL-6, although lower than the 5-days level, remained higher as compared to the other exposure groups. Taken together, the results suggest that exposure to a combination of LPS and glyphosate results in synergistic lung inflammatory effects that are distinguishable from the effects induced by individual exposures to LPS or glyphosate. These results have important implications for furthering our understanding of pulmonary inflammation induced from exposure to the complement of molecules common in agriculture.

The results of exposure to the combination of LPS and glyphosate suggest that the exposure induces an asthma phenotype comprised of a robust neutrophilic response, strong TLR-2 expression, IL-4 increases, MPO and IL-6 effects, and persistent T and B lymphocyte involvement specifically in the perivascular regions of the lungs. The robust influx of neutrophils after exposure to the combination of LPS and glyphosate are a distinguishing feature in the

asthma phenotype response to the combined exposure. While the analyses of the BAL fluid indicate cells that have migrated into the alveoli, we also quantified the leukocytes still trapped in the lung tissues, using MPO as a surrogate marker. Because neutrophils migrating out of blood vessels are usually activated, their presence in lung tissues and alveoli demonstrates an active inflammatory response. While critical for the host defense, the activated neutrophils also cause damage to tissues through their proteases and free oxygen radicals (192,198). These neutrophil findings support evidence from other agricultural exposure studies that showed neutrophil response after inhalation of grain dust extract (128) and swine dust extracts (129). However, when similar levels of glyphosate to our study were presented with an allergen (Ovalbumin), the neutrophilic response was not different from that of Ovalbumin alone (22). Taken together, these findings suggest that molecules presented to the respiratory system as a complex result in a differential inflammatory response and are influenced by the type of exposure.

Neutrophils are also important to the release of proinflammatory mediators. We found higher levels of TNF-α, IL-6, and KC in BAL fluid of animals treated short-term with combined LPS and glyphosate as compared to those given only LPS or glyphosate. Migration of neutrophils is mediated through a series of steps that involves their engagement with adhesion molecules expressed on vascular endothelium activated by cytokines such as TNF-α and IL-6 (198). The role of chemokines, such as KC, is central to the directed migration of neutrophils and other inflammatory cells (172). We also found increased expression of ICAM-1 after five days of exposure to the combination of LPS and glyphosate. The expression of ICAM-1 was significantly higher compared to all other exposures, including LPS. These results are similar to others studying agricultural exposures that have shown the increase in ICAM-1 in human bronchial epithelial cells exposed to swine dust extract (199). It is well known that ICAM-1 engage selectins to slow-down rolling neutrophils and its expression is upregulated by proinflammatory cytokines (100). Adhesion molecule response, such as ICAM-1, are likely important to the differential inflammatory effects of exposures. Taken together, these results suggest that repeated short-term exposure to the combination of LPS and glyphosate resulted in a synergism between the two agonists, release of cytokines, chemokines, and adhesion

molecule expression, resulting in a robust neutrophilic asthma response. The response was significantly greater, and distinguishable from, the inflammatory response resulting from the same level of exposure to the individual agonists.

We further found that repeated exposure (5-days) to the combination of LPS and glyphosate increased TLR-2 expression in lung tissues. Bacterial molecules activate target cells in the lungs through the engagement of pattern recognition receptors such as TLR-4 and TLR-2. To-date, data have shown involvement of both TLR-2 (201) and TLR-4 (169) in lung inflammation induced by exposure to swine dust. Further, polymorphisms in TLR-2 (202) and TLR-4 (41) genes have been shown to influence swine farmers' respiratory outcomes. TLR-4 not only activates immune and other cells in response to LPS, it also has a direct role in neutrophil recruitment in the lungs (135). In our findings the TLR-4 response from combined LPS and glyphosate exposure for five days was not different from the LPS exposure response; however, the 5-days exposure to the combination of LPS and glyphosate induced a strong TLR-2 expression, many folds higher than the LPS alone response. This TLR-2 response waned with longer-term repeated exposure (10-days), back to a level similar to that of the individual agent exposures. TLR-2 recognizes a wide range of microbes, and its role appears to be dependent on the dominant pattern recognition receptor (PRR) that is activated (203). In the combined LPS+glyphosate exposure group, we saw an increase in TLR-2 expression but not TLR-4 expression. The addition of a subsequent molecule exposure (glyphosate) to LPS may have altered the lungs' inflammatory susceptibility to LPS. It is possible that with a complex exposure, different PRRs strive for dominance in the inflammatory response, and this dominance alters depending on the molecules involved in the exposure. The results support that TLR-4 was activated in a similar manner whether LPS was presented as a single exposure, in the co-exposure with LPS+glyphosate, or with glyphosate alone; however, when an additional molecule, glyphosate, was added to the repeated LPS exposure, TLR-2 had a very robust response. Endogenous ligands or "alarmins" molecules contribute to host responses to infections (203); however, there has been no evidence to show that "alarmins" can modulate the course of microbial infections by engaging TLR-2. This work raises the possibility of such an effect.

Increasing exposures (10-days) resulted in an attenuation of responses of cytokines and chemokines, importantly IL-6, TLR-2, and ICAM-1. Attenuated response has been shown by others with exposures to organic dust and swine barn dust (130). A 2-week exposure to swine dust extract resulted in reduced TNF-α, IL-6, KC, and MIP-2 levels in BAL fluid while leukocytes remained accumulated in lungs as compared to 1-day and 1-week exposures (129), These findings are consistent with our results when we compare our combined LPS+glyphosate 10-day exposures to our 5-days exposures. The swine barn exposure is a complex of molecules, including LPS, likely glyphosate, but also many more molecules capable of inducing inflammatory responses. While some of the inflammatory response attenuated after our repeated longer-term (10-days) combined LPS and glyphosate exposure, intense expression of CD45+ B lymphocytes and CD3+ T lymphocytes, specifically around the lungs' perivascular region, and significantly increased lymphocyte counts in BAL fluid, persisted. Repetitive exposure to LPS alone (5 μg) has been shown to induce chronic lung inflammation with the recruitment of different subsets of lymphocytes and macrophages in the lungs (151). Our results are similar to the adaptive immune response shown after repetitive swine dust exposure, with resulting mixed mononuclear cellular aggregates comprising of CD45+ B lymphocytes, CD3+ T lymphocytes, and Mac3+ macrophages in the lungs (129). The mechanisms of the adaptive immune response from chronic exposure to the farming environment are not clear (202). Our results show that while some of the inflammatory response attenuated as exposure time increased, there were still significant differences in results between LPS exposed alone versus when LPS was co-exposed with glyphosate. While the strong TLR-2 response present at 5-days exposure attenuated by 10-days and returned back to similar levels to that of the individual LPS and glyphosate exposures, IL-6, IL-4 and MPO remained significantly higher in the co-exposed group, B and T lymphocytes persisted in the perivascular region, and respiratory measures were lower. These results suggest that the combined LPS and glyphosate exposure resulted in a differential inflammatory response in the longer-term, as compared to the individual exposures. The persistent cellular infiltration in the lungs may assist in explaining the persistence of respiratory symptoms and decreases in lung function that have been shown with repeated exposures in agricultural workers.

A strength of our study is that it utilized doses of LPS and glyphosate comparable to levels to which agricultural workers would be exposed and thought to represent levels of exposure for agricultural workers. That is, our LPS dose is similar to which grain farmers are exposed (166) and to a dose utilized in a grain dust extract exposure (128). The dose of glyphosate is also similar to that from airborne crop dust samples utilized by other exposure studies (22), as well as levels detected in the dust of farm houses (81). In addition, in our study, the doses of both LPS and glyphosate were kept constant for the individual and combined exposures. While the number of animals per group is not large, the sample size was judicious in keeping the number of animals small while still being able to discern significant differences between groups. The exposures in this study were intranasal and do not truly represent ambient exposures that would be experienced by exposed workers. While these findings add important information to the inflammatory response from complex agricultural exposures, there is a need to explore other characteristic features of chronic inflammation and adaptive immunity in response to agricultural exposures, including mucus cell metaplasia and tissue remodeling factors. Future studies aimed at understanding the inflammatory pathways induced by common agricultural co-exposures will be important to this research area.

This study adds to the data about the inflammatory effects induced by agricultural exposures by unravelling the inflammatory response of two common agricultural exposures. While the trends in our results are similar to those from more complex agricultural exposures, we furthered the work. We found that exposure of the lungs to the combination of LPS and glyphosate resulted in a greater inflammatory response and more robust histological changes, that could be differentiated from the responses to the single agent exposures. While the inflammatory response to the combined LPS and glyphosate exposure had some similarities to the LPS response, the responses were distinguishable, and the combined exposure response was stronger. This suggests that, while LPS may be important to the response, the introduction of a second molecule, such as glyphosate, can alter the response. TLR-2 may be important in this response and requires further investigation.

4.6 Conclusions

Repeated exposure to a combination of LPS and glyphosate, at exposure levels relevant for agricultural workers, enhanced lung inflammation as compared to individual exposure to LPS or glyphosate. The repeated exposure to a combination of LPS and glyphosate induced an asthma phenotype with robust neutrophilic response, and TLR-2, B, and T lymphocyte increases that were distinguishable from the individual LPS and glyphosate inflammatory responses. The work raises the possibility that endogenous ligand molecules may modulate the course of microbial infections by engaging TLR-2. Persistence in accumulation of inflammatory cells, specifically around the perivascular regions of the lungs was present after longer-term exposures, suggests an adaptative inflammatory response from longer-term exposure, which was different in the combined LPS and glyphosate exposure as compared to exposure to individual LPS and glyphosate. These results have important implications for furthering our understanding of enhanced pulmonary inflammation induced from exposures to the complement of molecules common in agricultural work environments.

CHAPTER 5

LUNG INFLAMMATION FROM REPEATED EXPOSURE TO LPS AND GLYPHOSATE

(MANUSCRIPT III)

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study methodology, results interpretation, reviewed and revised the manuscript. All authors read and approved the final manuscript.

5.1 ABSTRACT

Agricultural workplaces consist of multiple airborne contaminants and inhalation exposures induce respiratory effects in workers. Endotoxin (LPS) and glyphosate are two common airborne contaminants in agricultural environments. We have previously shown that exposure to a combination of LPS and glyphosate synergistically modulates immune reactions as compared to individual exposures. The immunopathogenesis of acute and chronic exposure to LPS and glyphosate is not known, therefore, we further investigated the lung histological differences in mice exposed to either a combination, or individual, LPS and glyphosate for one-day, five-days, and 10-days. Repeated exposure to a combination of LPS and glyphosate resulted in greater histological effects in lungs as compared to individual exposures to LPS or glyphosate. Repeated exposure to the combination of LPS and glyphosate resulted in robust infiltration of inflammatory cells in the perivascular, peribronchiolar and alveolar regions, and increases of alveolar septal thicknesses and perivascular spaces in the lungs with intense intercellular adhesion molecule (ICAM) -1 staining in the perivascular region, but minimal staining in the pulmonary artery endothelium.

Keywords: LPS, Glyphosate, Combination, Repeated exposure, Lung histology, ICAM-1

5.2 BACKGROUND

Agricultural workplace exposures are a known risk for respiratory effects in workers. Long-term agricultural exposures have been associated with chronic respiratory symptoms and accelerated decline in lung function of workers (4,194,204). Leukocyte accumulation has been shown in the bronchial epithelium and submucosal regions of lung biopsies (205) and in bronchoalveolar lavage (BAL) and nasal lavage fluid of agricultural workers (9,10,54,206). The immunopathogenesis in relation to agricultural exposures is not understood. Agricultural workplace exposures are complex and exposure to multiple airborne contaminants is common. Two of the most common agricultural workplace airborne contaminants are lipopolysaccharides (LPS) and glyphosate. Lipopolysaccharides are ubiquitous in agricultural workplaces and they mainly derive from gram negative bacteria (207). Glyphosate is a common active ingredient in herbicides (16) and is the most widely used herbicide ingredient worldwide. Exposure to glyphosate has been associated with wheeze in agricultural workers (47,90,208).

Acute (209,210) and chronic exposure (151) to LPS induces recruitment of inflammatory cells in the perivascular, peribronchiolar and alveolar regions of lungs. LPS exposure has been shown to increase the expression of intercellular adhesion molecule (ICAM) - 1 on vascular endothelial cells that is known to be important for the recruitment of inflammatory cells into lungs (155,156). Blocking of ICAM-1 expression by antibody treatment has been shown to inhibit the migration of leukocytes in lungs (211). Glyphosate exposure in mice for seven days has been shown to induce infiltration of leukocytes and release of type 2 cytokines (IL-5 and IL-13) in lungs (22). In our work, a synergistic inflammatory response was induced when mice were exposed to a combination of LPS and glyphosate (193). As compared to individual LPS or glyphosate exposure, exposure to the combination of LPS and glyphosate induced higher release of proinflammatory cytokines TNF-α, IL-6 and KC and the inflammatory response modulated after 10-days of exposure (193).

Detailed histological evidence of effects on the lungs from exposure to a combination of LPS and glyphosate has not been previously shown. We hypothesized that exposure to a combination of LPS and glyphosate would increase recruitment of inflammatory cells in lung

tissue and the effects on lung tissue would be enhanced as compared to that of singular LPS or glyphosate exposures. Given that chronic exposures in agricultural workers results in persistent respiratory effects we further hypothesized that after repeated exposures to the combination of LPS and glyphosate the lung histology would reveal persistent tissue effects. This work examines histological inflammatory differences and ICAM-1 expression in the lungs of mice exposed to a combination, and individual, LPS and glyphosate for one-day, five-days and 10-days.

5.3 MATERIALS AND METHODS

5.3.1 Mice exposures

The study design has been previously described (193). In short, the study involved four treatment groups: control, LPS alone, glyphosate alone, and combined LPS and glyphosate. Mice were intranasally given saline (control), LPS (0.5 μg), glyphosate (1 μg) or a combination of LPS (0.5 μg) and glyphosate (1 μg). The design involved three treatment lengths: once, or once daily for five-days, or once daily for 10-days. N = 5 mice per group. At the end of exposure periods, mice were sacrificed by CO_2 inhalation and lavaged before the collection of lung tissue. All experimental procedures were approved by the Animal Research Ethics Board of the University of Saskatchewan (AUP 20160106).

5.3.2 Lung collection and processing

The lungs of the mice were ligated with thread at the right bronchus and the left lung was inflated with 0.3 ml of 4 % paraformaldehyde (PFA) in-situ through a cannula inserted into the trachea. The inflated left lung was removed and immersed in 4 % PFA for 16 hours at 4^0 C. The fixed lung tissues were processed through a series of alcohols in an Intelsint RVG/1 Histology Vacuum tissue processor (Intelsint; Turin, Italy) and followed by embedding in paraffin blocks by using Tissue Tek II tissue embedding station (Sakura Finetek; Nagano, Japan). Lung sections of 5 μm thickness were cut from paraffin blocks on American Optical Rotary Microtome (Model 820, American Optical, Buffalo, NY USA) and placed onto pre-charged slides (ThermoFisher Scientific, Waltham, MA USA).

5.3.3 Histopathology and scoring for inflammation

Sections from all treatment groups were stained with hematoxylin and eosin stain and mounted using Surgipath MM24 Mounting Media (Leica Biosystems, Richmond, IL USA) before histopathological analysis. Photomicrographs were taken using a bright field microscope equipped with Infinity 5-5 Microscope camera (Teledyne Lumenera, ON Canada). The primary type of inflammatory cells (polymorphonuclear cells and/or monomorphonuculear cells) infiltrating the perivascular, peribronchiolar and alveolar septal regions were scored for each of the treatment groups and treatment lengths, as well as levels of alveolar thickness and perivascular space differences. The lung histologic sections were scored independently and in a blinded manner by four investigators using the criteria outlined in Table 5.1 (212). The scoring was performed on randomly selected five fields per section from each treated mouse under 40X objective lens (N = five mice per treatment group; five fields per section of each mouse in a group). Each investigator provided a lung histology score for each of the parameters from Table 1 for each of the treatment groups. The average lung histology score of each parameter and each group was used for statistical analysis.

5.3.4 Real time PCR

The right lung samples from each mouse were subjected to real-time PCR for ICAM-1 expression. Briefly, lung tissue was homogenized, and RNA was purified using the RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA USA) according to manufacturer instructions. Purified mRNA was quantified using a Take3 plate and Bio-Tek Synergy HT plate reader (BioTek Instruments, Winooski, VT USA). cDNA was generated from 0.5 µg of mRNA using the iScript Reverse Transcription Supermix (BioRad, Hercules, CA USA) following the manufacturer's instructions. PCR was performed with ribosomal RNA (Life Technologies, Grand Island, NY USA) as an endogenous control, and with probes for mouse ICAM-1 (Mm00516023_m1; Life Technologies, Grand Island, NY USA) using a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA). Reactions were carried out in duplicate. Reactions were incubated for two minutes at 50^0 C, 10 minutes at 95^0 C followed by 40 cycles at 95^0 C for 15 seconds and 60^0 C for one

minute. The cycle threshold of the target gene obtained from real-time PCR data was analyzed with the ΔΔCt method to determine its relative quantification.

5.3.5 Immunohistochemistry

Lung sections on slides were stained with an ICAM-1 antibody using immunohistochemistry as described previously (193). Briefly, lung sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol. The tissue sections were incubated with hydrogen peroxide (0.5 % H_2O_2 in methanol) for 20 minutes to quench the endogenous peroxidase activity. This was followed by incubation with pepsin (2 mg/ml of 0.01 N HCL) for 30 minutes to unmask the antigen and with 1 % bovine serum albumin for 30 minutes to block non-specific binding. Lung sections were incubated overnight at 4^0 C with a primary antibody against ICAM-1 (dilution: 1:100; clone: EPR16608, Rockland Immunochemicals, Gilbertsville, PA USA). Following overnight incubation, the secondary anti-rabbit antibody (dilution: 1:200; ThermoFisher Scientific, Waltham, MA USA) conjugated to horseradish peroxidase was added on sections for one hour at room temperature. The colored reaction was developed using a commercial kit (Vector laboratories, Burlington, ON Canada). Counterstaining of sections was performed with methyl green stain (Vector Laboratories, Burlington, ON Canada). Some sections were stained with positive control (von Willebrand factor antibody; dilution 1:200; ThermoFisher Scientific, Waltham, MA USA) and omitted with primary antibody or a secondary antibody to assess non-specific binding. The optimum primary antibody concentration was determined by varying concentration staining.

An experienced investigator blinded to the treatment groups semi-quantified the cell specific expression of ICAM-1 in the perivascular region, alveolar region and pulmonary arteries by using scoring criteria outlined in Table 5.2 (212). The images were captured with Infinity 5-5 Microscope camera (Teledyne Lumenera, ON Canada) mounted on a bright field microscope. Scoring was performed on randomly selected five fields per section of each treated mouse under the 40X objective lens of microscope (N = five mice per treatment group; five fields per section of each mouse in a group). The average score for each group was used for statistical analysis.

5.3.6 Statistical analysis

Data analysis and graph preparation was performed using GraphPad Prism software (Graph-Pad Software, San Diego, CA USA). Statistical differences were determined using one-way ANOVA followed by Tukey's multiple comparisons tests. A p-value of < 0.05 was considered significant. For graphing of data "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group; "1" indicates a significant difference compared with 1-day exposure group; "2" indicates a significant difference compared with 5-day exposure group.

Table 5.1: Scoring criteria for lung inflammation

Score	**Perivascular and peribronchial infiltration**	**Alveolar septa cell infiltration**	**Alveolar thickness**	**Perivascular space**
0	No inflammation	None or occasional cells	No thickened alveolar walls	No increase
1	One to two concentric rows of inflammatory cells	Few loosely arranged cells	Sporadic areas of alveolar wall thickening	Small increase
2	Three or more concentric rows of inflammatory cells	Many cells in the peripheral parts of the alveolar septa	Frequent areas of alveolar wall thickening	Medium increase
3	Continuous perivascular and peribronchial cell accumulation	Numerous cells in the alveolar septa	Thickened walls throughout	Large increase

Table 5.2: Scoring criteria for quantification of ICAM-1 expression

Score	**Criteria**
0	No expression
1	Minimal staining
2	Moderate staining
3	Intense staining

5.4 RESULTS

5.4.1 Histopathology and scoring for inflammation

The semi-quantification of cell infiltration in perivascular, peribronchiolar, and alveolar regions, increases of alveolar septal thickness and perivascular space was performed in sections to get the average lung histology score for each treatment group. It revealed that control mice in all exposure groups had average lung histology score close to 0 for all parameters indicating little or no lung inflammation (Figure 5.1). Whereas after 1 day of combined or individual LPS and glyphosate exposure there were noticeably greater inflammation in all parameters and this inflammation persisted after five and 10-days of exposure. Exposure to a combination of LPS and glyphosate for five-days and 10-days had significantly higher lung histology scores as compared to all other groups including the one-day combined LPS and glyphosate exposure group. Resulting in concentric rows of perivascular and peribronchial inflammation, cells in the alveolar septa, areas of alveolar wall thickening, and increases in the perivascular spaces. In addition, the one-day combined LPS and glyphosate exposure resulted in a higher lung histology score as compared to the one-day control exposure.

Control group images from one-day, five-day and 10-day exposures showed normal lung architecture without infiltration of leukocytes in any of the regions (Figure 5.2A-C; Figure 5.4A-C).

Individual exposure to LPS (Figure 5.2D-F; Figure 5.4D-F) or glyphosate (Figure 5.2G-I) for one-day, five-days, and 10-day showed inflammatory changes in lungs comprised of leukocyte infiltration in the perivascular, peribronchiolar and alveolar regions.

Exposure to the combination of LPS and glyphosate for one-day, five-day or 10-day showed greatest infiltration of leukocytes in the perivascular and peribronchiolar regions [Scores in table 5.3: 1 for one-day group (Figure 5.2J); 2.5 for five-day group (Figure 5.3A, B, D, E); 3 for 10-day group (Figure 5.2L)] and in the alveolar regions [Scores in table 5.3: 1 for one-day group (Figure 5.4G); 2.5 for five-day group (Figure 5.4H); 3 for 10-day group (Figure 5.4I)] as compared to individual LPS and glyphosate exposure groups. The infiltration of leukocytes

comprised of polymorphonuclear leukocytes (shown by arrows in figures) as well as monomorphonuclear leukocytes (shown by arrowheads in figures). Within the combined LPS and glyphosate exposure groups, infiltration of leukocytes was greater in five-day [Scores in table 5.3: 2.5 (perivascular and peribronchiolar infiltration; Figure 5.3A, B, D, E); 2.5 (alveolar septa cell infiltration; Figure 5.3C, F; Figure 5.4H] and 10-day [Scores in table 5.3: 3 (perivascular and peribronchiolar infiltration; 3 (alveolar septa cell infiltration; Figure 5.4I] exposure groups as compared to the one-day exposure group [Scores in table 5.3: 1 (perivascular and peribronchiolar infiltration); 1 (alveolar septa cell infiltration; Figure 5.4I]. Similarly, exposure to the combination of LPS and glyphosate for one-day, five-day or 10-day showed greater increases in the alveolar thickness [Scores in table 5.3: 1 for one day group (Figure 5.4G); 2.5 for five-day group (Figure 5.4H); 2.5 for 10-day group (Figure 5.4I)] and perivascular spaces [Scores in table 5.3: 1 for one day group; 2.5 for five-day group (Figure 5.3A, D); 3 for 10-day group] as compared to individual exposure groups. Within the combined LPS and glyphosate exposure groups, greater increases in alveolar thickness and perivascular spaces observed after five-day [Scores in table 5.3: 2.5 (alveolar thickness, Figure 5.4H); 2.5 (perivascular spaces, Figure 5.3A, D)] and 10-day [scores: 2.5 (alveolar thickness, Figure 5.3I); 3 (perivascular spaces)] as compared to one-day [Scores in table 5.3: 1 (alveolar thickness, Figure 5.3G); 1 (perivascular spaces)]. Additionally, exposure to a combination of LPS and glyphosate for one-day, five-days, and 10-days resulted in blood vessel congestion (Figure 5.2J-L) and sloughing of bronchial epithelium (Figure 5.3B, E). Interestingly, intraluminal leukocytes were not detected in the pulmonary arteries of any of the treated lung sections from one-day, five-days or 10-days (Figure 5.A-B).

5.4.2 ICAM-1 mRNA and protein expression

Combined LPS and glyphosate exposure for five-days showed significantly higher ICAM-1 mRNA in the lung tissue as compared to five-days individual LPS, glyphosate and control exposures and one-day combined LPS and glyphosate exposures (Figure 5.6). In the combined LPS and glyphosate 10-days exposure group, ICAM-1 mRNA was significantly higher compared to the 10-days glyphosate, and control groups and one-day combined LPS and glyphosate

exposure groups and it was significantly lower than the five-days combined LPS and glyphosate exposure group.

Lungs of control mice in one-day (Figure 5.7A), five-days (Figure 5.7B), and 10-days (Figure 5.7C) exposure groups showed ICAM-1 immunoreactivity in the alveolar region, while the pulmonary arteries were minimally stained. All combined LPS and glyphosate exposure groups showed intense ICAM-1 staining in the perivascular and alveolar regions of the lungs [one-day (Figure 5.7D, G), five-days (Figure 5.7E, H) and 10-days (Figure 5.7F, I)]; with minimal staining in the pulmonary arteries (Figure 5.7E, H). Semi-quantification of these results showed significantly greater ICAM-1 perivascular (Figure 5.8A) and alveolar (Figure 5.8B) staining in combined LPS and glyphosate exposure groups (one-day, five-days, and 10-days) compared to respective staining in the control group.

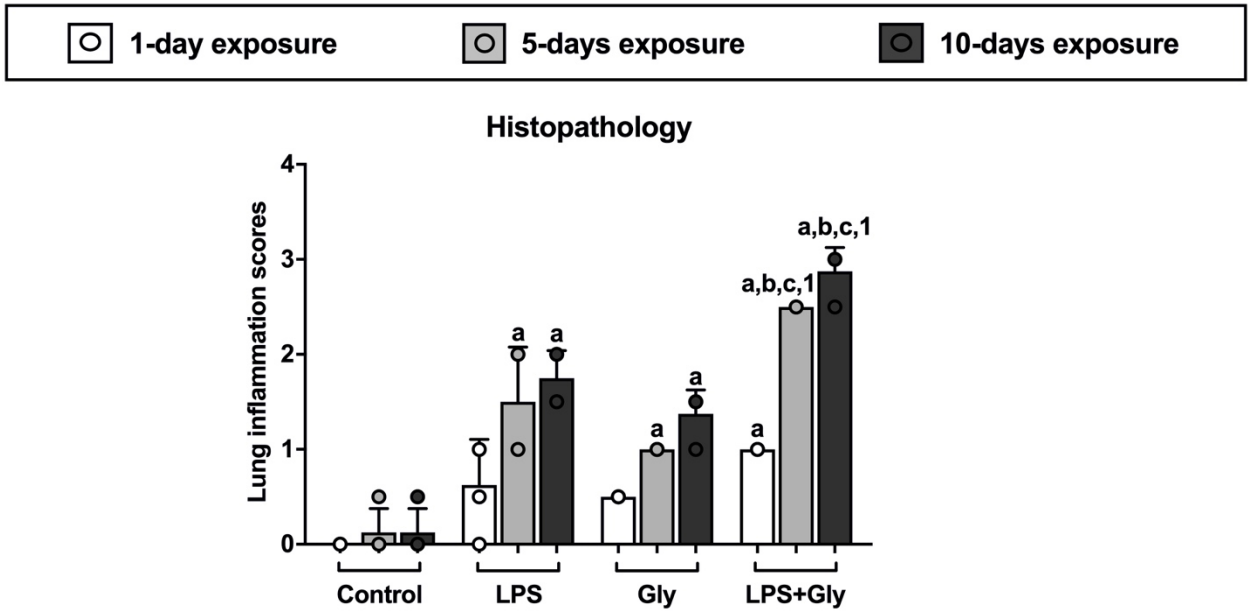

Figure 5.1: Lung inflammation scoring in stained lung sections. Mice were intranasally exposed to control, LPS, glyphosate (Gly), or combined LPS and glyphosate (LPS+Gly) for 1-day, 5-days, and 10-days. A lung histology score was computed using the sum of the 4 individual scores for each of the measured parameters generated by four investigators (N = 5 mice per treatment group; five fields per section of each mouse in a group). The average lung histology score of each group was used for statistical analysis. Data presented as mean ± SD. Significance ($p < 0.05$) is denoted as such: “a” indicates a significant difference compared with the control group; “b” indicates a significant difference compared with the LPS group; “c” indicates a significant difference compared with the glyphosate group; “1” indicates a significant difference compared with 1-day exposure group.

Table 5.3: Scoring results for lung inflammation

Duration	**Exposure**	**Perivascular & peribronchial infiltration**	**Alveolar septa cell infiltration**	**Alveolar thickness**	**Perivascular space**
One-day	Control	0	0	0	0
	LPS alone	1	1	0.5	0
	Glyphosate alone	0.5	0.5	0.5	0.5
	Combined LPS+Gly	1	1	1	1
Five-days	Control	0	0.5	0	0
	LPS alone	1	2	2	1
	Glyphosate alone	1	1	1	1
	Combined LPS+Gly	2.5	2.5	2.5	2.5
10-days	Control	0	0.5	0	0
	LPS alone	1.5	2	2	1.5
	Glyphosate alone	1.5	1.5	1	1.5
	Combined LPS+Gly	3	3	2.5	3

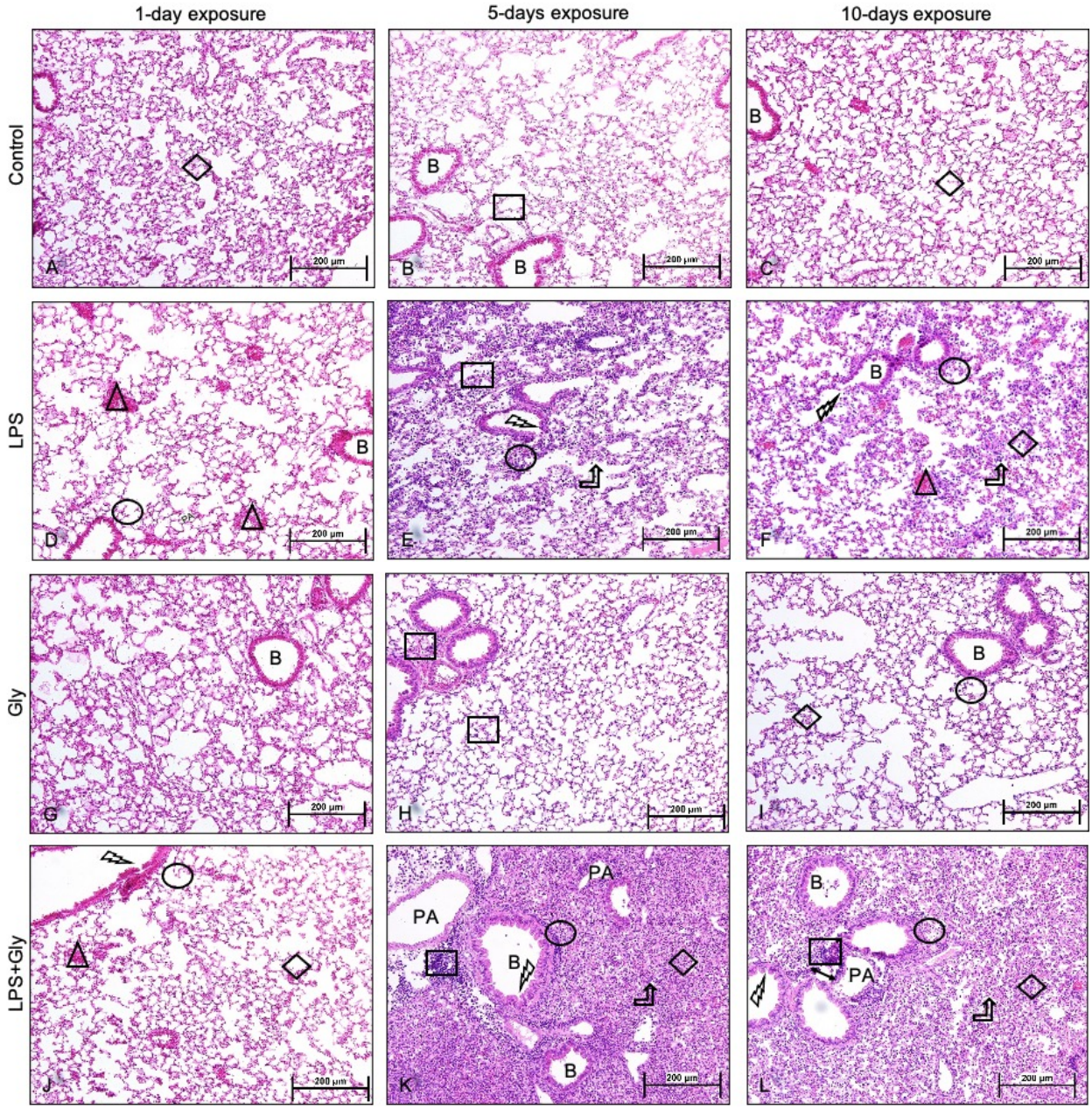

Figure 5.2: Hematoxylin and eosin-staining of lung sections. Lung sections of mice after exposure for 1-day, 5-days, and 10-days to control (A-C), LPS (D-F), glyphosate (Gly; G-I), or combined LPS and glyphosate (LPS+Gly; J-L). Group representative images showing perivascular infiltration (square), peribronchiolar infiltration (circle), alveolar infiltration (diamond), perivascular space increase (double arrow), blood vessels congestion (triangle), alveolar septa thickness increase (bent up arrow), and sloughing of bronchial epithelial surface (lightning bolt). Magnification: ×200 (A-L). Scale bar: 200 µm (A-L). PA: Pulmonary artery; B: Bronchus.

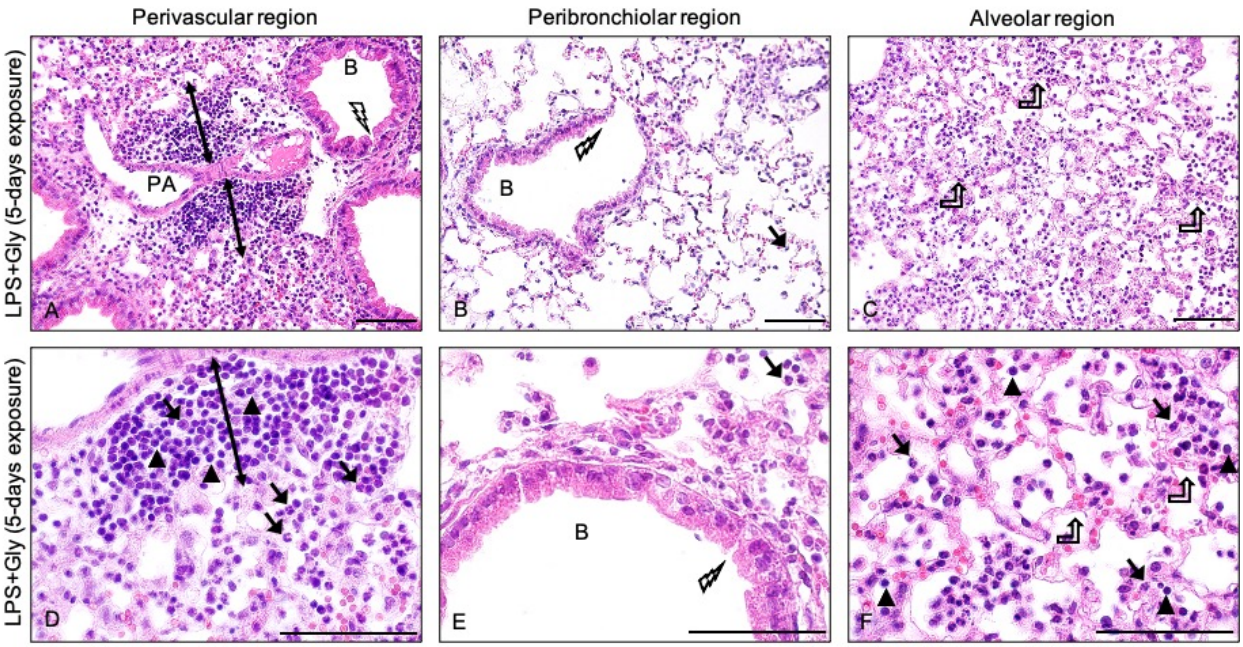

Figure 5.3: Perivascular, peribronchiolar and alveolar regions of lung sections. Lung sections of mice from 5-day combined LPS and glyphosate (LPS+Gly) exposure group showing perivascular (A, D), peribronchiolar (B, E), and alveolar (C, F) regions of hematoxylin and eosin-stained lung sections. Group representative images showing infiltration of polymorphonuclear (arrow) and monomorphonuclear (arrowhead) leukocytes, perivascular space increase (double arrow), alveolar septa thickness increase (bent up arrow), and sloughing of bronchial epithelial surfaces (lightning bolt). Magnification: ×400 (A-C); ×1000 (D-F). Scale bar: 50 μm (A-F); PA: Pulmonary artery; B: Bronchus.

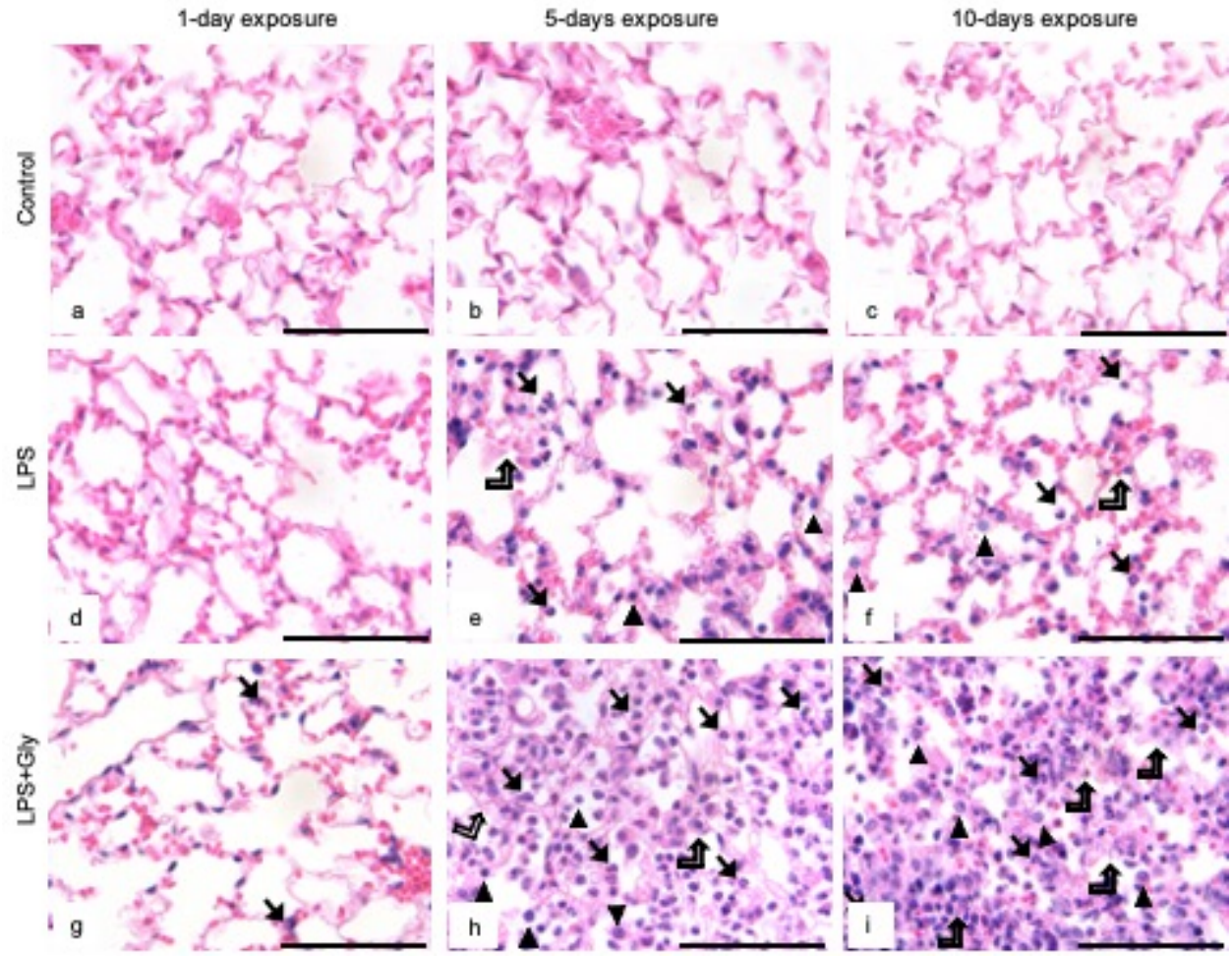

Figure 5.4: High magnification images of the alveolar region in stained lung sections. Mice were intranasally treated to control (a-c), LPS (d-f) or combined LPS and glyphosate (LPS+Gly; g-i) exposure groups for 1-day, 5-days and 10-days. Group representative images showing infiltration of polymorphonuclear (arrow) and monomorphonuclear (arrowhead) leukocytes and increase of alveolar septal thickness (bent up arrow). Magnification: ×1000 (a-i). Scale bar: 50 µm (a-i).

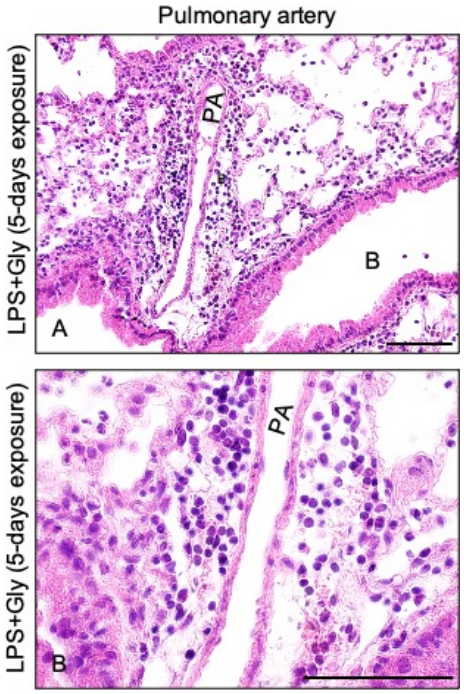

Figure 5.5: Pulmonary arteries in hematoxylin and eosin-stained lung sections. Lung sections of mice after 5-days exposure to combined LPS and glyphosate (LPS+Gly; A-B). Magnification: ×400 (A); ×1000 (B). Scale bar: 50 μm (A-B). PA: Pulmonary artery; B: Bronchus.

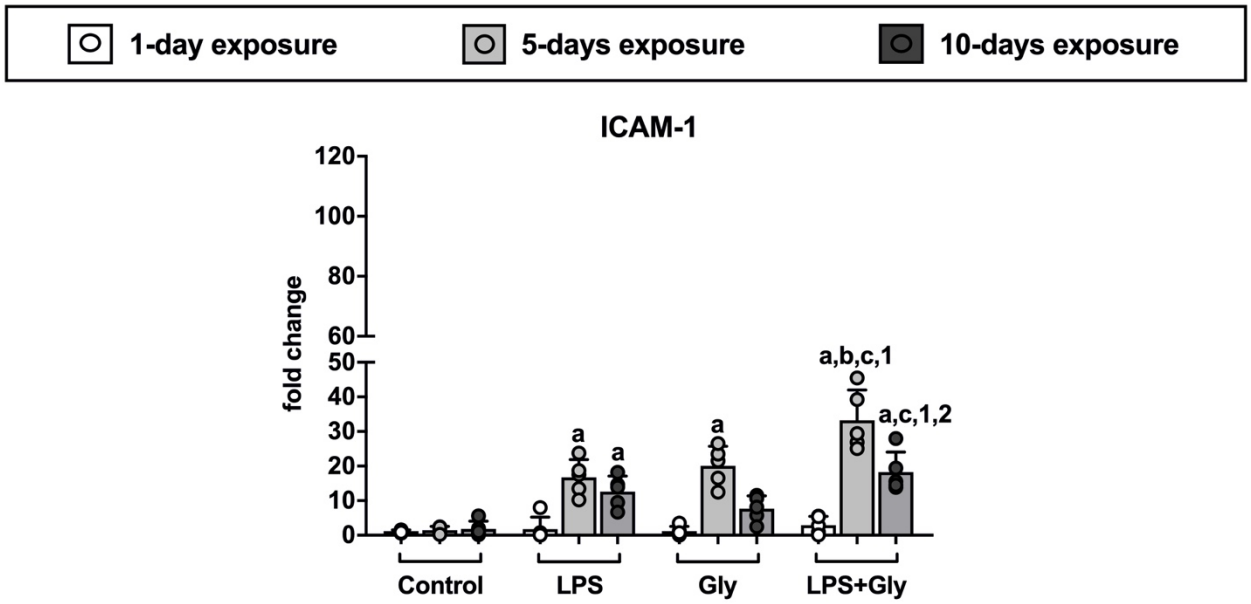

Figure 5.6: Real time PCR for ICAM-1 expression in lungs. Mice were treated for 1-day, 5-days or 10-days to control, LPS, glyphosate (Gly) or combined LPS and glyphosate (LPS+Gly). Data presented as mean ± SD (N = 5 mice/group). Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group; "1" indicates a significant difference compared with 1-day exposure group; "2" indicates a significant difference compared with 5-day exposure group.

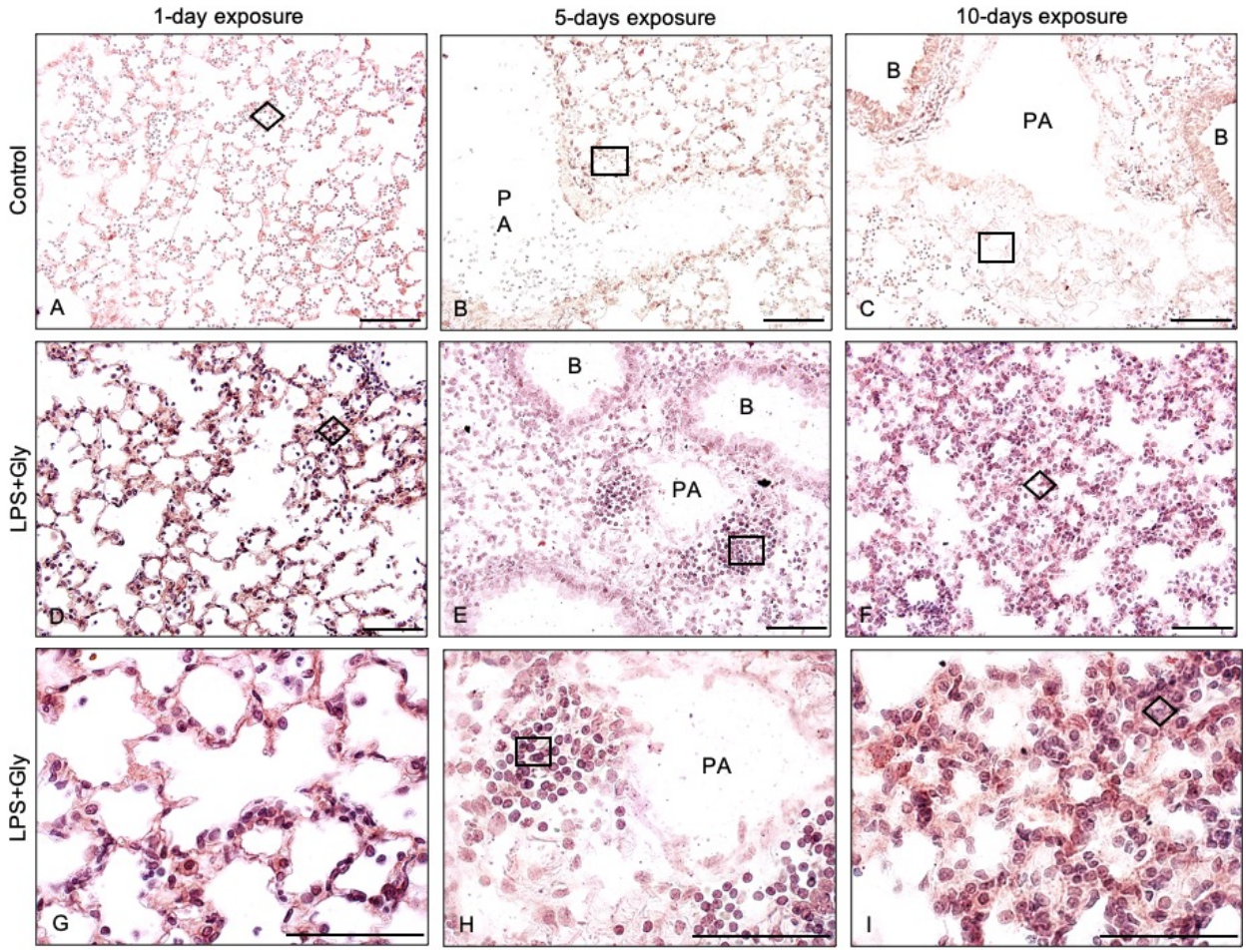

Figure 5.7: Immunohistochemical expression of ICAM-1 in lung sections. Mice were treated for 1-day, 5-days, and 10-days to control (A-C) or combined LPS and glyphosate (LPS+Gly; D-I). Group representative images showing ICAM-1 expression in perivascular region (square) and alveolar region (diamond). Magnification: ×400 (A-F); ×1000 (G-I). Scale bar: 50 μm (A-I). PA: Pulmonary artery; Br: Bronchus.

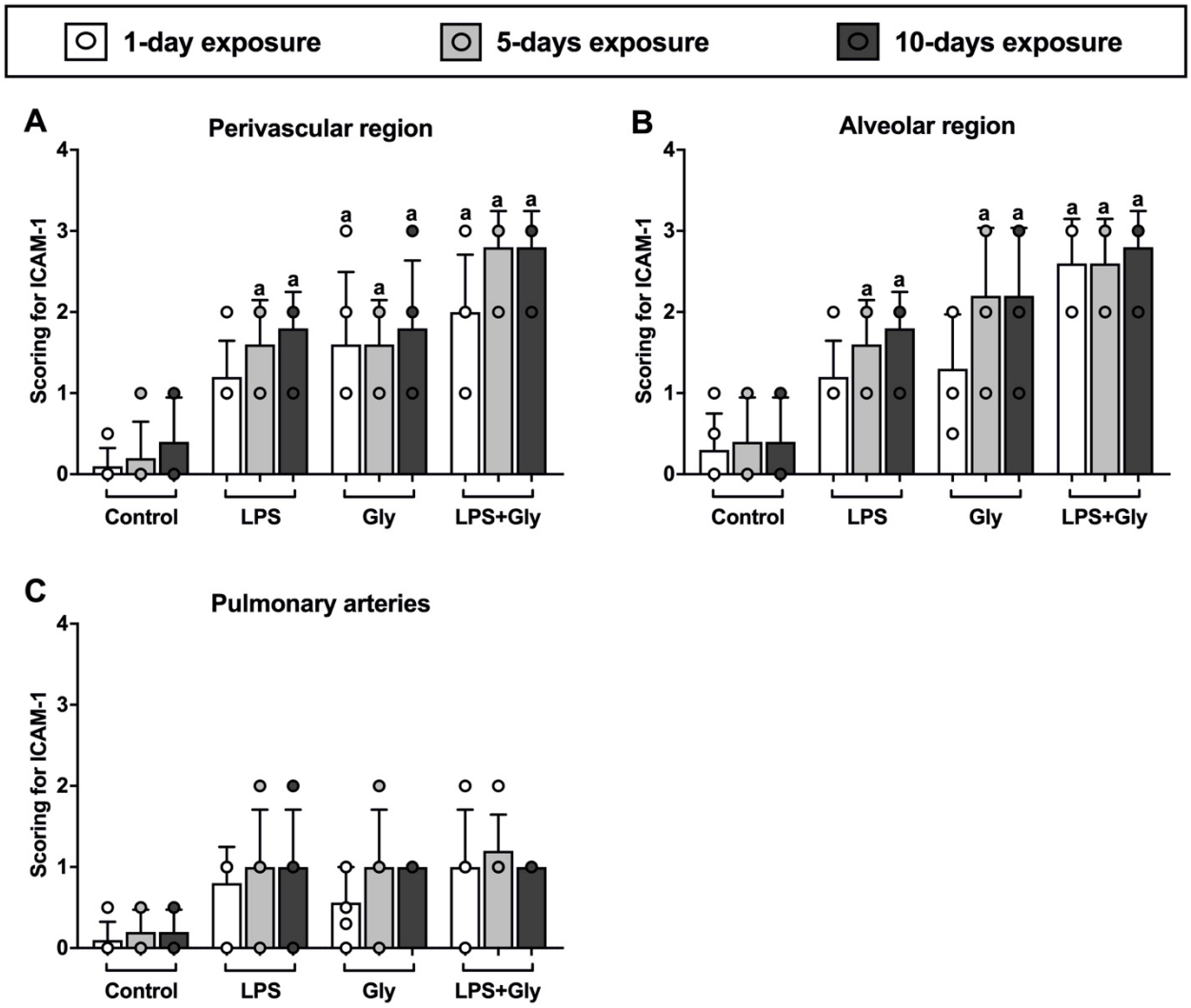

Figure 5.8: Quantification of ICAM-1 expression in lung sections. ICAM-1 expression quantified in perivascular region (A), alveolar region (B), and pulmonary arteries (C) of lung sections from control, LPS, glyphosate (Gly), or combined LPS and glyphosate (LPS+Gly) exposure groups for 1-day, 5-days, and 10-days. Data presented as mean ± SD (N = 5 mice/group; 5 fields per lung sections of each mice). “a” indicates a significant difference ($p < 0.05$) compared with the control group.

5.5 DISCUSSION

Exposure to a combination of LPS and glyphosate resulted in higher lung histology inflammatory scores as compared to scores from individual exposures to LPS or glyphosate. Repeated exposure to the combination of LPS and glyphosate resulted in robust infiltration of inflammatory cells in the perivascular, peribronchiolar and alveolar regions, and increases of alveolar septal thicknesses and perivascular spaces in the lungs. All exposures to the combination of LPS and glyphosate showed intense ICAM-1 staining in the perivascular and alveolar regions of the lungs with minimal staining in the pulmonary arteries. Further, there was significantly higher ICAM-1 mRNA in the lung tissue after repeated exposure to the combination of LPS and glyphosate, however, this expression waned with increasing exposure time.

Histological scoring revealed that exposure to a combination of LPS and glyphosate induced greater recruitment of inflammatory cells in lungs as induced by LPS alone or glyphosate alone exposures. Furthermore, repeated exposure to a combination of LPS and glyphosate resulted in robust infiltration of inflammatory cells in the perivascular, peribronchiolar and alveolar regions, and increases of alveolar septal thicknesses and perivascular spaces in the lungs. Migration of leukocytes is mediated by the release of proinflammatory cytokines from stimulated cells. Our previous work showed a synergistic release of TNF-α, IL-6, and KC cytokines with repeated exposure to the combination of LPS and glyphosate as compared to individual LPS or glyphosate exposures (193). Exposure to the same dose of glyphosate as used in our work, combined with the allergen, ovalbumin, did not result in greater lung inflammatory response than the ovalbumin or glyphosate alone treatments (22). This suggest that in combined exposures, a potent inflammatory stimulus such as LPS, could be critical for the induction of cellular and molecular inflammatory changes in the lungs. This agrees with previous work showing that individual LPS exposure, at agricultural relevant levels, shows only a fraction of the inflammatory response in lungs in comparison to complex agricultural exposures (11,23), suggesting a differential inflammatory effect when multiple contaminants are exposed to the pulmonary system.

Repeated exposure to the combination of LPS and glyphosate, for both five-days and 10-days, induced robust recruitment of inflammatory cells in the perivascular region as compared to the alveolar and peribronchiolar regions. Previous work has shown that multiple oral exposures to the pesticides fipronil, ethion or imidacloprid, followed by single intranasal LPS exposure, resulted in perivascular, alveolar and peribronchiolar infiltration of leukocytes (213–217). Currently, the relationship between peribronchiolar and perivascular recruitment of inflammatory cells remains unknown; however, our lung histology results showed a concomitant increase in the perivascular space around pulmonary arteries in both five-days and 10-days combined LPS and glyphosate exposure groups. This histological observation suggests an active role for the perivascular region in the recruitment of inflammatory cells into the lungs for intranasal exposures.

The characteristic feature of inflammation is the recruitment of inflammatory cells into tissue (218). Inflammatory cells are blood-borne and recruited to the site of stimulus through a series of cellular and molecular events (172). Recruitment of inflammatory cells is facilitated by a variety of adhesion molecules (100). One of the best-known adhesion molecules for inflammation is the ICAM-1. ICAM-1 is a cell surface glycoprotein, constitutively expressed on endothelial cells and its expression increases during inflammation (162). ICAM-1 mediates firm binding of inflammatory cells with the endothelial cells before their transmigration (162). Studies have shown upregulation of ICAM-1 on the pulmonary capillary endothelium and its important contribution for the recruitment of inflammatory cells into lungs (102,155,219,220). In lungs, the cellular adhesion and transmigration process primarily occurs in the capillaries (221,222). We examined the expression of ICAM-1 in lungs of our treated animals to elucidate further information on the perivascular recruitment of inflammatory cells in these exposures. Similar to other studies (102,219), in our study vascular ICAM-1 expression was observed in lungs from control mice. Further, ICAM-1 mRNA was significantly increased in the lungs after repeated exposure to the combination of LPS and glyphosate. Repeated exposure (five-days and 10-days) to the combination of LPS and glyphosate intensely increased the expression of ICAM-1 in lungs, predominantly in the perivascular region of the lungs. Our data showed a putative link between expression of ICAM-1 and the extent of perivascular recruitment of

inflammatory cells in lungs. Typically, pulmonary migration of leukocytes occurs in capillaries and is mediated by cytokines and adhesion molecules located on endothelial cells (222). Therefore, accumulation of inflammatory cells around thick-walled pulmonary arteries (perivascular region) as observed in our study, is intriguing. It is possible that in response to adhesion molecules and cytokines, inflammatory cells migrate across the thick-walled pulmonary arteries (187). However, this possibility seems very unlikely in our study, based on lung histology, as we did not observe transmigrating inflammatory cells in the pulmonary arteries of inflamed lungs; and we did not observe an increase of ICAM-1 expression in the endothelium of the pulmonary arteries in inflamed lung sections. The perivascular region also possesses capillaries (223,224) which is where we saw a predominant expression of ICAM-1. It is possible capillaries in the perivascular region of the lungs may be the important site for inflammatory cell recruitment in these complex intranasal exposures. In this work we did not elucidate the function of the perivascular leukocytes, but it is an important aspect to address in future studies.

Our results further showed that with repeated exposures to the combination of LPS and glyphosate leukocyte infiltration persisted in the lungs. This differs from the inflammatory cytokine findings of this same work, wherein there was a dampening of TNF-α, IL-6 and KC cytokines between five-days and 10-days of exposure to the combination of LPS and glyphosate (193). There can be several reasons for these findings. Exposure to the combination of LPS and glyphosate may impair the functional ability of macrophages to clear/phagocytose the inflammatory cells. Murine macrophages treated with LPS have shown a reduction in their ability to clear inflammatory cells through an imbalance of pro- and anti-inflammatory cytokines (225). Secondly, the combination of LPS and glyphosate may have direct chemotactic activity for leukocytes. The ability to directly attract human neutrophils has been shown on complex exposure such as grain dust extract that contains numerous contaminants including endotoxin (64,226). Thirdly, these exposures may be inducing ongoing low-grade chronic inflammation as inflammatory mediators TNF-α, KC and IL-6 were reduced but not completely diminished. Low levels of inflammatory mediators may keep the inflammatory process ongoing and prevent full recovery with repeated exposures.

There are several strengths and limitations to this study. This study examined both early (one-day) and repeated exposures to common agricultural exposures and is different dosing than studies by others (22). The treatments and doses of LPS and glyphosate were agriculturally relevant doses and the treatments mimicked agricultural worker exposure patterns. The glyphosate dose was similar to that utilized in another research (22). The histological scoring was blinded and carried out by four technicians well versed in histology, and agreement was very high between scorers. Serological analysis of lung cells may have provided additional support for these findings. Our analyses were limited to immunohistochemistry as lung tissues were fixed with paraformaldehyde and sectioned from paraffin blocks and therefore, we cannot perform electron microscopy which would clearly visualize the impact on capillaries. Future research to investigate the function of infiltered leukocytes in lungs after agricultural co-exposures could be important to furthering our understanding.

5.6 CONCLUSIONS

Exposure to a combination of LPS and glyphosate resulted in higher lung histology inflammatory scores as compared to scores from individual exposures to LPS or glyphosate. Repeated exposure to the combination of LPS and glyphosate resulted in robust infiltration of inflammatory cells in the perivascular, peribronchiolar and alveolar regions, increases of alveolar septal thicknesses and perivascular spaces in the lungs, and intense ICAM-1 expression in the perivascular region. Capillaries in the perivascular region may be important to the recruitment of inflammatory cells in complex intranasal exposures. These findings are important to further our understanding on agriculture-associated lung effects from exposure to the complex contaminants in the agricultural work environment.

CHAPTER 6 DISCUSSION

6.1 Overall discussion

The respiratory system responds to various environmental contaminants differently depending upon the nature of the contaminant, amount, incidence, and duration of exposure (4,6,7,40,43). It is known that respiratory inflammatory response in workers is attenuated after repeated exposures to the same agricultural environment (9,11,42). Agricultural exposures are complex in nature, include multiple contaminants and the workers are mostly exposed to many contaminants at the same time. It is not known if the respiratory inflammatory response changes when exposure to common agents occurs in a combination versus single agent exposure, nor is the inflammatory effect of repeated exposures clearly understood. This makes the study of lung responses in agricultural workers a highly complex area of investigation.

Endotoxin and glyphosate are two common contaminants to which agricultural workers are exposed. Glyphosate is the most common active ingredient in agricultural herbicides and the lung inflammatory potential of glyphosate is not clearly understood (15–17). Endotoxin is ubiquitous in agricultural workplace environments and induces an innate immune response mainly by binding to TLR-4 receptor (12,62,63,115). Airborne endotoxin and glyphosate have been detected in the same agricultural environment (22), suggesting the likelihood of co-exposure to endotoxin and glyphosate. Although LPS alone is known to stimulate an innate immune response in lungs, it is not known if addition of glyphosate can influence the LPS-generated immune response. The primary aim of this research was to evaluate the lung inflammatory potential of exposure to glyphosate alone and in combination with LPS, at agriculturally relevant doses, by using a mouse model.

My doctoral thesis characterized the differential inflammatory response in lungs after individual or combined exposure to LPS and glyphosate. The pulmonary inflammatory response induced by individual exposures to glyphosate and LPS was different from the inflammatory response induced when both individual agents, at the same doses, were given in a combination. As shown in chapter 3, exposure to glyphosate showed latent response with mixed eosinophilic

and neutrophilic infiltration in lungs. The cytokine response increased with repeated glyphosate exposure over time. Exposure to a combination of LPS and glyphosate, compared to individual exposures, resulted in a greater lung inflammation, as shown by the inflammatory markers in chapter 4 and histological changes in chapter 5. The combined LPS and glyphosate exposure resulted in a more significant synergistic neutrophilic response with TLR-2 immunomodulation than either the individual LPS or glyphosate responses. Further, the inflammatory response from the repeated combined LPS and glyphosate exposures dampened over time.

Glyphosate is a low molecular weight herbicide. Low molecular weight agents are less likely to induce an adaptive immune response due to inefficient presentation of these agents by antigen presenting cells to naïve T cells (227). If glyphosate is unable to induce adaptive immunity, perhaps glyphosate may stimulate an innate immune response. Epithelial cells in lungs are a major cellular component of innate immunity and are also the first point of contact for inhaled agents exposure (228). Airway epithelial cells can release Th2 cytokines (IL-4, IL-5 and IL-13) after stimulation and these cytokines can further upregulate endothelial adhesion molecules such as ICAM-1, VCAM-1 and vWF that mediate recruitment of inflammatory cells including neutrophils and eosinophils into lungs (182,229). As discussed in chapter 3, there was a latent response to glyphosate exposure with an increase of neutrophil counts, eosinophil markers and histological changes after short-term exposure (five-days) while inflammatory markers were not increased after a single glyphosate exposure (one-day). Upregulation of endothelial ICAM-1, VCAM-1, and vWF appeared to be important to regulate leukocyte infiltration in lungs after glyphosate exposure. Further, the immune response was modulated with an increase of IL-4, IL-5, and IL-13 cytokines after long-term (10-days) repeated glyphosate exposure. These findings suggest that, at a low agriculturally relevant dose, repeated glyphosate exposure appears to induce enough inflammatory stimulus in airway epithelial cells for induction of an innate immune response in lungs. These findings are further supported by our in-vitro experiment that showed dose-dependent effects of glyphosate treatments on the toxicity of human airway epithelial cells (Appendix A), suggesting that low dose glyphosate treatments are likely to induce stimulation of airway epithelial cells for cytokine release rather than toxic effects. Although we did not find an increase of IL-6 and IL-8 cytokines after 24 hours

of glyphosate treatment to cells, further cell work is needed to test for Th2 cytokines. In the agricultural environment, glyphosate exposure does not occur alone, it occurs in combination with other contaminants, including endotoxin (LPS). Glyphosate has been shown to be strongly associated with the inhalable fraction of organic dust that is enriched with endotoxin. Glyphosate does not appear to be a potent inducer of an innate immune response in the lungs; however, repeated glyphosate exposure may induce lung inflammatory changes.

The second aim of this work was to evaluate if glyphosate could modulate the innate immune response when presented with another agriculturally relevant inflammatory agent. We evaluated the inflammatory response when glyphosate was presented in combination with LPS. Five days of repeated exposure to the combination of LPS and glyphosate resulted in higher neutrophil counts, myeloperoxidase, TNF-α, IL-6, KC levels, and ICAM-1 and TLR-2 expression compared to the same length of treatment to LPS or glyphosate alone, suggesting that glyphosate potentiates the inflammatory response induced by LPS exposure. TLR-4 expression in the combined LPS and glyphosate group did not differ from the LPS alone exposure group. TLR-4 was tested as it activates on airway epithelial cells and other immune cells in response to LPS and TLR-4 activation has a direct role for neutrophil recruitment in the lungs (135–137). The findings revealed that TLR-4 did not respond differentially in the combined LPS+ Glyphosate exposure from that of the exposures to LPS alone, suggesting a different mechanism when agents were combined. The results revealed that, after five-days of exposure, the combined LPS and glyphosate exposure induced strong TLR-2 expression that was manyfold higher than LPS or glyphosate alone groups. TLR-2 recognizes a wide range of microbes and its role appears to be dependent upon the dominant pattern recognition receptor that is activated by exposure (203). The addition of glyphosate to LPS altered the immune response towards TLR-2 rather than TLR-4. Therefore, during complex agricultural exposure, it is possible that different pattern recognition receptors including TLR-2 and TLR-4, strive for dominance in the inflammatory response and this modulation of immune response depends upon the molecules involved in the exposure. Glyphosate exposure modified LPS-induced lung inflammatory responses and TLR-2 may be important in the modulated inflammatory response. Damaged lung tissue releases endogenous ligands for innate immune receptors (203,230); for example, “alarmins” for TLR-2

receptors. Alarmins may be important in modulating the course of microbial infections by engaging TLR-2. Endogenous ligands or “alarmins” molecules contribute to host responses to infections; however, there has been no evidence to show that “alarmins” can modulate the course of microbial infections by engaging TLR-2. This work raises the possibility that endogenous ligand molecules may modulate the course of microbial infections by engaging TLR-2.

Increasing exposures (10-days) resulted in an attenuation of responses of cytokines and chemokines, importantly IL-6, and TLR-2, and ICAM-1 expression (Chapter 4). This attenuated immune response has been shown by others after exposure to swine dust extract (129,130) and is similar to our results with the combined LPS and glyphosate exposure. Swine dust is a complex of molecules including LPS (167), while glyphosate has never been tested in swine dust. Despite a reduction in airway inflammatory mediators after long-term combined LPS and glyphosate exposure, infiltered leukocytes accumulated in BAL fluid and in perivascular, alveolar and peribronchiolar regions of lungs, including CD45+B and CD3 T lymphocytes (chapter 4 and chapter 5). Our results showed that, while some of the inflammatory response attenuated as exposure time increased, there were still significant differences in results from animals exposed to LPS alone versus when combined with glyphosate. While the strong TLR-2 response present at five days attenuated by 10-days and returned back to similar levels to that of the individual LPS and glyphosate exposures, IL-6, IL-4 and MPO remained significantly higher in the co-exposed group, B and T lymphocytes persisted in the perivascular region, and respiratory measures were lower. Repeated combined LPS and glyphosate exposure in mice could be used as a model to study the chronic inflammatory adaptation response. Because leukocytes such as the short-lived neutrophils migrating out of blood vessels are usually activated, their accumulation in lung tissue demonstrates a sign of ongoing inflammation albeit it may not be as acute as observed after short-term exposure (172). While critical for host defense, excessive neutrophil accumulation can damage the lung tissue by the production of reactive oxygen species that is central for progression of inflammatory diseases (172,198). Taken together, the results suggest that the combined exposure to LPS and glyphosate resulted in a differential inflammatory response in the longer-term, as compared to the individual

exposures. The persistent cellular infiltration in the lungs may assist in explaining the persistence of respiratory symptoms and decreases in lung function that have been shown with repeated exposures in agricultural workers.

6.2 Summary of the study

This research highlighted the ability of glyphosate to induce airway inflammation and lung pathology. Attempts to mimic agricultural exposure to glyphosate were made for the mice experiment by studying its agriculturally relevant dose, exposure pattern similar to a work shift, and involving repetitive exposure effects. Single exposure to glyphosate did not show a release of inflammatory meditators in lungs. Repetitive glyphosate exposure showed an increase of eosinophils and neutrophils, with Th2 cytokine release in lungs. Our study provides first data on upregulation of ICAM-1, VCAM-1, and vWF expression in lungs of glyphosate-treated mice, suggesting their role for inflammatory changes. Direct activation of airway epithelial cells may be important for the induction of an innate immune response by repetitive exposure to glyphosate.

A differential inflammatory response was elucidated after exposure to combined LPS and glyphosate than LPS or glyphosate individually. This study adds to the data of the inflammatory effects induced by agricultural exposures by unravelling the inflammatory response of two common agricultural exposures. While the trends in our results are similar to those from more complex agricultural exposures, we furthered the work. The studied doses of LPS and glyphosate were the same for both the combined and individual exposure groups. We found that exposure of the lungs to the combination of LPS and glyphosate resulted in a greater inflammatory response and more robust histological changes that could be differentiated from the responses to the single agent exposures. While the inflammatory response to the combined LPs and glyphosate exposure had some similarities to the LPS alone response, the responses were distinguishable, and the combined exposure response was stronger. This suggests that, while LPS may be important to the response, introduction of a second molecule, such as glyphosate, can alter the response. TLR-2 may be important in this response and requires further investigation. Agricultural exposure includes a combination of molecules.

Immunomodulation from combined exposure to common agricultural agents may be important to understand the pulmonary inflammatory response induced by complex agricultural exposures.

Attenuation in the inflammatory response was observed after repeated exposure to combined LPS and glyphosate. Results showed reduction in cytokine expression after 10-days of combined LPS and glyphosate exposure while leukocyte infiltration accumulated in lungs and showed intense immunostaining for CD45+ B and CD3+ T lymphocytes around the perivascular regions. Repeated exposure to combined LPS and glyphosate in mice could be used as a model to study the cellular mechanism underlying the adaptation response. The persistent cellular infiltration in the lungs may assist in explaining the persistence of respiratory symptoms and decreases in lung function that have been shown with repeated exposures in agricultural workers.

These results have important implications for furthering our understanding of enhanced pulmonary inflammation induced from exposures to the complement of molecules common in agricultural work environments.

6.3 Challenges, limitations, and future research

The exposures were performed via intranasal route in male, C57BL/6 mice. The sex and species of mouse, and exposure route was similar to previous studies on agricultural exposures including LPS or glyphosate exposure studies (22,129); however, differences in airway inflammatory response have been observed depending upon the sex (184,231), species of mouse model (212) and route of exposures (232). It is always challenging to mimic the real agricultural exposures in an animal model and each method of exposure route has its own advantages and disadvantages (233). An aerosol exposure route is more closely aligned with real-world agricultural exposure, but the use of an intranasal method is common and logistically viable for studying respiratory exposures. It would be interesting to compare the finding of our exposures on female C57BL/6 mice via an aerosol exposure route.

Another challenge for animal model research is the selection of dose for environmental agents. The doses of LPS and glyphosate in the current study were relevant agricultural exposures as their levels were intentionally selected to be close to detected levels in the agricultural environments in previous studies (22,166); however, data on glyphosate levels in an agricultural environment are lacking as there is only data from a single study (22). Research is needed on the estimation and measurement of glyphosate levels in grain and swine dust. As LPS levels are highly variable in agricultural dust, it may be better to estimate both LPS and glyphosate in the same agricultural dust samples.

Our glyphosate exposure study evaluated its inflammatory effects in lungs after one-day exposure or daily exposure for five days and 10-days. There remains a need to investigate other histological changes in lungs such as goblet cell metaplasia, collagen deposition, leukocyte infiltration etc. after repeated glyphosate exposure. Longer-term exposure to glyphosate in mice could show chronic inflammatory changes in the lungs of exposed mice. Research is needed to understand the mechanism of induction of an innate immune response in lungs after glyphosate exposure. Glyphosate exposure research by using TLR knock-out animal models could provide a clear picture on its role in the activation of TLR signaling and as a risk factor for higher susceptibility to microbial infections. In addition, glyphosate exposure has been associated with worsening of asthma in agricultural population-based studies. Future research using an asthma mouse model can elucidate whether glyphosate can worsen existing asthma or not.

Our combined LPS and glyphosate exposure showed reduction in airway inflammatory markers with persistent lung pathology after 10-days repetitive exposure. There remains a need to further characterize the different subsets of leukocytes infiltered within lungs and chronic histological changes in lungs. Future research on more prolonged exposure to combined LPS and glyphosate would help us understand the mechanism of a chronic inflammatory adaptation response. The intravital microscopy technique can provide a clear visualization of the route of leukocyte infiltration in various compartments of lungs.

The use of advanced molecular techniques would have strengthened the study results. Immunohistochemistry was used to characterize the expression of adhesion molecules and leukocyte infiltration in lungs. Protein expression results could be complemented with western blotting. Multi-marker flow cytometry would have better classify the different subsets of leukocytes infiltered into the lungs. The use of electron microscopy to visualize the perivascular capillaries in treated lungs would have strengthened the conclusion of chapter 5.

In this field of research, studies on combination of agents found in the agricultural environment appears important for illuminating the pulmonary inflammatory response. In addition, individual agent research will help to understand their specific role in pulmonary inflammation which may, in turn, further help to predict immunomodulatory role these individual agents could induce on its combined exposure with secondary stimulant. Lung immunomodulation from combination of exposures could help us explain the differential respiratory effects observed in agricultural workers.

CHAPTER 7

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APPENDIX A

HUMAN ALVEOLAR EPITHELIAL CELLS TREATMENT WITH COMBINED OR INDIVIDUAL LPS AND GLYPHOSATE: EFFECTS ON CELL VIABILITY AND CYTOKINE RELEASE

A.1 BACKGROUND

The alveolar epithelium is the major target in inhaled exposures of the lungs (234–236). Alveolar epithelial cells are located in the corners of alveoli. The alveolar epithelial surface is composed of alveolar type I and alveolar type II cells. Alveolar type I cells covers approximately 96% of the alveolar surface area and these cells are flat and extremely thin. Alveolar type II cells comprise approximately 4% of alveolar surface area but they represent majority of alveolar epithelial cells (about 60%). Alveolar type II cells play an important role for maintain the integrity and normal function of alveoli and they serve as progenitor of alveolar type I cells. Although both type I and II cells are constantly contributing to lung defense, type II cells are more active immunologically and secrete a broad variety of factors, such as cytokines and chemokines, involved in activation and differentiation of immune cells (234). Human lung carcinoma type II epithelial cells (A549 cells) are derived from alveolar type II cells and are considered useful to study the possible contribution of type II cells for inflammatory or toxic response of lungs (235).

Agricultural exposures are known to cause respiratory outcomes in workers and endotoxin and glyphosate are two common agents in the agricultural environment. In-vitro experiments on LPS treatments has been shown dose dependent effects on A549 cells viability and release of IL-6 and IL-8 cytokines (237,238). There is lack of data for glyphosate treatments. Li et al., 2013 treated A549 cells for 72 hours with different doses of glyphosate (between 15 mM to 50 mM) dissolved in DMEM media with fetal bovine serum and assessed cytotoxicity using a Cell titer Glu Luminescent Cell Viability assay (108). Glyphosate treatment at 50 mM showed a significant reduction (about 17 %) in viable A549 cells as compared to the untreated A549 cells (108). In addition, based on linear regression modelling, authors estimated a 136.5

mM concentration of glyphosate as the half maximal (50%) inhibitory concentration (IC50) for A549 cells (108). Treatment to combined LPS and glyphosate are never studied.

In this study, we treated A549 cells to different concentrations of LPS alone, glyphosate alone or combined LPS and glyphosate. Analysis were performed for cell's viability, IL-6, and IL-8 cytokines and TNFAIP3 expression.

A.2 MATERIALS AND METHODS

A.2.1 Cell culture experiment and treatments

A human alveolar epithelial cell line (A549 cells) was obtained from American Type Culture Collection (ATCC, Manassas, VA). A549 cells were cultured and grown in Dulbecco's Modified Eagle Media (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) containing penicillin and streptomycin (Gibco) at 37^0 C and 5% CO_2. After 80-90% confluency, cells were dissociated by treatment with Tryp-LE Select (Gibco) for 5 min. and counted using a hemocytometer. For treatments, A549 cells were seeded at a density of 0.5 x 10^6 per well in 6-well plates and allowed to attach overnight. Cells were washed with 1X PBS (ThermoFisher Scientific, Waltham, MA USA) before treatments. LPS (stock: 1 mg/ml, E. coli serotype 0111:B4; Sigma, St. Louis, MO USA) and glyphosate (stock: 0.85 M, analytical grade PESTANAL standard; Sigma, St. Louis, MO USA) stock solutions were prepared in DMEM serum free media and were syringe filtered (size: 0.22 µm; Millipore, Burlington, MA USA). Treatment with dimethyl sulfoxide (DMSO; 0.5%) in serum-free DMEM media was used as a positive control. Untreated cells in serum-free DMEM were used as a negative control. A549 cells were treated for 24 hours at 37^0 C and 5% CO_2 with different concentrations of LPS alone, glyphosate alone, and combined LPS and glyphosate.

A.2.2 Cytotoxicity

The viability of treated A549 cells was tested using a Cell Proliferation Kit 1 (MTT based, Roche, Catalog #11465007001) according to manufacturer's instructions. Briefly, cells were incubated with 10 µl of MTT labeling reagent for 4 hours at 37° C and 5% CO_2. Next, 100 µl of

solubilization buffer was added to each well and incubated for 24 hours in the incubator. Plates were read using a BioTek Synergy HT plate reader (BioTek, Winooski, VT USA) at 450 nm. The cell viability percentage was calculated using the following formula: Cell viability percentage: (average absorbance of treated cells / average absorbance of negative control cells) × 100.

A.2.3 ELISA

Cell culture media was collected and used for quantification of IL-6 and IL-8 protein using specific ELISA according to manufacturer's instructions (ThermoFisher Scientific, Waltham, MA USA). Proteins were tested in duplicate. Plates were read using a BioTek Synergy HT plate reader (BioTek, Winooski, VT).

A.2.4 RNA purification and RT-PCR analysis

RNA was purified from treated cells using the RNeasy Plus Mini kit (Qiagen, Chatsworth, CA USA) according to manufacturer instructions. Purified mRNA was quantified using a Take3 plate and BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT USA). cDNA synthesis was performed using iScript Reverse Transcription Supermix (BioRad Laboratories, Hercules, CA USA) by CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA USA).

RT-PCR was performed using mouse probe for A20/TNFAIP3 (Mm00437121_m1; Life Technologies, Grand Island, NY USA). Ribosomal RNA (Life Technologies, Grand Island, NY USA) was used as an endogenous control. PCR was conducted using a CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA USA). Reaction was carried out in duplicate and was incubated for 2 minutes at 500C, 10 minutes at 950C followed by 40 cycles at 950C for 15 seconds followed by 600C for 1 minute. Cycle threshold of each target gene obtained from real-time PCR data was analyzed with ΔΔCt method to determine its relative quantification.

A.3 RESULTS

A.3.1 Cytotoxicity

The dose dependent effect on toxicity of A549 cells was observed with treatments to glyphosate alone, LPS alone and combined LPS and glyphosate at different concentrations (Figures 1-3). The reduction in viable cell percentage was observed with the increase in glyphosate treatment concentration (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM, and 10 mM). Glyphosate alone treatment at 1 mM and 10 mM showed a significant cell death compared to the untreated cells. LPS alone at all used concentrations did not induce significant cell death. In relation to untreated cells, the significant cell death was observed on combination of LPS (0.5 µg/ml) with glyphosate at 10 µM, 100 µM, 1 mM, and 10 mM concentrations.

A.3.2 IL-8 and IL-6 levels

Combined LPS and glyphosate treatments (0.5 µg/ml LPS plus 0.1 mM Gly, 1 mM Gly or 10 mM Gly) significantly increased the IL-8 concentration as compared to untreated cells and respective individual LPS (0.5 µg/ml LPS) and Gly (0.1 mM, 1 mM or 10 mM) treatments (Figure 4).

None of the treatments shown a significant effect on IL-6 release in comparison to untreated cells (Figure 5).

A.3.3 TNFAIP3 expression

Combined LPS and Gly at 0.5 µg/ml LPS plus 10 mM Gly shown significant increase in TNFAIP3 as compared to untreated cells and 10 mM Gly treated cells (Figure 6).

A.4. DISCUSSION

Our invitro experiment showed dose dependent effect on A549 cells toxicity after treatments to glyphosate alone, LPS alone or combined LPS and glyphosate, suggesting lower concentrations of these treatments would possibly stimulate the lung epithelial cells. IL-8 levels were significantly higher after treatment to combined LPS and glyphosate than respective concentrations of individual treatments.

The concentrations of LPS and glyphosate were comparable between our experiments on A549 cells and mice. Mice experiment involved instillation of glyphosate at 1ug/40ul and LPS at 0.5 ug/40ul which suggest effective concentration to lungs would be at least equal to or less than 0.025 ug/ul of glyphosate and 0.012 ug/ul of LPS, respectively. For in-vitro experiment, highest concentration of glyphosate (M.W. 170 g/l or ug/ul) treatment was 10 mM (1.7 ug/ul) and of LPS treatment was 1 ug/ml (0.001 ug/ul). It suggests that both glyphosate and LPS concentrations in mice experiment were lower than their highest concentrations used in in-vitro experiment.

We found that glyphosate treatment at 10 mM in serum free media reduced about 30% of A549 viable cells. Li and colleagues used glyphosate mixed with serum containing media for treatments and found 50 mM of glyphosate reduced about 17 % A549 viable cells as compared to the control cells (108). Serum protein may bind to glyphosate and subsequently reduces its availability to A549 cells. Recent data show that isopropylamine salt of glyphosate did not contribute to apoptotic and genotoxic effects on A549 cells (76,77,110). Therefore, our results need to be interpreted with caution as they are based on pure glyphosate. Furthermore, glyphosate exposure in mice increased IL-4, IL-5 and IL-13 levels as shown in chapter 3. Although we did not test for these cytokines in in-vitro experiment but findings of less toxicity of A549 cells at lower doses of glyphosate, suggest the probable stimulatory effect on lung epithelial cells. Therefore, these cellular results support our lung inflammatory findings of mice experiment after exposure to a lower dose of glyphosate. Further studies are needed to examine not only the mechanism of glyphosate-induced inflammation but also the cytotoxicity in vivo. For example, we would like to examine apoptosis and cell death in the lungs of glyphosate-treated animals. In addition, no information is available on the absorption of glyphosate in lungs including lung epithelial cells. Glyphosate could be radiolabelled and used to investigate whether it's absorbs into epithelial cells or not.

As shown in chapter 4, exposure to combined LPS and glyphosate induced greater release of KC cytokine in lungs of mice than individual LPS and glyphosate. Murine KC cytokine is functional homologue of IL-8 in humans, and it is important for neutrophil recruitment (239).

Airway epithelial cells are one of the primary sources of IL-8 (239). Direct treatment of combined LPS and glyphosate to A549 cells showed dose dependent effect on cells viability and greater release of IL-8 than treatments to alone LPS or glyphosate. These results of A549 experiments complement the mice study and showing the differential response from lung epithelial cells after exposure to combined LPS and glyphosate. It suggests that lung epithelial cells may be important for greater inflammatory response including KC release in lungs after combined LPS and glyphosate exposure. Future A549 cellular research needs to investigate effects on other inflammatory markers after exposure to combined LPS and glyphosate.

A.5 CONCLUSIONS

Overall, our findings on A549 cells complement the results of mice experiment after exposure to alone or combined LPS and glyphosate. Results showed dose dependent effect on A549 cells ability. Combined LPS and glyphosate treatment to A549 cells significantly increased IL-8 levels. More A549 cellular research is needed to investigate other inflammatory changes after treatments to glyphosate alone or combined LPS and glyphosate.

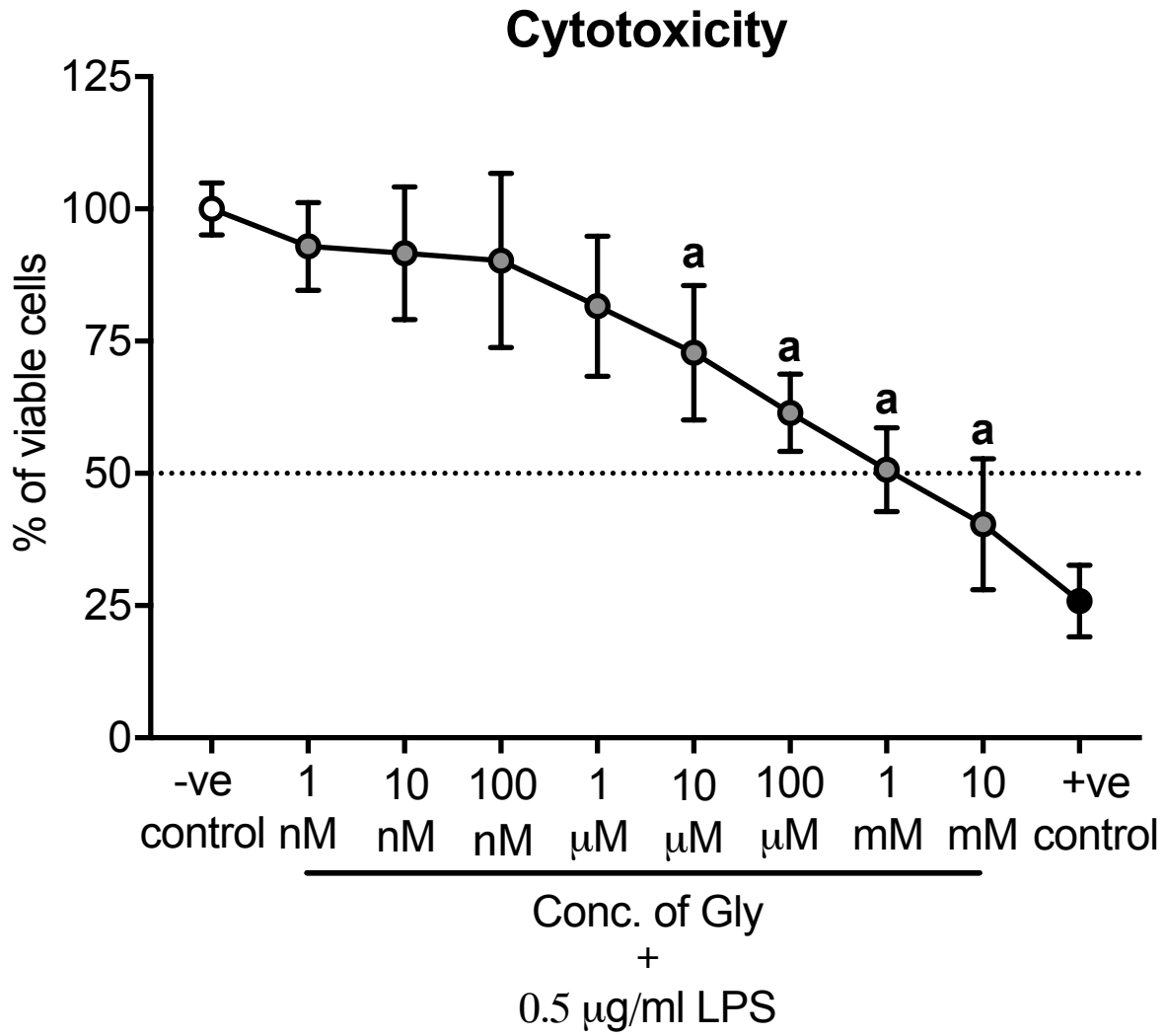

Figure A.1: Viability of combined LPS and glyphosate treated A549 cells. DMSO was used for positive control treatment. The cell viability was measured using Cell Proliferation Kit 1. The LD_{50} is shown as dotted line. Data presented as mean ± SD of three independent experiments with each treatment performed in duplicate. "a" indicates a significant difference ($p < 0.05$) compared with the negative control group.

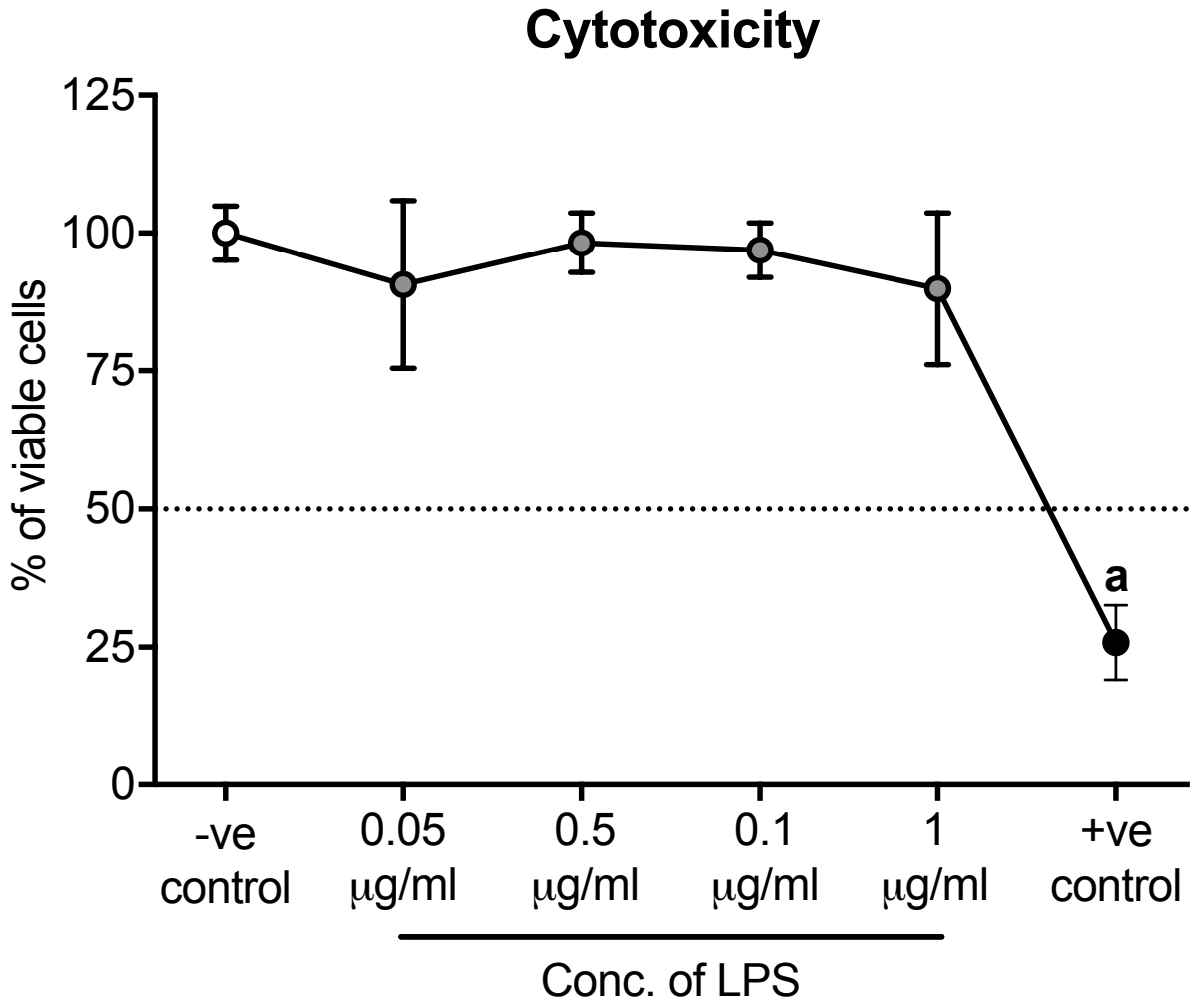

Figure A.2: Viability of LPS treated A549 cells. DMSO was used for positive control treatment. The cell viability was measured using Cell Proliferation Kit 1. The LD_{50} is shown as dotted line. Data presented as mean ± SD of three independent experiments with each treatment performed in duplicate. "a" indicates a significant difference ($p < 0.05$) compared with the negative control group.

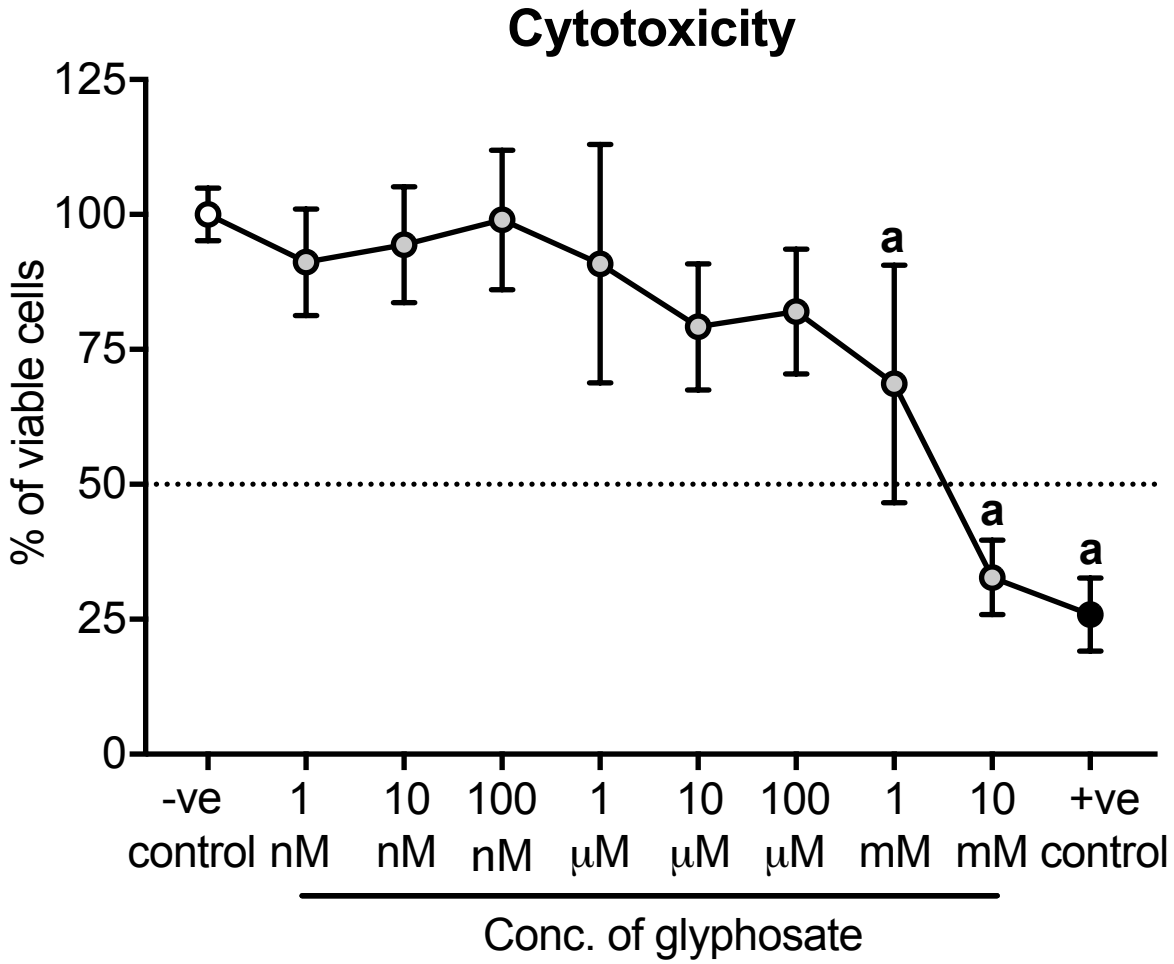

Figure A.3: Viability of glyphosate treated A549 cells. DMSO was used for positive control treatment. The cell viability was measured using Cell Proliferation Kit 1. The LD_{50} is shown as dotted line. Data presented as mean ± SD of three independent experiments with each treatment performed in duplicate. "a" indicates a significant difference ($p < 0.05$) compared with the negative control group.

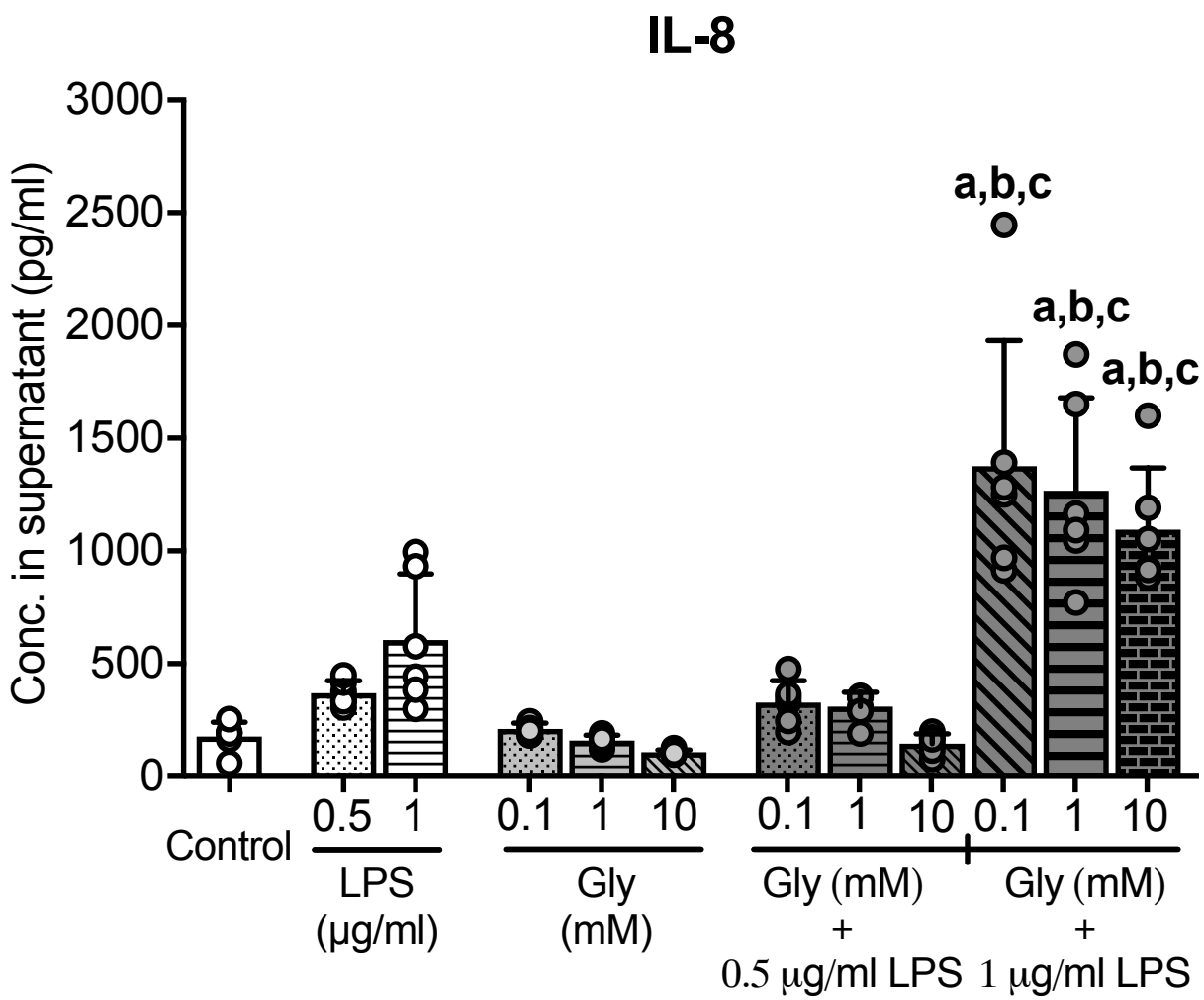

Figure A.4: Levels of IL-8 cytokine in supernatant of A549 treated cells. IL-8 levels after treatment of A549 cells to LPS alone, glyphosate alone or combined LPS and glyphosate at different concentrations. Media supernatant was assessed by ELISA. Data presented as mean ± SD of three independent experiments with each treatment performed in duplicate. Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "b" indicates a significant difference compared with the LPS group; "c" indicates a significant difference compared with the glyphosate group.

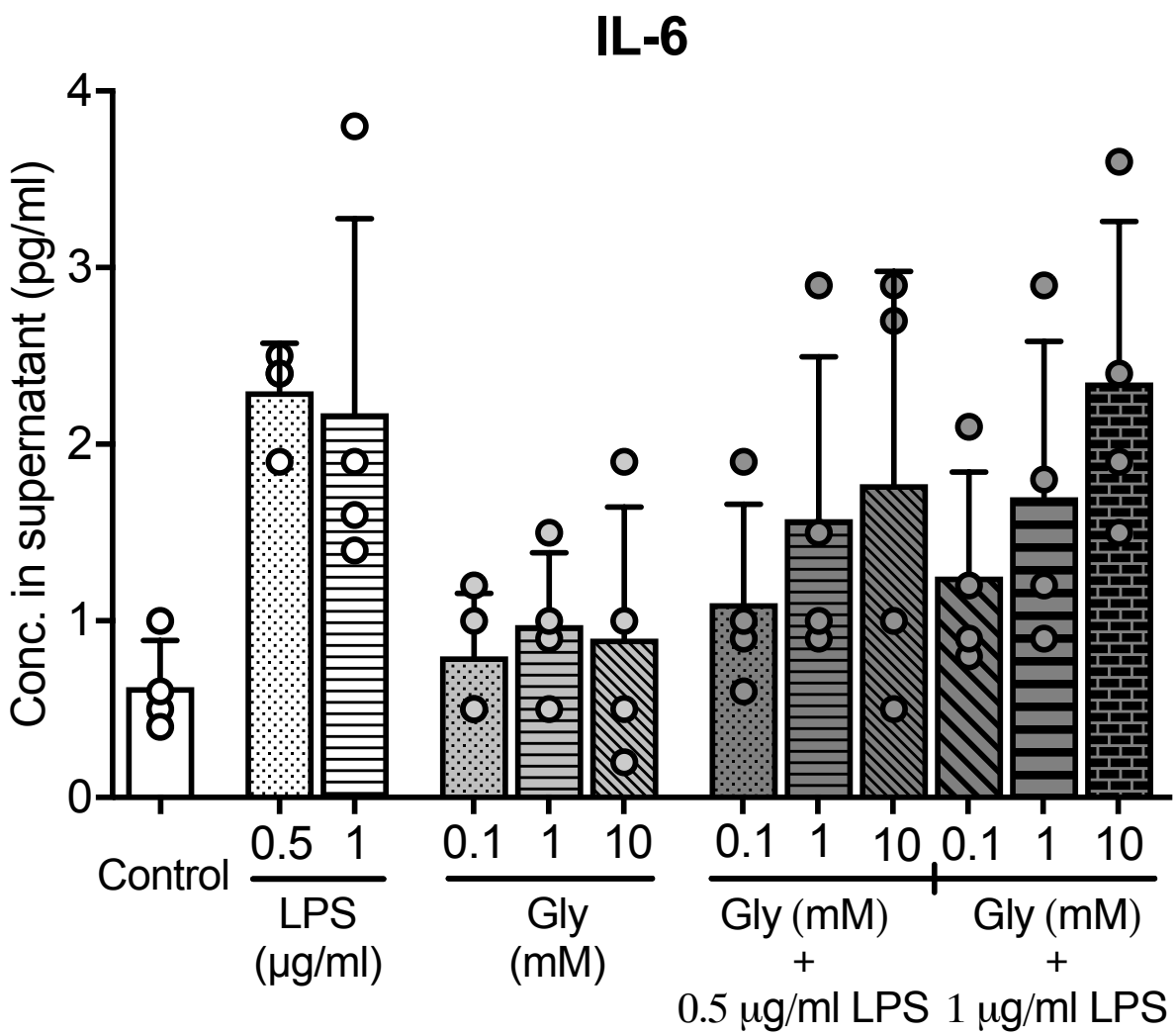

Figure A.5: Levels of IL-6 cytokine in supernatant of treated A549 cells. IL-6 levels after treatment of A549 cells to LPS alone, glyphosate alone or combined LPS and glyphosate at different concentrations. Supernatant was assessed by ELISA. Data presented as mean ± SD of two independent experiments with each treatment performed in duplicate.

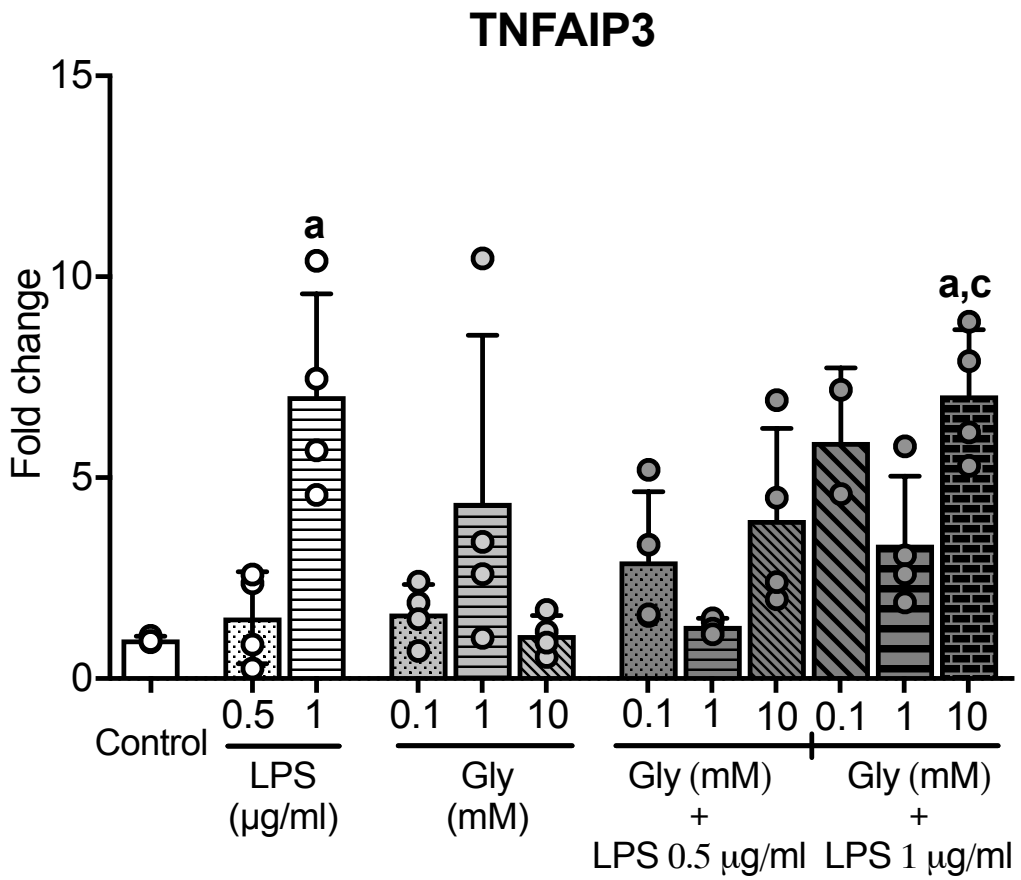

Figure A.6: Real time PCR for expression of TNFAIP3 after A549 cellular treatments. Fold change of TNFAIP3 after treatment of A549 cells to LPS alone, glyphosate alone or combined LPS and glyphosate at different concentrations. RT-PCR was conducted with mouse probe against TNFAIP3. Data presented as mean ± SD of two independent experiments with each treatment performed in duplicate. Significance ($p < 0.05$) is denoted as such: "a" indicates a significant difference compared with the control group; "c" indicates a significant difference compared with the glyphosate group.

APPENDIX B

GENOMIC PROFILE OF TOLL-LIKE RECEPTOR SIGNALING PATHWAYS IN LUNGS OF GLYPHOSATE EXPOSED MICE: RT^2 PROFILER PCR ARRAY

B.1 BACKGROUND

Glyphosate is a low molecular agent, used in herbicides (15). Population based studies have shown association of glyphosate exposure with respiratory outcomes in agricultural workers (47,90). However, the mechanism of glyphosate to induce airway inflammation is unknown. Low molecular weight agents are less likely to induce an adaptive immune response due to inefficient presentation of these agents by antigen presenting cells to naïve T cells (227). If glyphosate is unable to induce adaptive immunity, perhaps glyphosate may stimulate an innate immune response. Toll-like receptors (TLRs) play crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns derived from various microbes (192). TLRs signal through the recruitment of specific adaptor molecules, leading to activation of the transcription factors NF-κB and IRFs, which dictate the outcome of innate immune responses. As there is no data on innate immune signaling pathway after glyphosate exposure, we undertook a pilot project to study the TLR signaling pathway related gene expression changes in lungs of mice after exposure to glyphosate for 1-day, 5-days or 10-days.

B.2 MATERIALS AND METHODS

RNA was extracted from frozen lung samples of glyphosate and control mice for 1 day, 5- days and 10-daysexposure periods using RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA) as mentioned previously (Chapter 5; N = 2). cDNA was prepared from isolated RNA using an RT^2 First Strand Kit (Qiagen) following guidelines of RT^2 Profiler PCR Array kit. Reaction for cDNA preparation was run at 42^0 C for 15 min. followed by 95^0C for 5 min. Prepared cDNA was added to RT^2 SYBR Green qPCR Master Mix and dispensed into the RT^2 Profiler™ PCR Array following manufacturer's instructions (Qiagen; PAMM-018ZD-24). This mouse array profiles the expression of multiple genes central to TLR-mediated signal transduction and innate immunity. Each 96-well plate contains 84 pathway specific genes of interest, five housekeeping genes for

normalization of data, three positive PCR control for genomic DNA contamination and three reverse transcription control (table 1). A list of genes in this array along with their description is provided in table 2. Array PCR was run using CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). Program was set as following: 95°C for 10 min., 95°C for 15 sec followed by 60°C for 1 min. repeated for 40 cycles, and 95°C for 15 sec., 60°C for 15 sec. and 95°C for 15 sec. Software helped in the data acquisition including establishment of cycle threshold (C_T) of each gene.

Data analysis was performed using $2^{-\Delta\Delta CT}$ method and its detail is provided in RT² Profiler PCR Array handbook. Briefly, C_T value for each gene of interest (GOI) was first normalized to the average of 5 housekeeping genes (AVG HKG) as $\Delta C_T = (C_T^{GOI} – C_T^{AVG\ HKG})$. Average of ΔC_T value for each gene of interest was calculated by using ΔC_T from biological replicate of same gene. Fold change in each gene of interest in glyphosate group over control group ($2^{-\Delta\Delta CT}$) was calculated by using the formula: $\Delta\Delta CT = \Delta C_T$ (glyphosate group) - ΔC_T (control group).

B.3 RESULTS

Glyphosate exposure for 1-day, 5-days or 10-days showed the changes in expression of several genes compared to the control exposure (table 3). These results are shown in heat map as well (Figure 1). Glyphosate exposure resulted > 2-fold change in total of two genes in one-day group, six genes in 5-days group and two genes in 10-days groups over control exposure group. Glyphosate exposure for 1-day showed upregulation (> 2-fold change) in heat shock protein family A gene (Hspa1a; fold change: 3.47) and TNF-α induced protein 3 gene (TNFAIP3; fold change: 2.07) genes. Glyphosate exposure for 5-days changed (> 2-fold change) the gene expression of TNF-α (fold change: 3.21), CXCL10 (fold change: 3.11), IL-1β (fold change: 2.09), MYD88 (fold change: 4.55), NF-KB (fold change: 14.22) and reticuloendotheliosis oncogene (Rel; fold change: 5.71). After glyphosate exposure for 10-days, HSPa1a (fold change: 8.70) and translation related gene eif2ak2 (fold change: 5.32) expression were changed (> 2-fold change). No gene was downregulated among all profiled genes after glyphosate exposure for 1-day, 5-days, or 10-days in comparison to the control exposure.

B.4 DISCUSSION

As respiratory data is limited on glyphosate exposure, this broad-spectrum expression profiling of TLR pathway related genes was undertaken on lung samples of mice after exposure to glyphosate for 1-day, 5-days, or 10-days. The primary aim of this broad-spectrum expression profiling was to help us select the few genes and further confirming their expression with gene specific PCR as shown in chapter 3. Our results showed that glyphosate exposure for 5-days increased the expression of MyD88 and NF-KB in lungs. MyD88 is an accessory molecule and NF-KB is a transcription factor involved in TLR signaling pathways. Our gene specific PCR findings in chapter 3 showed that glyphosate exposure significantly increased the TLR-2 and TLR-4 (5-days and 10-days exposure) compared to control exposure. As MyD88 and NF-KB are involved in TLR-2 and TLR-4 signaling pathway, these findings suggest that TLR-2 and TLR-4 signaling pathway could be important to investigate the mechanism of glyphosate induced lung inflammation. In addition, as TLR signaling pathways gets activated by microbes (192), our study suggest glyphosate exposure could enhance susceptibility to microbial infection. Future research on glyphosate exposure in TLR knock out mice could provide better understanding on activation of TLR signaling pathways.

The major limitation of this analysis was less sample size (N = 2). More sample size could have strengthened the results of this pilot project.

B.5 CONCLUSIONS

This pilot project showed upregulation in few TLR signaling related genes in the lungs of mice after exposure to glyphosate. This primary data will help us in selection of genes for further confirmation by gene specific PCR in glyphosate research.

Table B.1: A list of genes in RT² Profiler PCR Array

Position	UniGene	GenBank	Symbol	Description
A01	Mm.392569	NM_010472	Agfg1	ArfGAP with FG repeats 1
A02	Mm.4475	NM_013482	Btk	Bruton agammaglobulinemia tyrosine kinase
A03	Mm.336851	NM_009812	Casp8	Caspase 8
A04	Mm.290320	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2
A05	Mm.3460	NM_009841	Cd14	CD14 antigen
A06	Mm.89474	NM_009855	Cd80	CD80 antigen
A07	Mm.1452	NM_019388	Cd86	CD86 antigen
A08	Mm.439656	NM_009883	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta
A09	Mm.3996	NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase
A10	Mm.248327	NM_019948	Clec4e	C-type lectin domain family 4, member e
A11	Mm.4922	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
A12	Mm.1238	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)
B01	Mm.877	NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10
B02	Mm.378990	NM_011163	Eif2ak2	Eukaryotic translation initiation factor 2-alpha kinase 2
B03	Mm.405823	NM_007922	Elk1	ELK1, member of ETS oncogene family
B04	Mm.5126	NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain
B05	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene
B06	Mm.313345	NM_010439	Hmgb1	High mobility group box 1
B07	Mm.334313	NM_008284	Hras1	Harvey rat sarcoma virus oncogene 1
B08	Mm.6388	NM_010479	Hspa1a	Heat shock protein 1A
B09	Mm.1777	NM_010477	Hspd1	Heat shock protein 1 (chaperonin)

B10	Mm.1245	NM_010510	Ifnb1	Interferon beta 1, fibroblast
B11	Mm.240327	NM_008337	Ifng	Interferon gamma
B12	Mm.277886	NM_010546	Ikbkb	Inhibitor of kappaB kinase beta
C01	Mm.874	NM_010548	Il10	Interleukin 10
C02	Mm.103783	NM_008351	Il12a	Interleukin 12A
C03	Mm.15534	NM_010554	Il1a	Interleukin 1 alpha
C04	Mm.222830	NM_008361	Il1b	Interleukin 1 beta
C05	Mm.896	NM_008362	Il1r1	Interleukin 1 receptor, type I
C06	Mm.14190	NM_008366	Il2	Interleukin 2
C07	Mm.1019	NM_031168	Il6	Interleukin 6
C08	Mm.2856	NM_010559	Il6ra	Interleukin 6 receptor, alpha
C09	Mm.38241	NM_008363	Irak1	Interleukin-1 receptor-associated kinase 1
C10	Mm.152142	NM_172161	Irak2	Interleukin-1 receptor-associated kinase 2
C11	Mm.105218	NM_008390	Irf1	Interferon regulatory factor 1
C12	Mm.3960	NM_016849	Irf3	Interferon regulatory factor 3
D01	Mm.275071	NM_010591	Jun	Jun oncogene
D02	Mm.87787	NM_010735	Lta	Lymphotoxin A
D03	Mm.2639	NM_010745	Ly86	Lymphocyte antigen 86
D04	Mm.116844	NM_016923	Ly96	Lymphocyte antigen 96
D05	Mm.18494	NM_008928	Map2k3	Mitogen-activated protein kinase kinase 3
D06	Mm.412922	NM_009157	Map2k4	Mitogen-activated protein kinase kinase 4
D07	Mm.15918	NM_011945	Map3k1	Mitogen-activated protein kinase kinase kinase 1
D08	Mm.258589	NM_172688	Map3k7	Mitogen-activated protein kinase kinase kinase 7
D09	Mm.21495	NM_016700	Mapk8	Mitogen-activated protein kinase 8
D10	Mm.43081	NM_013931	Mapk8ip3	Mitogen-activated protein kinase 8 interacting protein 3

D11	Mm.68933	NM_016961	Mapk9	Mitogen-activated protein kinase 9
D12	Mm.3177	NM_010739	Muc13	Mucin 13, epithelial transmembrane
E01	Mm.213003	NM_010851	Myd88	Myeloid differentiation primary response gene 88
E02	Mm.256765	NM_008689	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105
E03	Mm.102365	NM_019408	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
E04	Mm.170515	NM_010907	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
E05	Mm.220333	NM_010908	Nfkbib	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
E06	Mm.300795	NM_010909	Nfkbil1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like
E07	Mm.238146	NM_172766	Nfrkb	Nuclear factor related to kappa B binding protein
E08	Mm.442385	NM_011630	Nr2c2	Nuclear receptor subfamily 2, group C, member 2
E09	Mm.28957	NM_023324	Peli1	Pellino 1
E10	Mm.21855	NM_009402	Pglyrp1	Peptidoglycan recognition protein 1
E11	Mm.212789	NM_011144	Ppara	Peroxisome proliferator activated receptor alpha
E12	Mm.292547	NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2
F01	Mm.4869	NM_009044	Rel	Reticuloendotheliosis oncogene
F02	Mm.249966	NM_009045	Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
F03	Mm.112765	NM_138952	Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2
F04	Mm.34580	NM_019786	Tbk1	TANK-binding kinase 1

F05	Mm.203952	NM_174989	Ticam1	Toll-like receptor adaptor molecule 1
F06	Mm.149280	NM_173394	Ticam2	Toll-like receptor adaptor molecule 2
F07	Mm.23987	NM_054096	Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein
F08	Mm.273024	NM_030682	Tlr1	Toll-like receptor 1
F09	Mm.87596	NM_011905	Tlr2	Toll-like receptor 2
F10	Mm.33874	NM_126166	Tlr3	Toll-like receptor 3
F11	Mm.38049	NM_021297	Tlr4	Toll-like receptor 4
F12	Mm.116894	NM_016928	Tlr5	Toll-like receptor 5
G01	Mm.42146	NM_011604	Tlr6	Toll-like receptor 6
G02	Mm.23979	NM_133211	Tlr7	Toll-like receptor 7
G03	Mm.196676	NM_133212	Tlr8	Toll-like receptor 8
G04	Mm.44889	NM_031178	Tlr9	Toll-like receptor 9
G05	Mm.1293	NM_013693	Tnf	Tumor necrosis factor
G06	Mm.116683	NM_009397	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3
G07	Mm.1258	NM_011609	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a
G08	Mm.103551	NM_023764	Tollip	Toll interacting protein
G09	Mm.264255	NM_001033161	Tradd	TNFRSF1A-associated via death domain
G10	Mm.292729	NM_009424	Traf6	Tnf receptor-associated factor 6
G11	Mm.371667	NM_080560	Ube2n	Ubiquitin-conjugating enzyme E2N
G12	Mm.278783	NM_023230	Ube2v1	Ubiquitin-conjugating enzyme E2 variant 1
H01	Mm.328431	NM_007393	Actb	Actin, beta
H02	Mm.163	NM_009735	B2m	Beta-2 microglobulin
H03	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H04	Mm.3317	NM_010368	Gusb	Glucuronidase, beta
H05	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1

H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Table B.2: Gene expression changes in mice lungs after glyphosate exposure. Gene expression changes in lungs of glyphosate exposed mice over control mice for 1-day, 5-days or 10-days exposure. RNA analyzed by RT² Profiler PCR Array and data was presented as fold change (N = 2 per exposure group). Genes showing > 2-fold change are highlighted.

	Fold change after glyphosate exposure (N = 2 per exposure group)		
Gene label	**1-day exposure**	**5-days exposure**	**10-days exposure**
Agfg1	1.05	1.67	0.92
Btk	0.84	1.67	1.09
Casp8	1.08	1.67	1.04
Ccl2	0.71	1.67	0.64
CD14	0.77	1.67	1.09
CD80	1.33	1.67	1.19
CD86	1.14	1.67	0.95
Cebpb	1.19	1.67	0.69
Chuk	0.67	1.67	0.78
Clec4e	0.59	1.67	0.44
Csf2	1.01	1.67	1.07
Csf3	0.59	1.67	1.01
CXCL10	0.57	**3.11**	0.89
Eif2ak2	1.03	1.67	**5.32**
Elk1	1.75	1.34	0.86
FADD	0.75	0.68	0.84
FOS	1.90	0.76	1.05
HMGB1	0.71	0.68	0.70
Hras	0.80	1.18	1.07
HSP1A	**3.47**	0.99	**8.70**
HSPd1	0.97	0.78	0.96
IFNb1	0.59	1.67	1.01
IFNg	0.59	1.67	1.01

IKbKb	1.25	0.89	1.13
IL10	0.59	1.67	1.01
IL12a	0.77	1.11	0.68
IL1a	0.57	1.29	0.88
IL1b	1.07	**2.09**	0.35
IL1r1	1.32	0.96	0.95
IL2	0.59	1.67	1.01
IL6	0.59	1.25	0.75
IL6ra	1.34	0.79	0.80
IRAK1	1.06	0.68	1.02
IRAK2	0.99	0.95	1.14
Irf1	1.02	1.60	0.96
Irf3	0.59	0.69	0.79
Jun	1.17	**14.22**	0.73
Lta	0.59	1.67	1.01
Ly86	0.59	1.18	1.36
Ly96	0.75	0.87	0.82
MAP2K3	1.34	1.51	1.01
MAP2K4	1.24	0.84	1.07
MAP3K1	1.21	0.72	0.88
MAP3K7	1.29	0.81	0.92
MAPK8	1.30	0.77	1.03
MAPK8ip3	0.72	1.37	1.27
MAPK9	1.49	0.66	0.75
Muk13	0.59	1.67	1.01
MyD88	0.90	**4.55**	1.26
NFkB1	1.16	1.58	0.99
NFkB2	1.58	1.36	1.33
NFkBia	1.29	1.04	0.95
NFkBiB	0.59	1.67	0.86

NFkBil1	0.77	1.23	1.26
NFrkB	1.03	0.78	1.06
Nr2c2	1.09	0.67	0.77
Peli1	1.00	0.65	0.91
Pglyrp1	0.55	1.18	1.32
Ppara	0.64	0.64	1.31
PTGS2	1.83	0.57	0.89
Rel	1.18	**5.71**	0.92
RelA	0.66	1.62	1.39
Ripk2	0.83	0.81	0.83
TBK1	1.12	0.88	0.87
TiCAM1	0.61	0.49	1.23
TiCAM2	0.74	0.85	1.51
TIRAP	0.66	1.00	0.99
TLR1	0.69	0.52	0.96
TLR2	1.68	1.21	1.35
TLR3	1.01	0.62	0.73
TLR4	1.02	0.71	1.05
TLR5	1.20	0.59	0.82
TLR6	0.58	1.67	0.88
TLR7	0.98	1.67	0.83
TLR8	0.57	0.46	0.94
TLR9	0.70	1.23	1.63
TNF	0.72	**3.21**	0.93
TNFAIP3	**2.07**	0.80	1.14
TNFrsf1a	0.88	0.68	1.05
TOLLIP	1.27	0.98	0.82
TRADD	0.60	0.86	0.88
TRAF6	1.13	0.61	0.93
UBE2n	0.86	0.59	0.79

UBE2V1	0.89	0.70	1.27

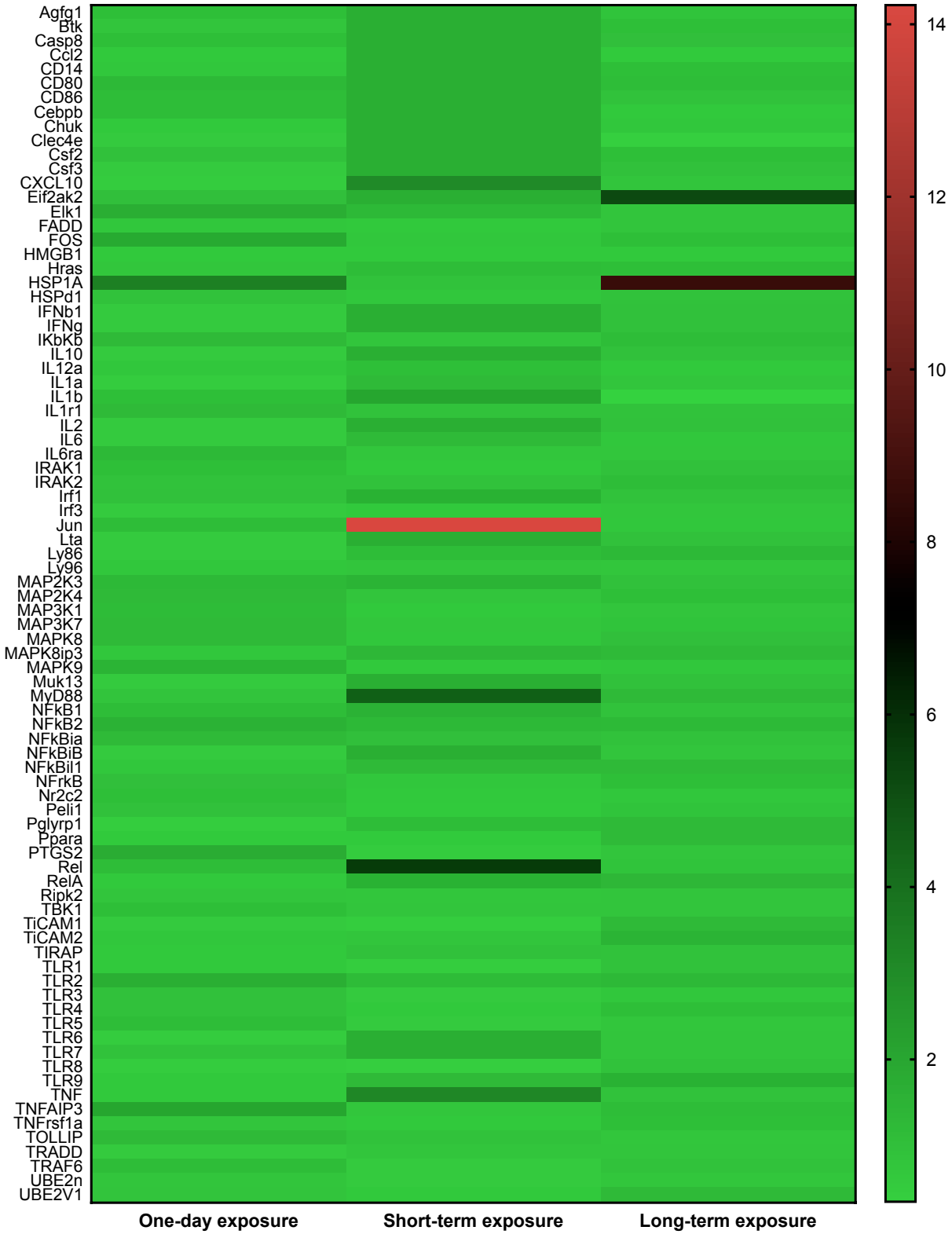

Figure B.1: Heat map for gene expression changes in mice lungs. Heat map for gene fold change in mice lungs after glyphosate exposure for one day, five day (short term) or 10 day (long term).

APPENDIX C

SUPPLEMENTARY FIGURES

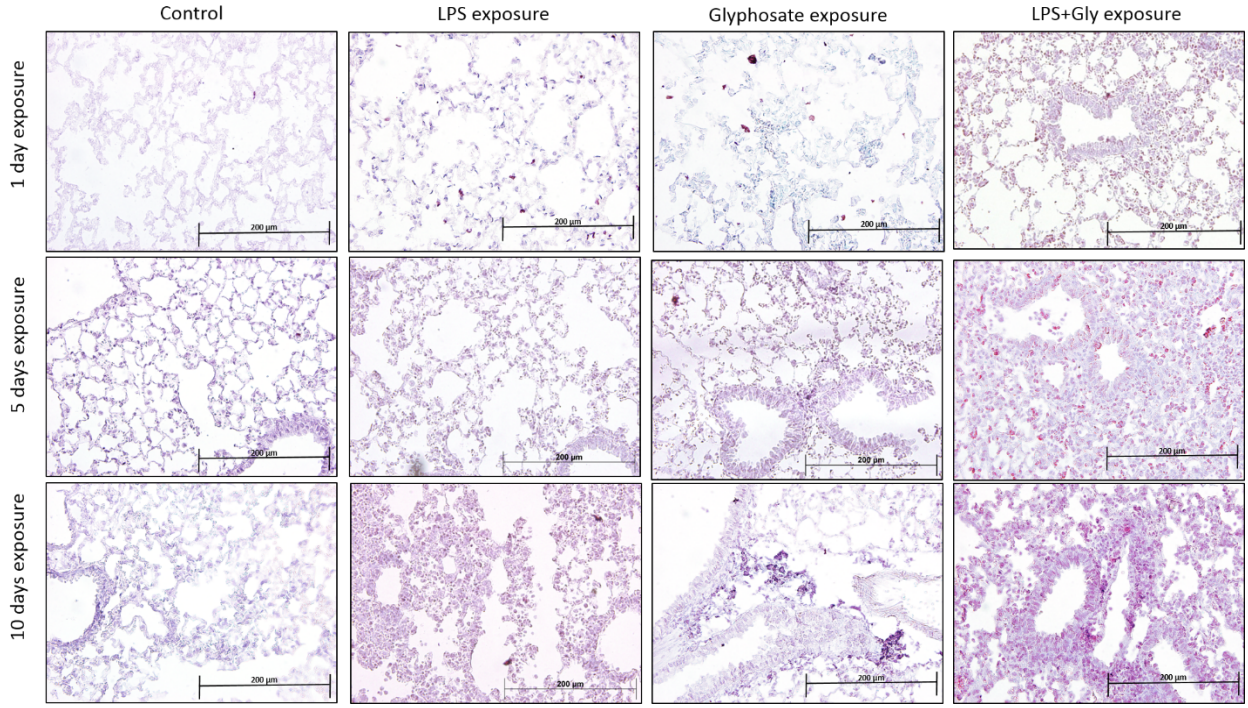

Figure C.1: Immunohistochemistry for Gr-1 expression in lung sections of treated mice. Mice were treated to control (A-C), LPS (D-F), Gly (G-I) or combined LPS+Gly (J-L) for 1-day, 5-days and 10-days. Gr-1 is a marker for neutrophil. Group representative images showing Gr-1 positive staining (N = 5 mice per group; 5 random fields per section). Magnification: ×400 (A-L). Scale bar: 200 µm (A-L). PA: Pulmonary artery; B: Bronchus.

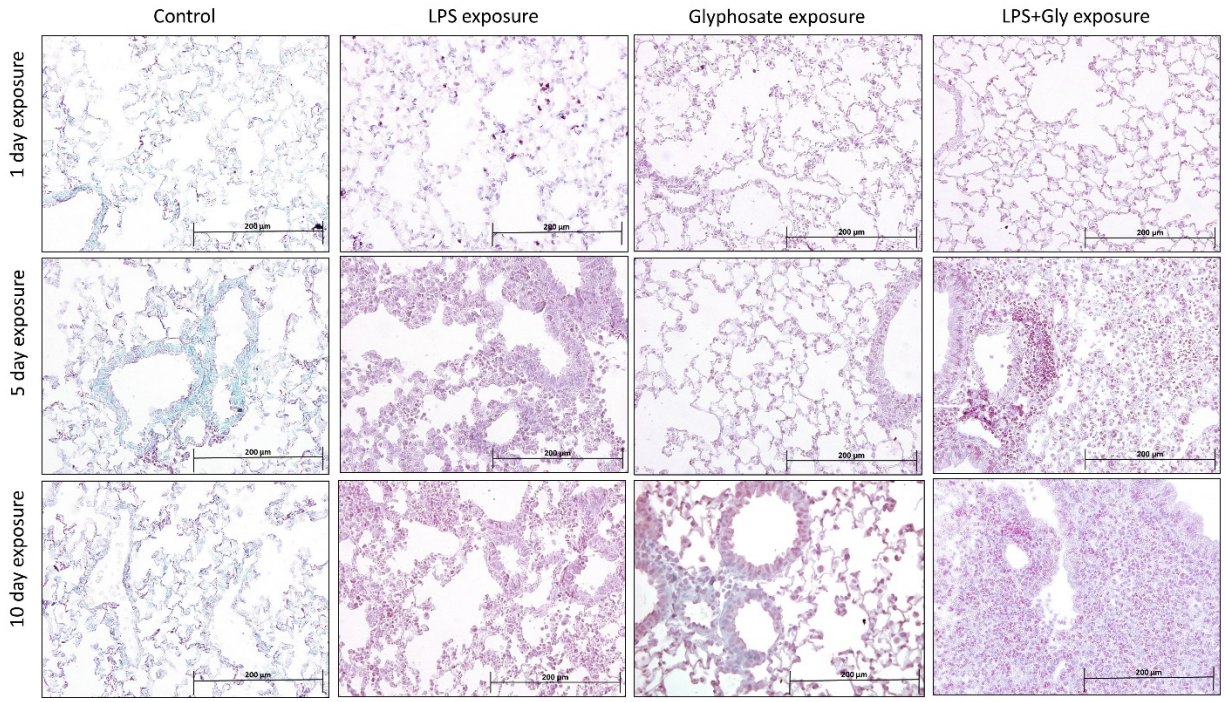

Figure C.2: Immunohistochemistry for Siglec-F expression in lung sections of treated mice. Mice were treated to control (A-C), LPS (D-F), Gly (G-I) or combined LPS+Gly (J-L) for 1-day, 5-days and 10-days. Siglec-F is a marker for macrophage. Group representative images showing Siglec-F positive staining (N = 5 mice per group; 5 random fields per section). Magnification: ×400 (A-L). Scale bar: 200 µm (A-L). PA: Pulmonary artery; B: Bronchus.

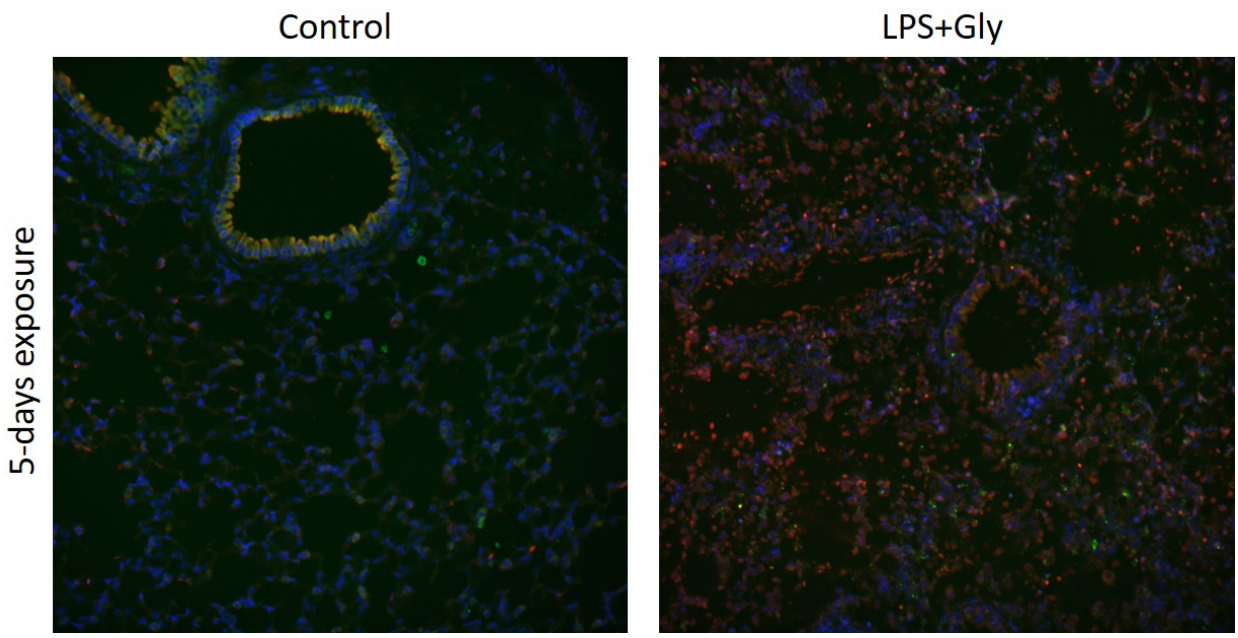

Figure C.3: Immunofluorescent staining for Gr-1 and CX3CR1 in lung sections. Mice were treated to control or combined LPS+Gly for 5-days. Combined LPS+Gly exposure induced robust Gr-1+ neutrophil infiltration into lungs. Gr-1 is a marker for neutrophil (red color). CX3CR-1 is a marker for macrophages (green color). Nucleus visualizes by DAPI staining in blue color.

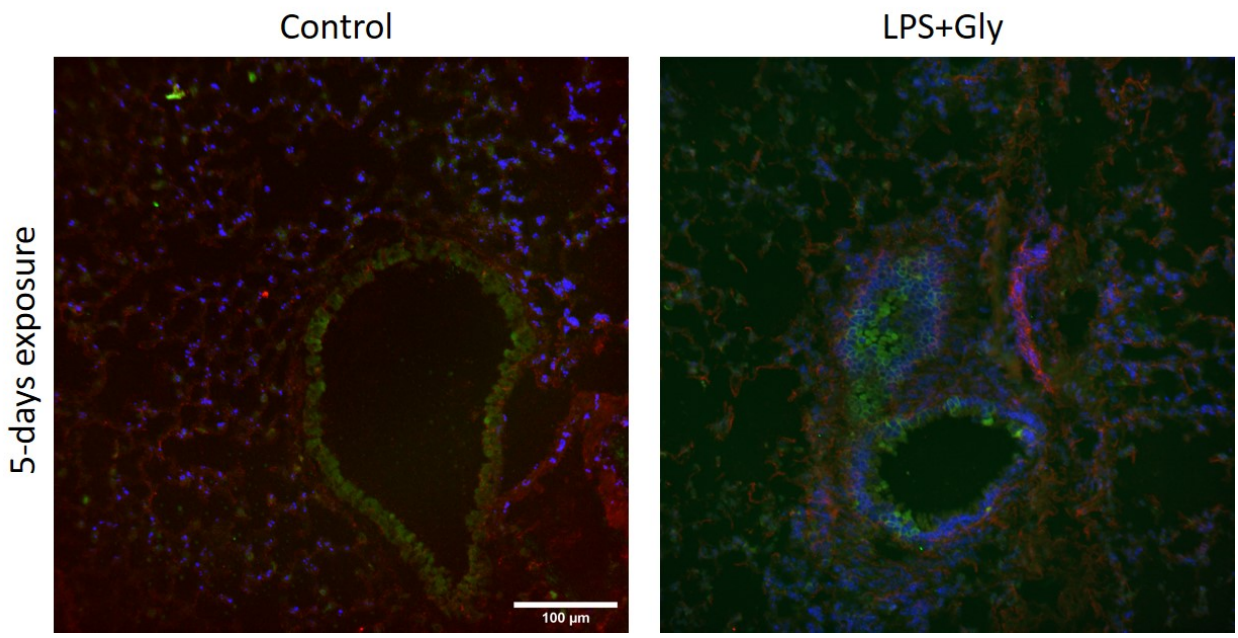

Figure C.4: Immunofluorescent staining for CD3 and vWF expression in lung sections. Mice were treated to control or combined LPS+Gly for 5-days. Combined LPS+Gly exposure induced CD3+ macrophage infiltration into lungs. vWF is a marker for blood vessel endothelium (red color). CD3 is a marker for macrophages (green color). Nucleus visualizes by DAPI staining in blue color.

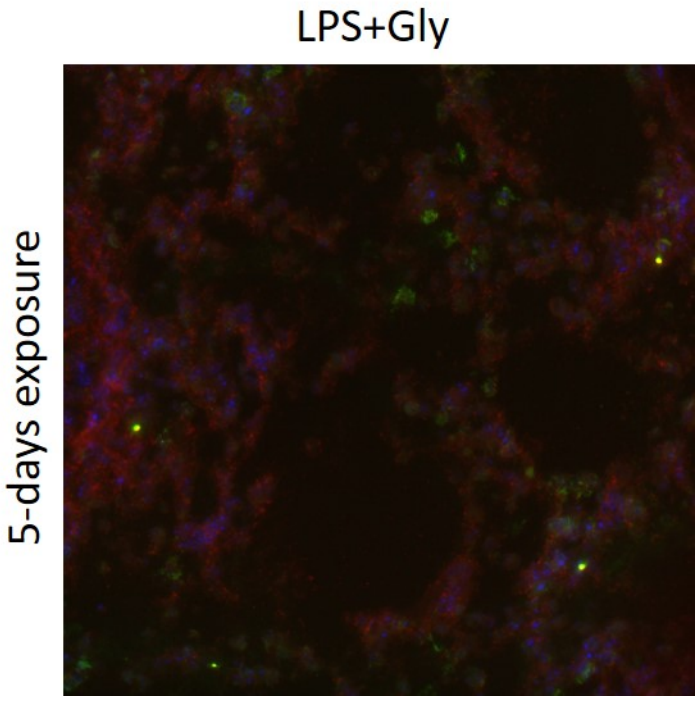

Figure C.5: Immunofluorescent staining for Ly6C and vWF expression in lung sections. Mice were treated to combined LPS+Gly for 5-days. Combined LPS+Gly exposure induced Ly6C+ monocyte infiltration into lungs. vWF is a marker for blood vessel endothelium (red color). Ly6C is a marker for monocyte (green color). Nucleus visualizes by DAPI staining in blue color.