RECRUITMENT AND FUNCTION OF PULMONARY INTRAVASCULAR

MACROPHAGES IN RATS

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ABSTRACT

Humans with biliary cirrhosis are highly susceptible to acute pulmonary dysfunction and suffer from hepato-pulmonary syndrome. The mechanisms of this enhanced susceptibility remain unknown. It is well established that pulmonary intravascular macrophages (PIMs) are present in cattle, horses, goat and sheep and increase susceptibility for lung inflammation. Species such as rat and mouse also recruit PIMs especially in a bile duct ligation model of biliary cirrhosis. The contributions of recruited PIMs to lung inflammation associated with liver dysfunction remain unknown. Therefore, I characterized a bile duct ligation (BDL) model in rats to study role of recruited PIMs in lung inflammation. First, Sprague Dawley rats were subjected to BDL (N=6) or sham surgeries (N=3) and were euthanized at 4 weeks post-surgery. Five rats were used as the controls. Lung tissues were collected and processed for histology, immunohistology, immuno-electron microscopy, enzyme-linked immunosorbant assay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR). Light microscopy demonstrated normal lung morphology in sham-operated and control rats but showed septal recruitment of mononuclear cells, which were positive for anti-rat monocytes/macrophage antibody ED-1, in BDL rats. (*p=0.002*). Immuno-electron microscopy confirmed localization of ED-1 in PIMs. BDL rats showed increased lung expression of monocyte chemoattractant protein-1 (MCP-1) protein and mRNA compared to the controls (*p=0.017*) but not of IL-1β, TNF-α, TGF-β and IL-10. Then, I treated BDL rats (N=5) with gadolinium chloride (GC; 10 mg/Kg body weight intravenous) and found reduced numbers of PIMs (*p=0.061*) at 48 hours post-treatment along with increased expression of TGF-β and IL-10.

I challenged control rats (N=5) and BDL rats (N=6) with *Escherichia coli* lipopolysaccharide (*E. coli* LPS; 0.1 mg/Kg body weight intravenous). All the BDL rats died within 3 hours of LPS challenge (100% mortality) while the normal LPS-treated rats were euthanized at 6 hours post-treatment. Histology and ED-1 staining showed dramatic increase in the number of septal monocytes/macrophages in BDL+LPS rats compared to normal LPS-treated rats (*p=0.000*). Staining of lung sections with an LPS antibody localized the LPS in lungs. RT-PCR analyses showed no differences in IL-1β transcript levels between LPS challenged BDL rats and LPS challenged control rats (*p=0.746*) but ELISA showed increase in IL-1β concentration in LPS

challenged BDL rats compared to LPS challenged control rats ($p=0.000$). TNF-α mRNA ($p=0.062$) and protein ($p=0.000$) was increased in BDL+LPS rats compared to the control+LPS rats. Immuno-electron microscopy showed IL-1β and TNF-α in PIMs. BDL rats challenged with LPS showed increased expression of IL-10 mRNA and protein ($p=0.000$ & 0.002 respectively) in lungs compared to LPS challenged control rats. TGF-β mRNA did not change ($p=0.128$) but lower protein concentrations ($p=0.000$) were observed in LPS-treated control rats compared to BDL+LPS.

To further address the role of PIMs, I treated rats with GC at 6 hours or 48 hours (N=5 each) before LPS challenge. The mortality in the 6 hour group was 20% while all the rats in 48 hour group survived till 6 hours. Histology and ED-1 staining showed decrease in the number of intravascular cells in these groups compared to LPS treated BDL rats ($p=0.039$ for 6 hour group; $p= 0.002$ for 48 hour group). There were no differences in IL-1β mRNA in both 6 hour and 48 hour groups compared to the LPS challenged BDL rats ($p=0.712$ & 0.509 respectively). ELISA showed no decrease in IL-1β concentration in 6 hour GC-treated group but a decrease was noticed at 48 hours compared to LPS challenged BDL rats ($p=0.455$ & 0.008 respectively). TNF-α mRNA levels were not different between LPS-challenged GC-treated BDL rats and LPS-challenged BDL rats ($p=0.499$ & 0.297 for 6 hour & 48 hour GC groups respectively). But TNF-α concentration in 48 hour GC group ($p=0.001$) but not in 6 hour GC group ($p=0.572$) was lower in comparison to BDL+LPS group. IL-10 mRNA was decreased in both 6 hour and 48 hour GC groups ($p=0.038$ & 0.000 respectively) compared to LPS challenged BDL rats. ELISA showed decrease in IL-10 concentration in 48 hour GC group ($p=0.030$) but not in 6 hour GC group ($p=0.420$). TGF-β mRNA expression was decreased in 48 hour GC group ($p=0.000$) but not in 6 hour GC group ($p=0.182$). But GC treatment did not affect TGF-β concentrations.

The data from these experiments characterize a BDL model to study PIM biology, show PIMs' pro-inflammatory potential and their possible role as a therapeutic target in lung inflammation.

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LIST OF ABBREVIATIONS

BDL: Bile duct ligation

CINC: Cytokine- induced neutrophil chemoattractant

ELISA: Enzyme linked immunosorbent assay

GC: Gadolinium chloride

G-CSF: Granulocyte- Colony stimulating factor

GM-CSF: Granulocyte macrophage- Colony stimulating factor

HPS: Hepatopulmonary syndrome

ICAM-1:   Intracellular adhesion molecule-1

IFN-γ: Interferon gamma

IL-1β: Interleukin 1-beta

IL-10:   Interleukin-10

iNOS: inducible-Nitric oxide synthase

MCP-1:   Monocyte chemoattractant protein-1

MIP-2:   Monocyte inhibitory protein-2

N: Number

NF-κB: Nuclear factor-kappa B

P: Probability

PIMs: Pulmonary intravascular macrophages

RT-PCR: Reverse transcription- polymerase chain reaction

TLR-4:   Toll-like receptor-4

1. INTRODUCTION

1.1. Inflammation:

Inflammation is the body's reaction to invasion by an infectious agent, antigen challenge or physical, chemical or traumatic damage. It is a complex reaction involving a number of cellular and molecular components with accompanying changes in the physiological systems. During inflammation, blood supply to the inflamed tissue increases and the inflamed tissue appears to contain greater number of blood vessels. Capillary permeability increases, which is partly caused by retraction of endothelial cells (Thickett et al., 2001). This permits large molecules to escape from the capillaries and thus allow the soluble mediators of inflammation to reach the site of inflammation. There is also increased leukocyte migration out of the capillaries into the surrounding tissues. In the earliest stages of inflammation, neutrophils are particularly prevalent, but later monocytes and lymphocytes migrate towards the site of infection (Kaplanski et al., 2003), (Larsen and Holt, 2000). The development of inflammatory reaction is controlled by cytokines (Dinarello, 1997), by products of the plasma enzyme system (Barrington et al., 2001), by lipid mediators (Robinson1987), and by vasoactive mediators released from the mast cells (Wasserman, 1987), basophils (Holgate, 2000) and platelets (Weyrich et al., 2003). Because inflammation is a complex interplay involving a variety of cells and molecules, the outcomes are usually determined by the balance between pro- and anti-inflammatory forces. For example excessive accumulation of activated neutrophils usually results in profound tissue damage and mortality. However, the inflammation process has inbuilt checks to tightly regulate the neutrophil recruitment and expression of various mediators. Therefore, the inflammation can at best be described as an essential reaction designed to protect the host.

1.2. Lung inflammation:

The epithelial surface area of the lung is large compared to epithelial surface area of other organs in the body. Lung epithelium is continuously exposed to the external environment and is always in direct contact with different types of injurious agents. Because of the

circulation of blood from whole of the body through the lungs, lungs are also continuously exposed to blood-borne bacteria, viruses or endotoxins. This makes lungs highly vulnerable to both air-borne and blood-borne infectious agents. Although there are many causes for lung inflammation, bacteria and their endotoxins play very important role in inducing acute lung inflammation. Endotoxemia is defined as the presence of endotoxins in the blood in sufficient amounts to cause disease. Endotoxin-induced systemic activation of the immune system includes cytokine production, complement activation, platelet aggregation and activation of immune cells. Endotoxin activates immune cells such as macrophages through a pathway which makes use of a receptor known as TLR-4 (Nomura *et al.*, 2000). Interaction of TLR-4 on the cell surface with two other surface proteins, CD14 and MD2, leads to the recruitment of adaptor proteins (Myd88, IRAK, and TRAF-6) that ultimately induces activation of transcription factor complex NF-kB (Aderem and Ulevitch, 2000).

Translocation of NF-kB into cell nucleus promotes expression of inflammatory mediators such as TNF-α, IL-1β, IL-6 and IL-8 (Sweet and Hume, 1996). Pro-inflammatory mediators such as TNF-α and IL-1β cause up-regulation of adhesion molecules on the pulmonary vascular endothelium (E-selectins, ICAM-1) as well as alveolar epithelium (ICAM-1) (Strieter *et al.*, 2002). Vascular adhesion molecules enhance adhesiveness of polymorphonuclear cells such as neutrophils and facilitate their transmigration into the interstitium and distal airways (Bevilacqua, 1993). Alveolar epithelial ICAM-1 enhances adhesiveness of alveolar macrophages to the epithelial cells, resulting in intensified production of cytokines and chemokines (Beck-Schimmer *et al.*, 2002).

1.3. Pro-inflammatory cytokines:

1.3.1. TNF-α and IL-1β

TNF-α and IL-1β are two of the most important "acute response" cytokines induced most notably via TLR-4 signaling pathway (Strieter *et al.*, 2002). TNF-α is a hormone like polypeptide that possesses a wide range of immunologic, metabolic and inflammatory activities. TNF-α is mainly produced by monocytes and macrophages in bacterial

infections or endotoxemia (Beutler and Kruys, 1995). TNF-α is initially expressed as a 233-amino-acid membrane-anchored precursor, which is proteolytically processed to yield the mature, 157-amino-acid cytokine (Gearing *et al*., 1994). TNF-α is released from a cell membrane-anchored precursor by proteolytic cleavage by metalloproteinase enzyme (Gearing *et al*., 1995). Mature TNF-α is a trimeric molecule, each subunit of which consists of an antiparallel beta-sandwich (Jones *et al*., 1989).

 IL-1β is a protein synthesized by a variety of cells, but primarily by monocyte-macrophages, in response to various stimuli such as bacteria, endotoxins and other cytokines (Cybulsky *et al*., 1987). IL-1β also exists in the form of precursor protein (pro-IL-1β), which is then cleaved by the enzyme into the mature form (Boraschi *et al*., 1996). TNF-α and IL-1β are produced by activated macrophages in response to a variety of stimuli such as endotoxin. Macrophages recognize endotoxin through receptor known as TLR-4 present on their surface (Nomura *et al*., 2000). TLR-4 through interaction with other proteins activates transcriptional factor complex NF-kB within the activated macrophage and leads to the expression of genes for TNF-α and IL-1β (Aderem and Ulevitch, 2000). TNF-α and IL-1β upregulates expression of adhesion molecules on pulmonary vascular endothelium and alveolar epithelium to promote recruitment of neutrophils into inflamed lungs (Strieter *et al*., 2002) (Bevilacqua1993).

1.3.2. MCP-1

Monocyte chemotactic protein-1 (MCP-1) is derived from mononuclear cells and other cell sources including macrophages. It is a potent chemoattractant for monocytes and macrophages. There are two isoforms of MCP-1 (13kD & 9kD), which can be distinguished by lectin binding (Jiang *et al*., 1991). MCP-1 plays a key role in monocyte recruitment by both integrin activation and by promoting migration of monocytes to the blood vessels (Ashida *et al*., 2001). Therefore, MCP-1 helps in recruitment of leukocytes to lung (Warren *et al*., 1993). MCP-1 gene expression is regulated by oxidation-reduction sensitive mechanism and reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl nitrate and peroxynitrite and TNF-α (Chen *et al*., 2004). MCP-1

expression in the lungs increases in response to silica, smoke and endotoxins. MCP-1 has also a crucial role in the resolution and repair processes of acute bacterial pneumonia by enhancing the removal of dying neutrophils and hepatic growth factor (HGF) production by alveolar macrophages (Amano *et al*., 2004). MCP-1 concentrations increase early in the course of multiple organ failure and this suggest that this chemokine is important in the early inflammatory changes that lead to multiple organ failure later in the course of this illness (Thomas *et al*., 2005). MCP-1 gene expression increases in bleomycin induced lung fibrosis in rats and infiltrating eosinophils represent the major cellular source of increased MCP-1 expression (Zhang *et al*., 1994). Therefore, MCP-1 appears to be a critical regulator of acute inflammation including that in the lungs.

1.4. Anti-inflammatory cytokines:

The anti-inflammatory cytokines constitute a group of immunoregulatory molecules that control the response of pro-inflammatory cytokines. The anti-inflammatory cytokines along with other specific cytokine inhibitors and soluble cytokine receptors helps to limit the potentially injurious effects of sustained or excess inflammatory reactions (Opal and DePalo, 2000). There exists a fine balance between anti-inflammatory cytokines and pro-inflammatory cytokines.

1.4.1. IL-10

IL-10 is primarily synthesized by monocytes, CD4+ Th2 cells and B cells. It circulates as a homodimer consisting of two tightly packed 160 amino acid proteins (Howard *et al*., 1992). In addition to its Th2 inhibitory activity, it is also a potent deactivator of monocyte/macrophage pro-inflammatory cytokine production (Brandtzaeg *et al*., 1996). IL-10 inhibits cell surface expression of major histocompatibility complex class-II molecules and the LPS recognition and signaling molecule CD14 (Opal *et al*., 1998). It inhibits nuclear translocation of NF-kB after LPS stimulation and promotes degradation of mRNA for the pro-inflammatory cytokines (Clarke *et al*., 1998). IL-10 suppresses cytokine production by neutrophils and natural killer cells. IL-10 also attenuates surface expression of TNF receptors and also promotes the shedding of TNF receptors into the

systemic circulation (Dickensheets *et al*., 1997). Addition of IL-10 to the cultures of monocytes, which were activated by IFN-γ, LPS or combination of both resulted in the inhibition of production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF and G-CSF at the transcriptional level (de Waal *et al*., 1991). Also activation of monocytes by LPS in the presence of neutralizing anti-IL-10 monoclonal antibodies resulted in the production of higher amounts of cytokines as compared to LPS treatment alone (de Waal *et al*., 1991). In a similar study in mice, where endotoxemia induced by the injection of LPS resulted in the rapid release of IL-10 and neutralization of endogenously produced IL-10 by administration of anti-IL-10 monoclonal antibody prior to LPS challenge resulted in a marked increase in the serum levels of TNF-α and IFN- γ (Marchant *et al*., 1994). Low concentrations of IL-10 in patients with early adult respiratory distress syndrome (ARDS) indicates poor prognosis and accounts for increased mortality rate (Donnelly *et al*., 1996). Similarly, administration of IL-10 in humans after LPS challenge resulted in fewer systemic symptoms, neutrophil responses and chemokine production (MIP-1, MCP-1) than placebo-treated control subjects (Olszyna *et al*., 2000). These data demonstrate innate anti-inflammatory actions of IL-10.

1.4.2. TGF-β

TGF-β belongs to a superfamily of dimeric proteins that share a similar structure. It is synthesized as an inactive precursor and requires activation to exert its effects. The active molecule is a 25-kd homodimer of two 12.5-kd disulphide-linked monomers. There are three isoforms of TGF-β designated as TGF-β1, TGF-β2 and TGF-β3 (Roberts, 1998). TGF-β is produced by lymphocytes, macrophages and dendritic cells and its expression control the differentiation, proliferation and state of activation of these immune cells (Letterio and Roberts, 1998). TGF-β is an important regulator of cell proliferation, differentiation and formulation of the extracellular matrix. Release and activation of TGF-β stimulates the production of various extracellular matrix proteins and thus contribute to tissue repair (Branton and Kopp, 1999).

TGF-β modulates the expression of adhesion molecules which in turn provides a

chemotactic gradient for leukocytes and other inflammatory cells and TGF-β then inhibits them once they become activated (Letterio and Roberts, 1998). TGF-β stimulates several processes that are important for tissue repair following lung injury which includes, reducing production of pro-inflammatory cytokines from macrophages, promoting recruitment of fibroblasts, differentiating myofibroblasts and stimulating the production of extra-cellular matrix proteins (Lasky and Brody, 2000). TGF-β signaling and regulation are mediated by a family of *Smad* proteins. TGF-β induces phosphorylation of *Smad* proteins, which then translocate to the nucleus and regulate transcription of target genes (Massague, 1998). Over-expression of TGF-β enhances synthesis and deposition of extra-cellular matrix components including collagen and also alters the balance of matrix metalloproteinase and their inhibitors. This signals a very important role for TGF-β in the pathogenesis of pulmonary fibrosis (Broekelmann *et al*., 1991). IL-7 inhibits TGF-β signaling by up-regulating *Smad7* protein, which is a major inhibitor of *Smad* family and thus reduces TGF-β mediated collagen synthesis which has a beneficial effect on pulmonary fibrosis (Zhang *et al*., 2004). IL-13 released from infiltrating cells following injury to airway epithelium and increased secretion of TNF-α in early stages of inflammation stimulates production of TGF-β (Strieter, 2002; Hira *et al*., 2003). Integrin αvβ6 is also associated with activation of latent TGF-β (Strieter, 2002). TGF-β1 is the most abundant isomer and plays a central role in remodeling and repair of injured lung tissue (Zhang *et al*., 1995). Myofibroblasts, fibroblasts and eosinophils are the major sources of TGF-β1 in bleomycin-induced lung fibrosis (Zhang *et al*., 1995). Activated fibroblasts contain TGF-β1, which in turn express increased levels of fibronectin and procollagen and increased deposition of extra-cellular matrix (Broekelmann *et al*., 1991). In addition to promoting differentiation in myofibroblasts, TGF-β1 also protects myofibroblasts against apoptotic stimuli (Phan, 2002). Therefore, TGF-β1 is crucial for repair associated with inflammation.

1.5. Pulmonary Intravascular Macrophages:

Pulmonary intravascular macrophages (PIMs) are present in the lumen of pulmonary capillaries and are tightly adhered to their endothelium (Warner, 1996). PIMs are large

cells (10-80 μm diameter) with an indented nucleus, phagolysosomes, pseudopodia and adhesive structure holding them firmly to the endothelium (Warner, 1996). PIMs develop from circulating monocytes sequestered in the pulmonary capillaries (Staub, 1994). PIMs are normally present in certain species such as ruminants, horses, pigs and cat (Atwal *et al.*, 1992) (Singh *et al.*, 1994) (Staub, 1994). In these species, PIMs play very important role in the phagocytosis of bacteria, endotoxin or foreign particles. There is evidence that blood borne bacteria and endotoxins are very efficiently removed by pulmonary intravascular macrophages in these species (Warner *et al.*, 1987) (Singh and Atwal,1997). Intravenous injection of radio-labeled gold colloid and magnetic iron oxide particles in sheep resulted in predominant uptake by pulmonary intravascular macrophages (Warner *et al.*, 1986). PIMs participate in the clearance of effete erythrocytes, neutrophils, fibrin and cellular debris (Winkler, 1988). PIMs produce various inflammatory mediators to initiate or modulate lung inflammation (Watson *et al.*, 1994). PIMs also show some immune activity by playing important role in antigen presentation i.e., presenting antigen to T-lymphocytes (Chitko-McKown *et al.*, 1991). Species that normally contain PIMs are highly susceptible to lung inflammation (Longworth, 1997).

Neonatal lambs that normally have few PIM at birth were less responsive to the injection of liposomes or Monastral blue particles as compared to 2 week old lambs that normally have large number of PIMs and showed pulmonary responses to liposomes or Monastral blue (Longworth *et al.*, 1992). Inhibition of PIMs by administration of detergent resulted in less pulmonary responses to *E. coli* endotoxin in horses (Longworth *et al.*, 1996). PIM depletion in horses by intravenous administration of gadolinium chloride substantially reduced recruitment of inflammatory cells in alveolar septa following LPS administration (Parbhakar *et al.*, 2005). Similarly, removal of PIMs before inoculation of *Mannheima hemolytica* in calves caused significant reduction in the number of IL-8 and TNF-α positive cells and thus inhibition of acute lung inflammation (Singh *et al.*, 2004). Pro-inflammatory nature of PIMs in endotoxin induced lung injury in sheep was also shown by depleting PIMs which resulted in decreased pulmonary hypertension and edema (Sone *et al.*, 1999). PIMs secrete TNF-α, IL-1β, leukotriene-B, thromboxane and arachidonic acid metabolites which in turn promote inflammatory response (Chitko-McKown *et al.*,

1991). Recent evidence from *in vivo* studies showed presence of TNF-α and IL-1β in cattle and horse PIMs (Singh *et al*., 2004) (Parbhakar *et al*., 2005). These studies have established a critical proinflammatory role for PIMs in acute lung inflammation in domestic animal species.

1.6. Recruitment of pulmonary intravascular macrophages

Species that do not harbor PIMs include humans, dogs, rats and mice (Staub, 1994). These species can tolerate large doses of endotoxin without showing significant pulmonary vascular responses and edema as compared to species that normally contain PIMs (Chang *et al*., 1987), (Berczi *et al*., 1966). These species are also less susceptible to acute lung inflammation as compared to ruminants (Staub, 1994), (Longworth, 1997). However, these species can recruit PIMs under certain experimental conditions such as liver injury induced by chronic bile duct ligation, infusion of endotoxin and inhalation of 100% oxygen (Chang and Ohara, 1994), (Singh *et al*., 1998). Bile duct ligation in rats resulted in the appearance of a large number of mononuclear phagocytes morphologically similar to intravascular macrophages (Chang and Ohara, 1994). It was further supported by the retention of significant quantities of fluorescent microspheres in bile duct ligated rats. Exact mechanism of PIM recruitment in bile duct ligated rats is not known, however, it is believed that disruption of liver mononuclear phagocyte system in liver injury induced by bile duct ligation causes appearance of intravascular macrophages in the lung as a compensatory mechanism. Single intraperitoneal injection of *E. coli* bacteria in rats resulted in the appearance of PIMs with in 48 hours followed by their disappearance at 96 hours (Singh *et al*., 1998). There is also evidence of increase in the numbers of intravascular macrophages in the lungs of baboons exposed to 100% oxygen. Recently, PIM recruitment has been reported following exposure to the pig barn air and recruited PIMs increased sensitivity to *E. coli* LPS-induced inflammatory response (L. Gamage, MSc thesis). Recruitment of PIMs in rats resulted in increased sensitivity to endotoxin (Chang and Ohara, 1994). These studies show that under certain physiological stresses, PIMs are recruited in species that normally do not possess these cells. However, there is a lack of understanding of recruited PIMs' contributions to lung physiology.

1.7. Hepatopulmonary Syndrome

Hepatopulmonary syndrome (HPS) is characterized by intrapulmonary vascular dilatation and increased alveolar to arterial oxygen tension difference which leads to systemic and pulmonary vascular abnormalities in humans with advanced liver cirrhosis (Herve *et al.*, 1998). Rats with cirrhosis induced by bile duct ligation also develop HPS with intrapulmonary vascular dilatation and an increased alveolar to arterial oxygen difference (Nunes *et al.*, 2001). It is mainly related to excessive production of nitric oxide which in turn depends primarily on increased expression and activity of iNOS within PIMs in cirrhotic rats (Nunes *et al.*, 2001). TNF-α is considered to be a potent iNOS activator in macrophages, levels of which increases in the blood of cirrhotic rats (Gross *et al.*, 1991). Inhibition of TNF-α in cirrhotic rats prevented development of hyperdynamic circulatory state and thus HPS mainly by decreasing the vascular overproduction of nitric oxide (Sztrymf *et al.*, 2004). Cirrhotic animals showed overgrowth of bacteria in the intestine, impairment of the host defense and disruption of mucosal barrier resulting in vascular translocation of bacteria (Wiest *et al.*, 1999). Translocation of bacteria from gut to pulmonary circulation is believed to activate intravascular macrophages which contribute excessive amounts of nitric oxide (Panos and Baker, 1996). The role of recruited PIMs in nitric oxide production is further strengthened by the data that prevention of translocation of bacteria decreases production of nitric oxide from intravascular macrophages and thereby reduced the severity of HPS (Rabiller *et al.*, 2002). Therefore, PIMs may be an important regulator of development of HPS in rats with end stage liver disease and inhibition of their activity may have beneficial effect.

1.8. Bile duct ligation model

Although rats and humans normally do not contain PIMs, they can recruit PIMs under certain conditions such as intra-peritoneal injection of bacteria (Singh *et al.*, 2004), (Singh *et al.*, 1998) or induction of biliary cirrhosis by performing bile duct ligation (Chang and Ohara, 1994). Intra-peritoneal injection of *E. coli* resulted in the recruitment of large number of activated mononuclear phagocytes, resembling PIMs, in the lung microvasculature within 48 hours of treatment with bacteria (Singh *et al.*, 1998). These mononuclear phagocytes were transient because they disappeared by 4 - 7 days after

treatment with bacteria. On the other hand, biliary cirrhosis induced by bile duct ligation resulted in the appearance of PIMs, resembling PIMs of domestic animal species, within 1 week after bile duct ligation and progressively increased by 2 weeks and 4 weeks (Chang and Ohara, 1994). The recruited PIMs were closely adhered to the capillary endothelium. These data show that bile duct ligation induces permanent recruitment of PIMs compared to the transient accumulation observed following a single intraperitoneal injection of bacteria. Rats with ligated bile ducts may be a reliable model to study PIMs' roles in pulmonary diseases in cattle and horses in addition to their recent use as a model for HPS.

2. HYPOTHESES:

a. Bile duct ligation induces recruitment of PIMs.

b. Recruited PIMs increase sensitivity to acute lung inflammation.

c. Depletion of PIMs protects against the severity of acute lung inflammation

3. OBJECTIVES:

a. To investigate recruitment of PIMs following bile duct ligation.

b. To study functions of recruited PIMs in acute lung inflammation.

4. RATIONALE:

Lung inflammatory diseases continue to be a major cause of mortality and morbidity in humans as well as domestic animals. Lung inflammation could be an outcome of direct microbial insult or indirect outcomes of disease in other organs. HPS is a recognized clinical problem, which is associated with liver dysfunction. Human patients with end stage liver diseases, such as advanced liver cirrhosis, are highly susceptible to HPS. There is growing evidence that PIMs, recruited in biliary dysfunction, play important roles in HPS. PIMs are normally present in domestic animal species such as cattle, horses, sheep and goat and make these host species highly susceptible to lung inflammation and diseases. Because PIMs can be recruited in species such as rats following bile duct ligation or during general physiological stress, it is critical to establish their precise contributions in lung inflammation. Therefore, I propose to conduct a series of experiments to characterize recruited PIMs in a rat model of bile duct ligation and use this model to investigate PIMs' contributions to lung inflammation associated with biliary cirrhosis.

My first experiment is designed to understand the PIM recruitment in rats following ligation of their bile ducts and to establish a method to deplete recruited PIMs. This experiment also includes examination of lung tissues for the expression of selected inflammatory cytokines to assess direct effects of PIM recruitment on lung physiology. The second experiment is designed to understand the contributions of PIMs in LPS-induced lung inflammation. This will be accomplished by removing recruited PIMs with gadolinium chloride before LPS challenge and compare LPS-induced lung inflammation in rats with intact PIMs. The proposed experiments are expected to yield data that will enhance our understanding of basic biology and inflammatory functions of PIMs.

5. MATERIALS and METHODS

Table 5.1. Reagents and their sources

Reagents	Source
Anti-rat goat polyclonal TNF-α antibody	Santa Cruz Biotec., USA
Anti-rat goat polyclonal IL-1β antibody	Santa Cruz Biotec., USA
Anti-porcine mouse monoclonal TGF-β antibody	R & D Systems, Inc., USA
Anti-mouse goat polyclonal IL-10 antibody	Santa Cruz Biotec., USA
Anti-rat mouse monoclonal macrophage antibody	Serotec, USA
Purified hamster anti mouse/rat TNF- α (ELISA)	BD Pharmingen,
Biotin rabbit anti-mouse/rat TNF-α (ELISA)	BD Pharmingen
Recombinant rat TNF-α (ELISA)	BD Pharmingen
Anti-rat IL-1β antibody (ELISA)	R & D Systems, Inc., USA
Biotinylated anti-rat IL-1β antibody (ELISA)	R & D Systems, Inc., USA
Recombinant rat IL-1β (ELISA)	R & D Systems, Inc., USA
Monoclonal anti-TGF-β2 antibody (ELISA)	R & D Systems, Inc., USA
TGF-β2 biotinylated antibody (ELISA)	R & D Systems, Inc., USA
Recombinant human TGF-β2 (ELISA)	R & D Systems, Inc., USA
Purified anti-rat IL-10 (ELISA)	BD Pharmingen
Biotin mouse anti-rat IL-10(ELISA)	BD Pharmingen
Recombinant rat IL-10 (ELISA)	BD Pharmingen
Purified anti-rat MCP-1 (ELISA)	BD Pharmingen
Biotin anti-rat MCP-1 (ELISA)	BD Pharmingen
Recombinant rat MCP-1 (ELISA)	BD Pharmingen
Peroxidase color development kit (immunostaining)	Vector laboratories, USA
Methyl green	Vector laboratories, USA
Ketamine hydrochloride	Vetrepharm, Canada Inc.
Xylazine	Bayer, Canada
Gadolinium chloride	Sigma Co.

5.1. Animals

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance. Specific pathogen free 350-400 gram rats were procured from Charles River Laboratories, Canada. Rats were acclimatized for a period of one week before the experiment.

5.2. Experimental Design

Table 5.2. Treatment groups

Group		Treatment
Control	(N=5)	No surgery and no treatment
Sham	(N=3)	Sham operation and no treatment
Bile duct ligated (BDL)	(N=4)	Bile duct ligation and no treatment
BDL & Gadolinium chloride (GC)	(N=5)	Bile duct ligation and administration of gadolinium chloride (10 mg/kg) intravenously
Control + *E. coli* LPS	(N=6)	Control rats injected with *E. coli* LPS
BDL+ *E. coli* LPS	(N=6)	Bile duct ligation and administration of *E. coli* LPS (0.1mg/kg iv)
BDL+ GC + *E. coli* LPS	(N=5)	Bile duct ligation, administration of GC and then treatment with LPS (0.1mg/kg iv) 6 hrs after GC (10 mg/kg) intravenously
BDL+ GC + *E. coli* LPS	(N=5)	Bile duct ligation, administration of GC and then treatment with LPS (0.1mg/kg iv) 48 hrs post-GC treatment (10 mg/kg) intravenously

5.3. Operating Procedures

By using sterile techniques, a mid line incision was made, the common bile duct was identified and ligated at two points 5 mm apart by using non absorbable suture material (Nylon, 4-0). The common bile duct was then resected in the middle of two ligatures. In sham operated animals, the common bile duct was identified and separated from the

surrounding soft tissue with out ligation and resection. The surgery was done using intraperitoneal anesthesia induced with the combination of ketamine (80 mg/kg) and xylazine (10 mg/kg). After surgeries, rats were allowed free access to water and rat chow and allowed to survive for 4 weeks. Rats were observed every 6 hours and really sick rats were humanely euthanized.

5.4. Sampling and Processing

5.4.1. Lung tissue processing:

5.4.1.1. Light Microscopy:

A. Histopathology: Blocks from left lung were fixed in 4% paraformaldehyde for 24 hours at 4°C. The tissues were transferred to PBS, dehydrated in ascending concentrations of ethanol and cleared in xylene followed by embedding in paraffin. Five micron sections were cut from each tissue block and placed on silane-coated slides. They were dewaxed in 2 changes of xylene and stained with hematoxylin-eosin for histologic examination.

B. Immunohistology: Sections were de-waxed and rehydrated in graded ethanol series followed by incubation with 5% hydrogen peroxide in methanol to remove endogenous peroxide. Sections were treated with pepsin (2 mg/ml 0.01 N hydrochloric acid) for 45 minutes for antigen retrieval and then incubated with 1% bovine serum albumin to block non-specific sites. Sections were treated with primary [TNF-α, IL-1β, MCP-1, IL-10, TGF-β2 and anti-macrophage (ED-1)] antibodies and with appropriate horseradish peroxide-conjugated secondary antibodies for 60 and 30 minutes respectively followed by color development using a commercial kit (Vector Laboratories, Canada). Controls included incubation of sections with only secondary antibody or with only color development reagent. Another control was to stain some of the sections with anti-von Willebrand Factor (vWF) antibody which recognizes vascular endothelium. Finally, sections were counter-stained with methyl green.

5.4.1.2. Immuno-electronmicroscopy:

Lung pieces of approximately 1mm³ sizes were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde for 3 hours at 4°C. The fixed tissues were rinsed in 0.1 M sodium cocadylate buffer and dehydrated in graded ethanol series. These pieces were infiltrated with LR White Resin (EM Sciences, USA) and polymerized in polythene beam capsules under ultraviolet light for 48 hours at -1°C.One micron sections were prepared and stained with toluidine blue to select appropriate areas for ultra-microtomy. Ultra thin (0.1 micron) sections were cut from the tissue blocks and placed on nickel grids.

Nickel grids with sections were floated on a blocking buffer (1% bovine serum albumin and 0.1% Tween-20 in tris-buffered saline; pH 7.9) for 30 minutes followed by incubation with primary (ED-1, TNF-α, IL-1β and MCP-1) antibodies for one hour. Tissue sections were rinsed three times for five minutes each in Tris-buffered saline and incubated with appropriate gold conjugated secondary antibodies (1:100) for one hour followed by staining with 2% aqueous uranyal acetate and then lead citrate.

5.4.1.3. RT-PCR

A. RNA isolation

Total RNA was isolated from lung tissues of rat by sequential extraction with TRIzol reagent (Invitrogen) followed by treatment with RNase-free DNase I (Qiagens) and purification by RNeasy mini columns (Qiagens) according to manufacturer's instructions. RNA integrity was confirmed by agarose gel electrophoresis, and RNA was quantified by spectrophotometric analysis.

B. Reverse transcription – PCR

Superscript III one-step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen) was used for semi-quantitative analysis of the levels of expression of MCP-1, pro-inflammatory cytokines (IL-1β, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β) in lung tissues of variously treated rats. Reactions were performed as directed by the manufacturer, using the oligonucleotide pairs as below:

Gene	Forward primer	Reverse primer
MCP-1	5'-CGCTTCTGGGCCTGTT-3' and 5'-GAAGTGCTTGAGGTGGT-3'	
IL-1β	5'-TTGCCCGTGGAGCTTC-3' and 5'-CGGGTTCCATGGTGAAC-3'	
TNF-α	5'-GCACAGAAAGCATGATCC-3'and 5'-GTGGGTGAGGAGCACAT-3'	
IL-10	5'-GCTGCGACGCTGTCAT-3' and 5'-GCGCTGAGCTGTTGCT-3'	
TGF-β2	5'- CATCCCGCCCACTTTC-3' and 5'-CATGCCCCAGCACAGA-3'	

Each reaction was performed using 10 ng of total RNA and thermocycler was programmed for reverse-transcription at 55 °C for 30 min, initial denaturation of the cDNA at 94 °C for 2 min, 30 amplification cycles, each of which consisted of 94 °C for 15 sec, 59 °C for 30 sec, and 68 °C for 1 min followed by a final extension at 68 °C for 5 min. To ensure lack of DNA contamination 2 units of Platinum Taq DNA polymerase was substituted for the Superscript III RT/Platinum *Taq* mixture in the reaction. A negative control reaction consisted of all the components of the reaction mixture except RNA. Amplified PCR products were electrophoresed on a 1 % TAE-agarose gel, stained with ethidium bromide and photographed under UV light.

5.4.1.4. Enzyme-linked immunosorbent assay (ELISA):

Lung tissues were homogenized in 10X HBSS solution containing proteinase-K inhibitor cocktail. Homogenates were collected in eppendorf tubes and centrifuged at 4°C for 20 minutes at 25, 000 RCF. Supernatants were collected in eppendorf tubes and stored at - 80°C. Cytokine concentrations in supernatants were determined using ELISA. For the ELISA, wells of microtitre plate were coated with 50 μl of capture antibody (diluted with sodium phosphate buffer) and incubated at 4°C overnight. Next day, capture antibody was removed and 200 μl of blocking buffer (1% BSA in PBS) was added to each well and incubated at 37°C for one hour. Blocking buffer was removed and wells were washed with PBST (PBS containing Tween-20). Standards and samples were diluted in blocking buffer-Tween and 100 μl of standards and samples were added to the wells and incubated at 37°C for 2 hours. After removing standards and samples, wells were washed with PBST and 100 μl of detection antibody was added to each well after dilution with blocking buffer-Tween. Plate was incubated at 37°C   for one hour. Detection antibody

was removed and wells were washed and 100 μl of streptavidin-HRP (1:2500 in blocking buffer-Tween) was added to each well and incubated at room temperature for 30 minutes. Wells were washed thoroughly with PBST and 100 microlitres of substrate (TMB) was added to each well and incubated in dark at room temperature for 20 minutes. On color development, reaction was stopped by adding 50 μl of 0.5 M sulphuric acid to each well. Readings were then taken at 450 nm wavelength.

5.5. Statistical analysis:

Analysis was done using a statistical software package (SPSS 12.0 for windows). Mean differences among groups were compared by using one-way analysis of variance followed by post-hoc analyses with Fisher's LSD test.

6. RESULTS

6.1. Results (Experiment-1)

6.1.1.Clinical response to bile duct ligation: Initially, I encountered a mortality rate of 50% in rats subjected to bile duct ligation. However, with improvement in my surgical and post-operative skills, the mortality rate came down to 15%, which is lower than the rate reported by other investigators. All bile duct ligated rats became dull and inactive up to two days after bile duct ligation.Their food and water intake decreased and all rats lost up to 50-100 grams of weight over the period of one week. After one week, rats started eating and drinking normally and started gaining weights and reached their original weights (sometimes exceeding original weights) in 3-4 weeks after bile duct ligation. However, their urine and mucous membranes became icteric. Nearly four weeks after the ligation, a swelling was evident upon palpation in anterior part of the abdomen in most of the rats. Sham operated rats continued to grow normally even after the surgery.

6.1.2. Histopathology: Control rats and sham operated rats showed normal lung morphology (Figure 6.1.1.A-B, F). In contrast, bile duct ligated rats showed increased number of mononuclear cells in the alveolar septa (Figure 6.1.1.C-E). Gadolinium chloride treatment of BDL rats resulted in decrease in the number of mononuclear cells (Figure 6.1.1.D). Because lungs of sham-operated rats appeared similar to the controls, I used only the control rats in the analyses.

6.1.3. ED-1 Immunocytochemistry

6.1.3.1. Immunohistology for ED-1: Immunohistology demonstrated staining of septal cells with ED-1. Bile duct ligated rats showed increase in the number of ED-1 positive cells in the alveolar septa (Figure 6.1.2.B) compared to the sham-operated rats (Figure 6.1.2.A). However, BDL rats treated with gadolinium chloride showed a decrease in number of ED-1 positive cells (Figure 6.1.2.C) compared to that in BDL rats.

6.1.3.2. Immuno-electron microscopy for ED-1: Lung sections stained with ED-1 antibody and gold-conjugated secondary antibodies showed labeling in large mononuclear cells resembling macrophages (Figure 6.1.3). These ED-1 positive cells were attached to capillary endothelium and contained lysosomes.

6.1.3.3. Numerical counts of ED-1 positive cells: Following confirmation that ED-1 antibody recognizes sequestered PIMs, we performed counts of cell stained with ED-1 antibody. Bile duct ligation induced increase in the number of ED-1 positive cells compared to that in sham operated rats ($p=0.002$) (Figure 6.1.4). There were very few ED-1 positive cells in the alveolar septa of sham-operated rats. Gadolinium chloride treatment of BDL rats decreased the numbers of ED-1 positive cells compared to those in BDL rats ($p=0.061$) (Figure 6.1.4).

6.1.4. MCP-1 Expression

6.1.4.1. MCP-1 mRNA expression: Total RNA extracted from the lungs of variously treated rats was subjected to RT-PCR for MCP-1. Samples devoid of RNA as well as those treated with DNase did not show any amplified product. RT-PCR for GAPDH showed bands of equal size indicating uniform loading of RNA samples. PCR products separated on 1% agarose gel showed differential expression of MCP-1 transcripts. Gel densitometry showed that bile duct ligation increased MCP-1 mRNA expression compared to control rats ($p=0.017$). Gadolinium chloride treatment of BDL rats also resulted in increase in MCP-1 transcript levels in comparison to the control and BDL rats ($p=0.000$ & 0.000 respectively) (Figure 6.1.7.A).

6.1.4.2. MCP-1 protein expression: MCP-1 protein expression in lungs was measured with ELISA. MCP-1 concentrations were higher in BDL rats compared to that in control rats ($p=0.089$). Gadolinium chloride treated BDL rats showed higher concentrations of MCP-1 compared to the control rats ($p=0.048$) (Figure 6.1.7.B).

6.1.4.3. Immunohistochemistry for MCP-1: Staining of lung sections with MCP-1 antibody showed only focal staining in the alveolar septa of control rats and sham operated rats (Figure 6.1.5.A & B). However, lung sections from rats after 2 weeks of bile duct ligation showed uniform MCP-1 staining in the alveolar septa (Figure 6.1.5.C). Although pattern of MCP-1 staining at 4 weeks post-BDL was similar to the 2 week rats, the staining intensity was increased (Figure 6.1.5.D).

6.1.4.4. Immuno-electron microscopy for MCP-1: Lung sections stained with MCP-1 antibody and then gold conjugated secondary antibodies showed specific staining for MCP-1 in PIMs (Figure 6.1.6.A) and endothelial cells (Figure 6.1.6.B).

6.1.5. IL-1β expression

6.1.5.1. IL-1β mRNA expression: Total RNA extracted from rat lungs was amplified with RT-PCR for IL-1β. Densitometric analyses showed no differences in the levels of IL-1β transcripts between different groups (Figure 6.1.8.A).

6.1.5.2. IL-1β protein expression: There were no differences in IL-1β concentration between control rats, BDL rats and gadolinium chloride treated BDL rats (Figure 6.1.8.B).

6.1.6. TNF-α expression

6.1.6.1. TNF-α mRNA expression: Densitometry of gel bands showed no differences in the levels of TNF-α transcript between control and BDL rats. However, lungs of rats treated with gadolinium chloride showed increase in TNF-α transcript levels compared to the BDL and control rats (*p=0.004* in both groups) (Figure 6.1.9.A).

6.1.6.2. TNF-α protein expression: TNF-α protein concentrations were not different between control rats, BDL rats and gadolinium chloride treated BDL rats (Figure 6.1.9.B).

6.1.7. IL-10 expression

6.1.7.1. IL-10 mRNA expression: Gel densitometry showed increased levels of IL-10 transcripts in lungs of BDL rats compared to control rats (*p=0.000*). Gadolinium chloride treatment of rats increased IL-10 expression in BDL rats compared to that in BDL or control rats (*p=0.000* in both groups) (Figure 6.1.10.A).

6.1.7.2. IL-10 protein expression: There was no difference in IL-10 concentration between control rats, BDL rats and gadolinium chloride treated BDL rats (Figure 6.1.10.B).

6.1.8. TGF-β expression

6.1.8.1. TGF-β mRNA expression: Densitometry of gels showed no difference in TGF-β transcripts between BDL or control rats. However, gadolinium chloride treated rats showed increase in TGF-β mRNA compared to the BDL or control rats (*p=0.000 & 0.01* respectively) (Figure 6.1.11.A).

6.1.8.2. TGF-β mRNA expression: There was no difference in TGF-β concentration between control rats, BDL rats and gadolinium chloride treated BDL rats (Figure 6.1.11.B).
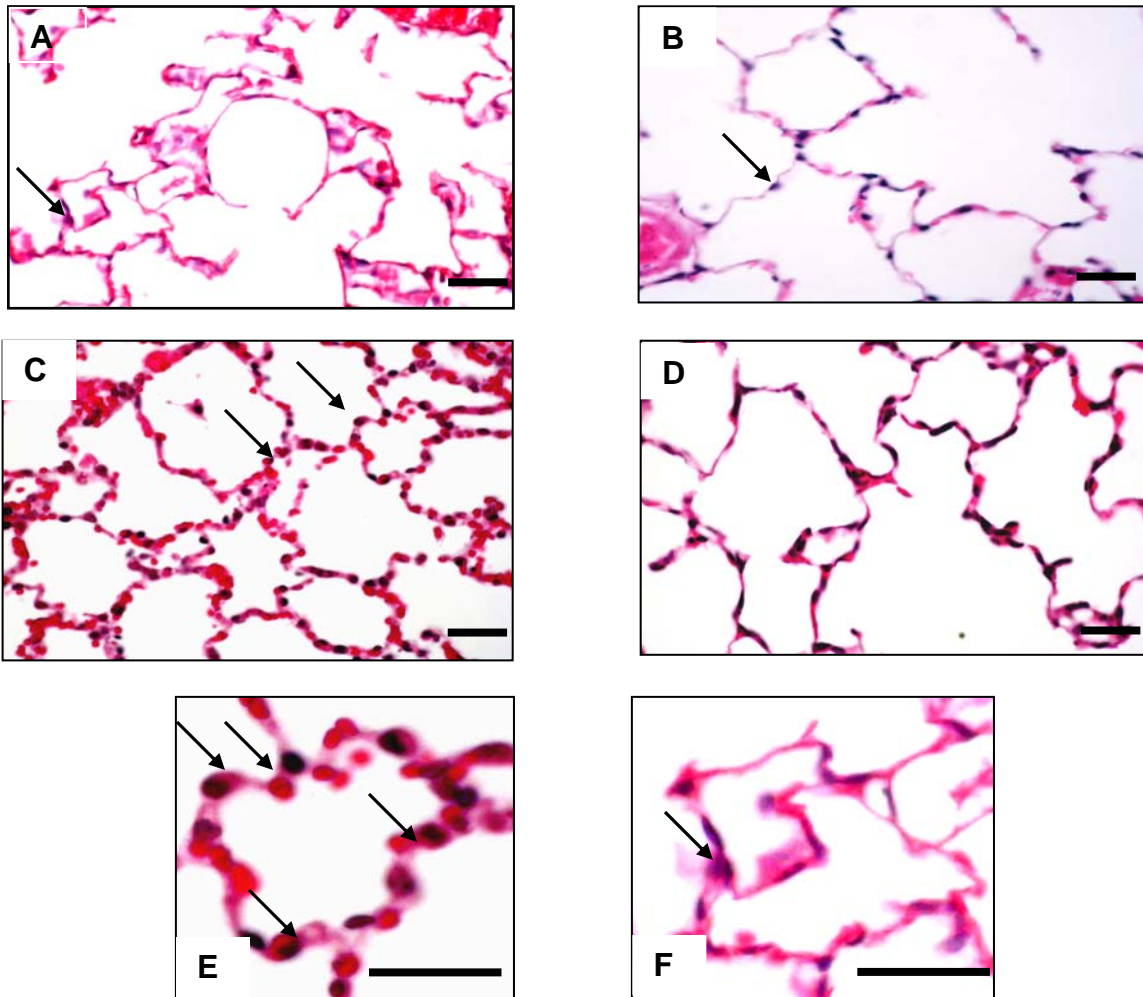
Histopathology:

Figure 6.1.1: H & E staining. There is extensive infiltration of mononuclear cells (arrows) in the alveolar septa of bile duct ligated (BDL) rats (C) compared to that in control (A) and sham operated (B) rats. However, number of mononuclear cells in the alveolar septa decreases in BDL rats after treatment with gadolinium chloride (D). High magnification views of lung sections from BDL (E) and a control (F) rat clearly show accumulation of large mononuclear cells in the septa of the former. Bar = 25µm.
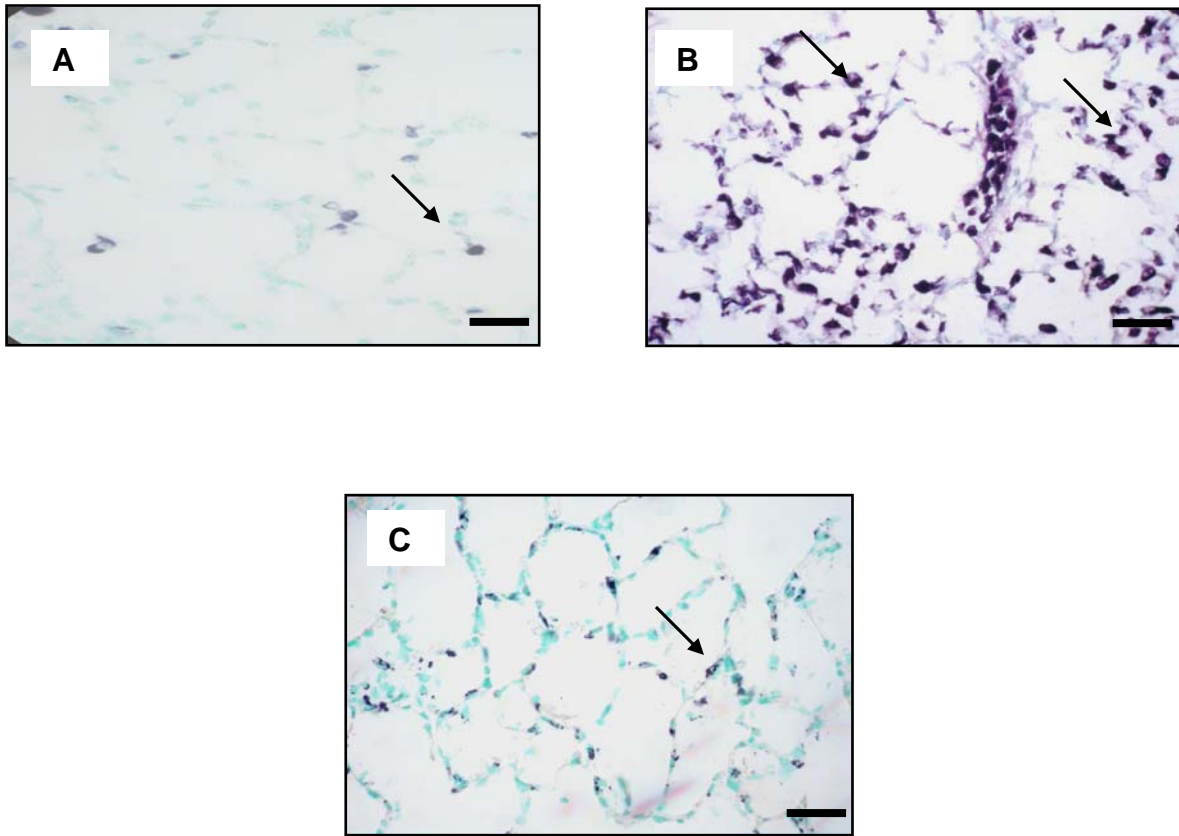
Immunohistology for ED-1:

Figure 6.1.2: Immunohistology for macrophages (ED-1 staining). There is increase in the number of ED-1 positive cells (arrows) in bile duct ligated (BDL) rats (B) compared to that in sham operated rats (A).However, number of ED-1 positive cells decrease in BDL rats after gadolinium chloride treatment (C). Bar = 25 µm.
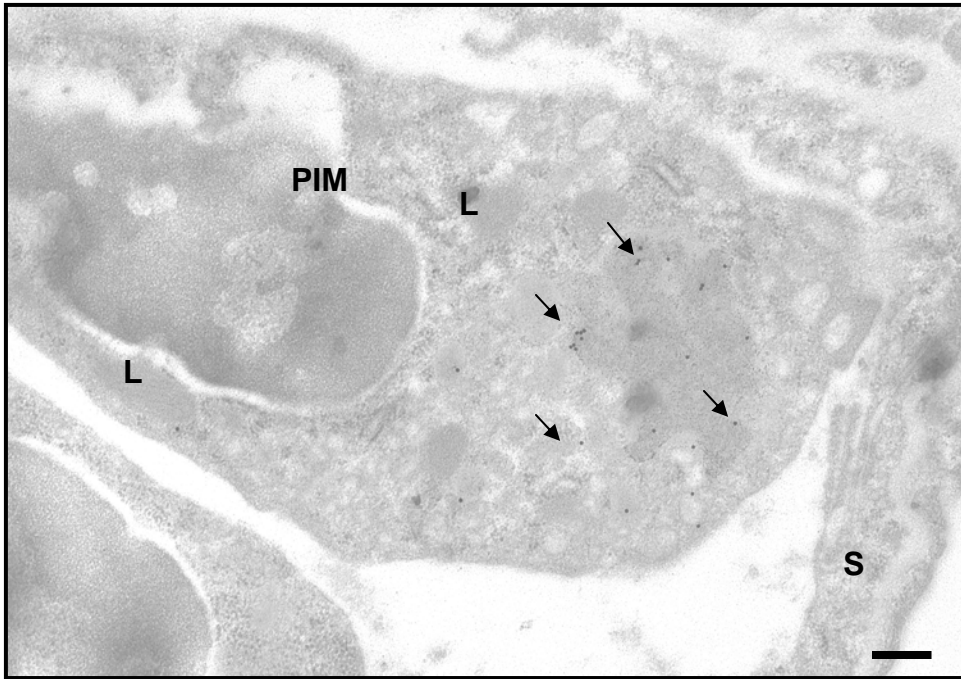
Immuno-electron microscopy for ED-1:

Figure 6.1.3: An immuno-electron micrograph showing ED-1 labeling (arrows) in a pulmonary intravascular macrophage (PIM) but not in alveolar septum (S) of a BDL rat. L: lysosome. Bar = 1 micron

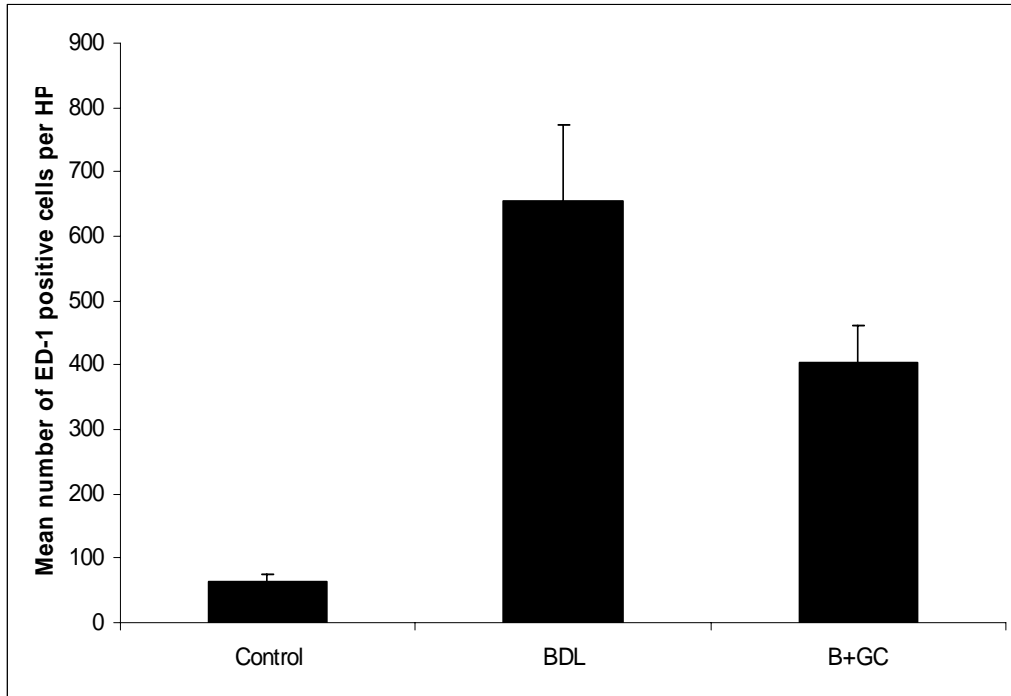**ED-1 Positive Cell Counts:**

Figure 6.1.4: ED-1 counts: Number of ED-1 cells in BDL and BDL+GC groups are higher compared to the control (*p<0.002*). BDL rats also contain higher numbers of ED-1 cells compared to BDL rats treated with GC (*p=0.06).*

Immunohistology for MCP-1:
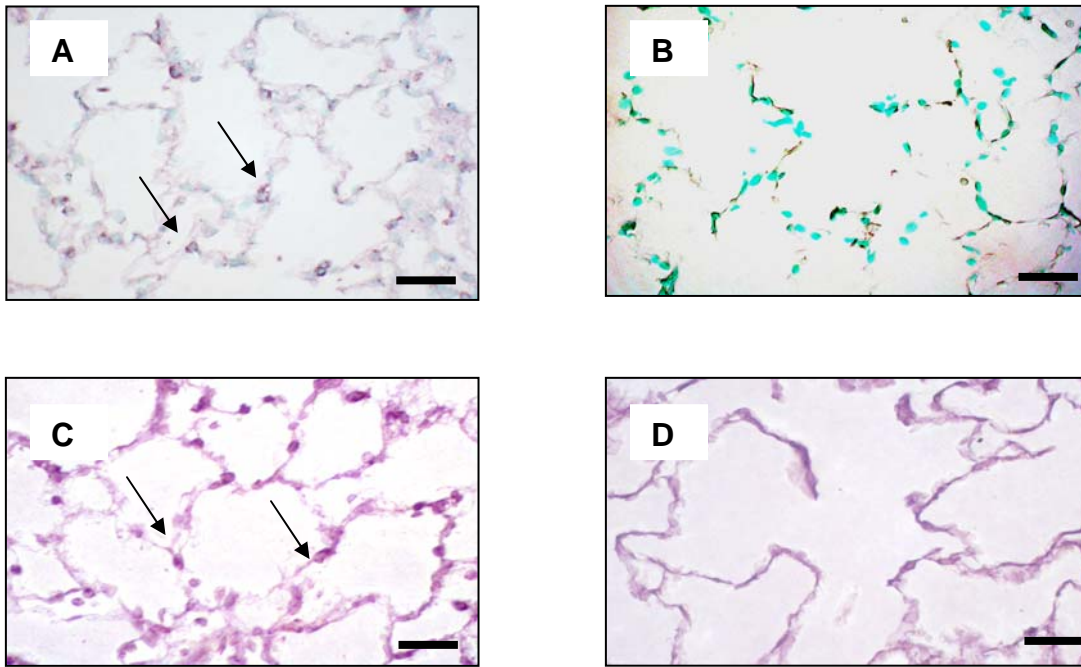
Figure 6.1.5: MCP-1 immunohistochemistry: Lung sections stained with MCP-1 antibody show focal purple staining (arrows) for MCP-1 in the alveolar septa in the control (A) and the sham-operated rats (B).However, there is uniform staining for MCP-1 in 2 week BDL rats (C) and 4 week BDL rats (D). Bar = 25 µm.

Immuno-electron microscopy for MCP-1:
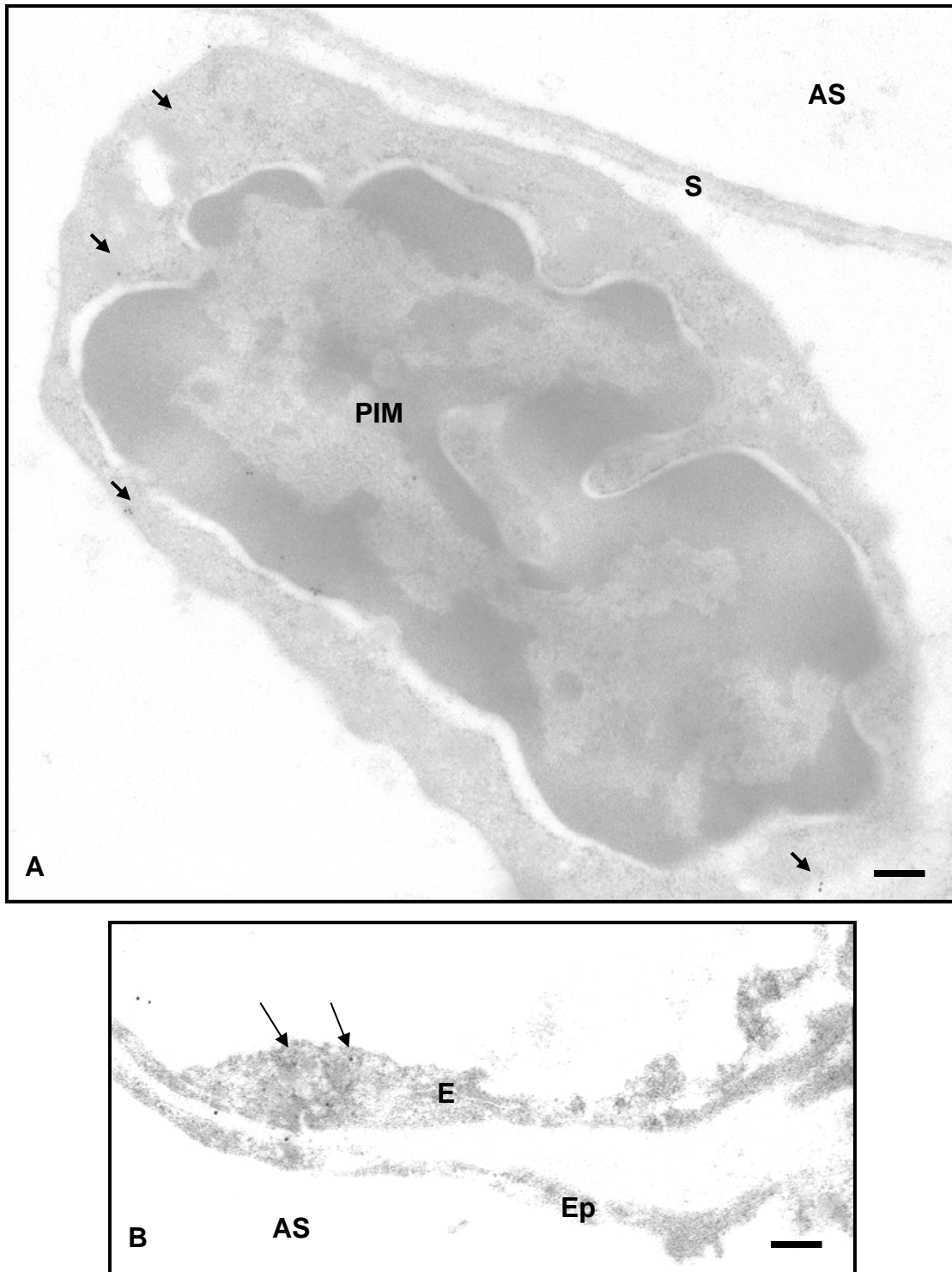
Figure 6.1.6: MCP-1 immuno-electron microscopy: An electron micrograph showing MCP-1 labeling (arrows) in a PIM (A) and an endothelial cell (E; B). Ep: epithelium; S: alveolar septum; AS: alveolar space. Bar = 1 micron

MCP-1 Expression:
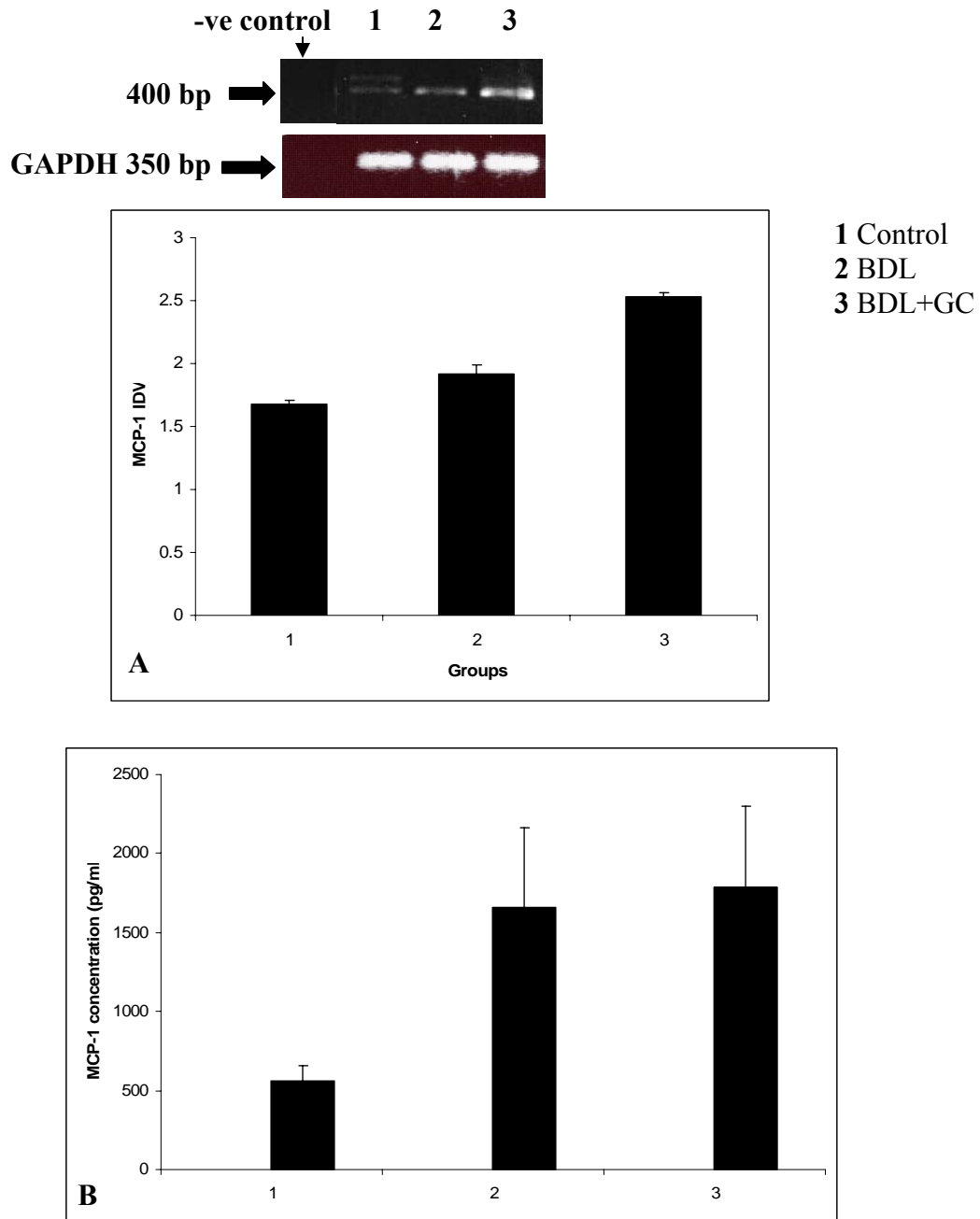
Figure 6.1.7: RT-PCR (A) detected higher levels of MCP-1 mRNA in BDL+GC rats compared to the controls and BDL (*p=0.000* for both) and in BDL rats in comparison to the controls (*p=0.017*). ELISA (B) detected higher concentrations of MCP-1 protein in BDL (*p=0.089*) and BDL+GC (*p=0.048*) rats compared to the controls.

IL-1β Expression:
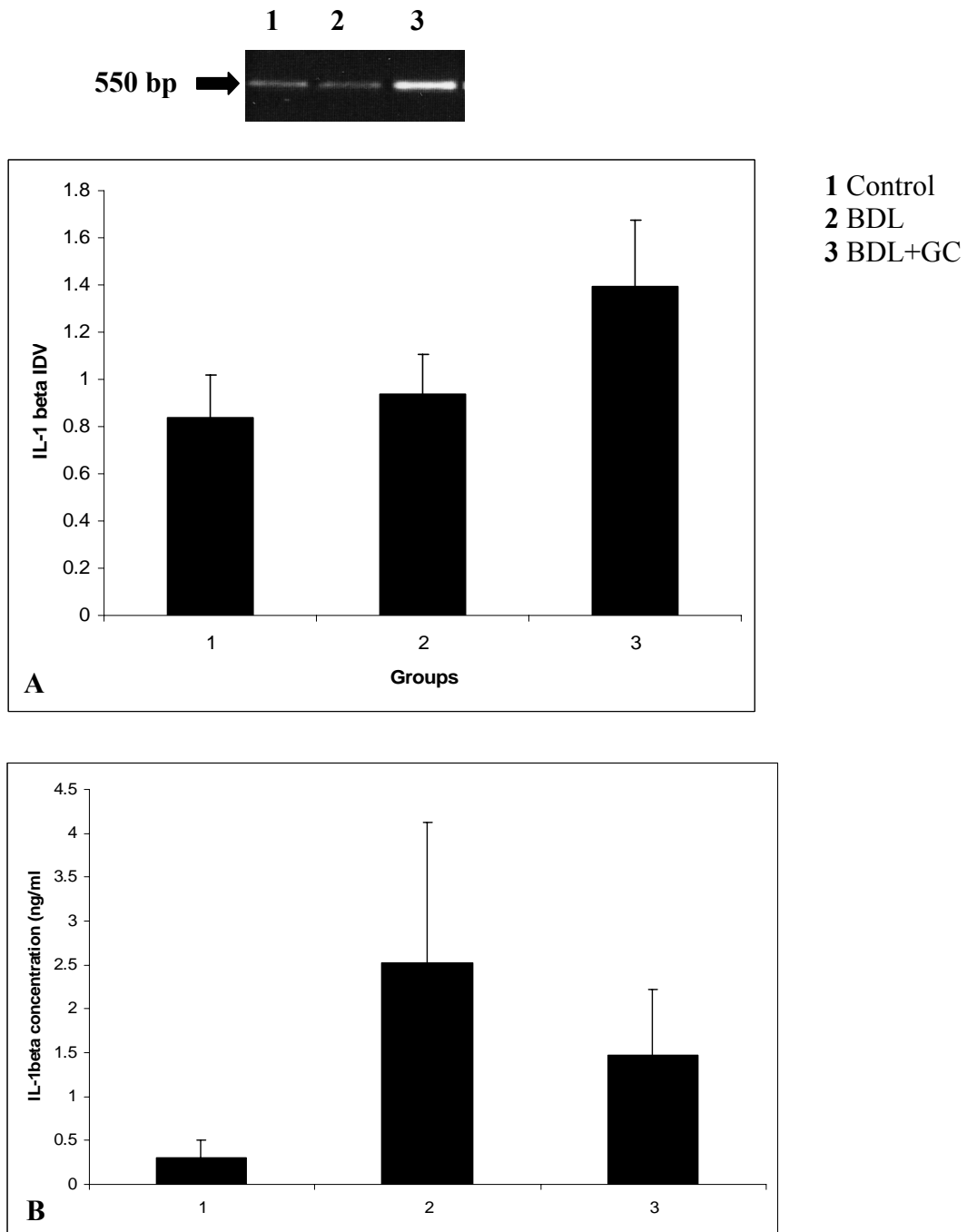
Figure 6.1.8: RT-PCR (A) detected IL-1β mRNA and semi-quantitative densitometry showed no differences between the groups (*p=0.76 and p=0.11*). ELISA (B) detected no differences in concentrations of IL-1β protein between control and BDL (*p=0.122*) or BDL and BDL+GC (*p=0.443*) groups.

TNF-α Expression:
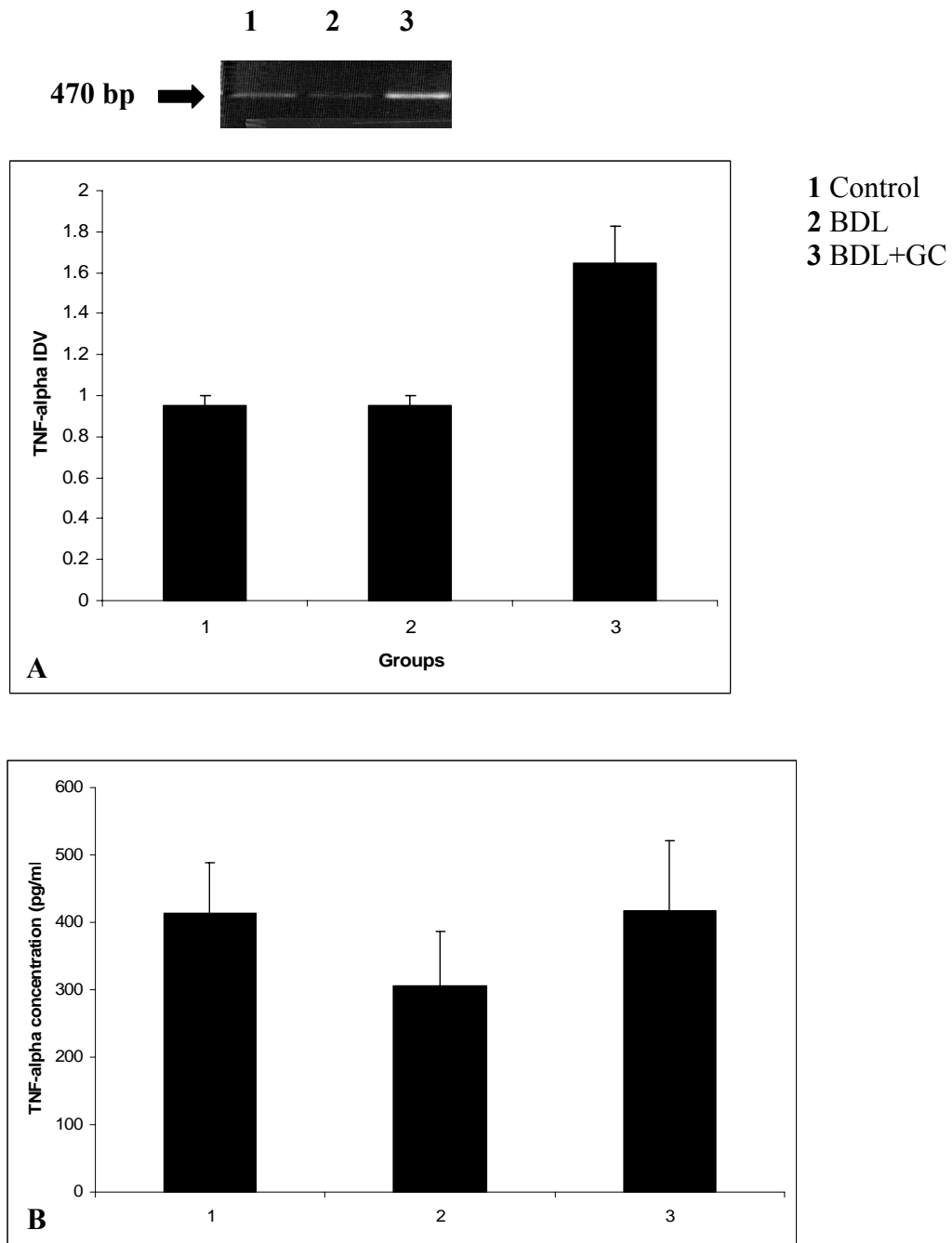
Figure 6.1.9: RT-PCR (A) detected TNF-α mRNA and semi-quantitative densitometry showed higher levels in BDL+GC rats compared to the controls ($p=0.004$) and the BDL rats ($p=0.004$). ELISA (B) detected no differences in concentrations of TNF-α protein between control and BDL ($p=0.398$) or BDL and BDL+GC ($p=0.405$) groups.

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IL-10 Expression:

1 Control
2 BDL
3 BDL+GC
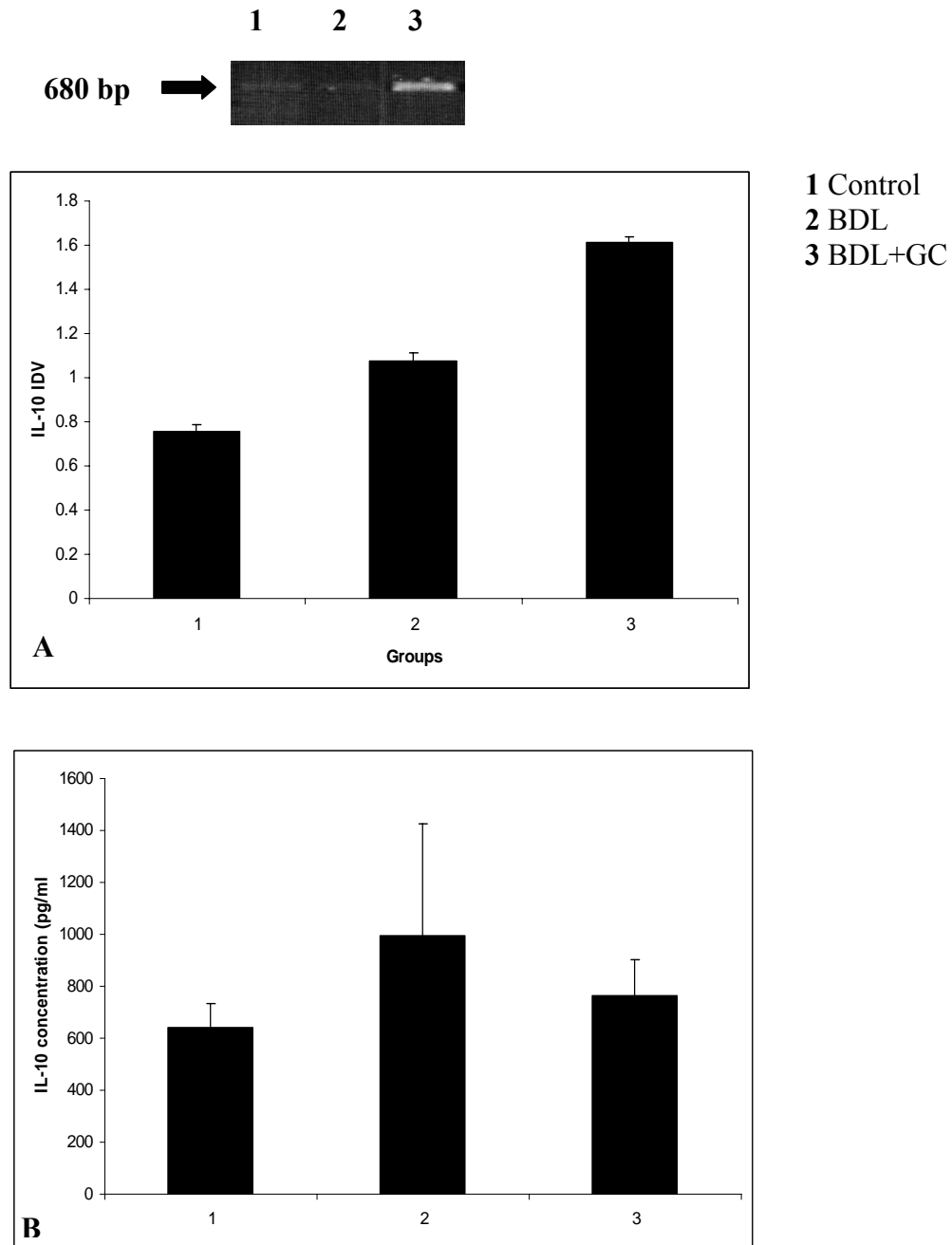
Figure 6.1.10: RT-PCR (A) detected higher levels of IL-10 mRNA in BDL+GC rats compared to the control (*p=0.000*) or BDL (*p=0.000*) rats. ELISA (B) detected no differences in concentrations of IL-10 protein between control and BDL (*p=0.331*) or BDL and BDL+GC (*p=0.539*) groups.

TGF-β Expression:

Figure 6.1.11: RTPCR (A) detected increased expression of TGF-β mRNA in BDL+GC rats compared to the control (*p=0.01*) or BDL (*p=0.000*) rats. ELISA (B) detected no differences in concentrations of TGF-β protein between control and BDL (*p=0.672*) or BDL and BDL+GC (*p=0.282*) groups.
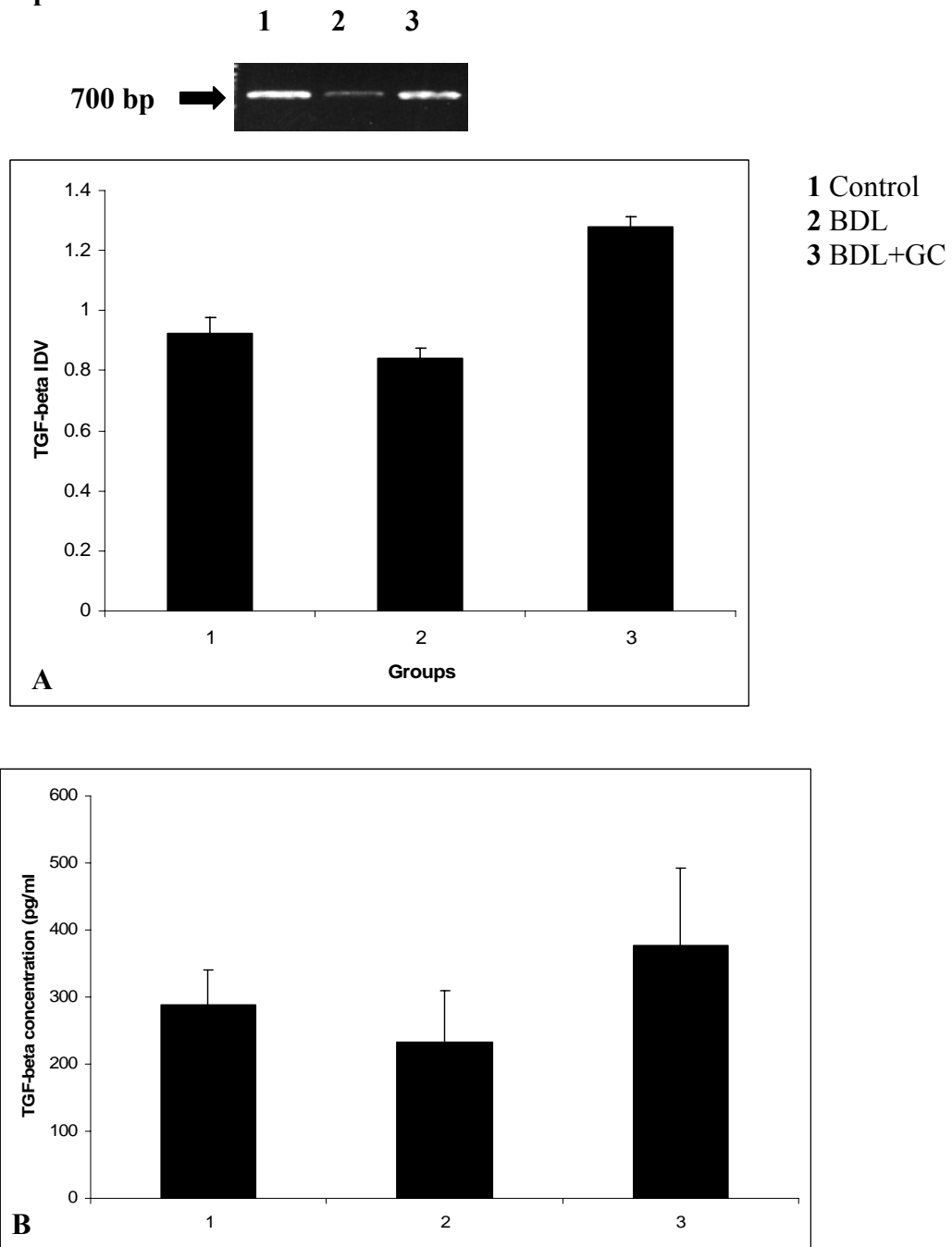
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6.2. Results (Experiment-2)

6.2.1.Clinical response to bile duct ligation: All bile duct ligated rats became dull and inactive up to two days after bile duct ligation.Their food and water intake decreased and all rats lost up to 50-100 grams of weight over the period of one week. After one week, rats started eating and drinking normally and started gaining weights and reached their original weights (sometimes exceeding original weights) in 3-4 weeks after bile duct ligation. However, their urine and mucous membranes became icteric. Close to four weeks after bile duct ligation, a swelling was evident up on palpation in anterior part of the abdomen in most of the rats. Sham operated rats continued to grow normally even after the surgery.

6.2.2. Clinical response to LPS challenge: The data are depicted in Table 6.1. Rats challenged with LPS after 4 weeks of the ligation became very dull and inactive immediately after the LPS injection. These rats showed increased and labored respiration and piloerection. All rats started to defecate and urinate. There was vocalization and convulsions before death. All of the rats with bile duct ligation died within three hours of the LPS challenge. In gadolinium chloride treated BDL rats only one rat died within three hours of the LPS challenge and 9 rats survived till 6 hours after LPS challenge (10% mortality). These rats also showed much mild clinical signs after the LPS challenge.

6.2.3. Histopathology: LPS challenge of control and BDL rats showed accumulation of mononuclear cells in the alveolar septa as well as increase in the thickness of the septa (Figure 6.2.1.A & B). It appeared that gadolinium chloride treatment of BDL rats prior to LPS challenge resulted in reduced infiltration of mononuclear cells and near normal alveolar septal thickness compared to that in LPS challenged BDL rats (Figure 6.2.1.C & D).

6.2.4. ED-1 Immunocytochemistry:

6.2.4.1. Immunohistology for ED-1: Immunohistology demonstrated staining of lung septal cells with ED-1. LPS treatment of BDL rats showed increase in the number of ED-1 positive cells in the alveolar septa (Figure 6.2.2.B) compared to the LPS-treated control

rats (Figure 6.1.2.C). Control rats challenged with LPS showed near normal number of ED-1 positive cells (Figure 6.2.2.A). Gadolinium chloride treatment of BDL rats prior to LPS challenge resulted in decrease in the number of ED-1 positive cells (Figure 6.2.2.C & D) compared to that in LPS challenged BDL rats.

6.2.4.2. ED-1 positive cell count: Intravenous infusion of LPS in BDL rats caused an increase in the number of ED-1 positive cells in comparison to the control or LPS-challenged control rats (*p=0.000* in both groups). Gadolinium chloride treatment of BDL rats 6 hours or 48 hours prior to LPS challenge resulted in decrease in the number of ED-1 positive cells as compared to that in LPS challenged BDL rats (*p=0.039 & 0.002* respectively; Figure 6.2.3).

6.2.5. Immunohistochemistry for *E. coli* LPS: Anti-LPS antibody was used to confirm the LPS in the lung sections from rats in all the LPS-treated groups. There was less staining for LPS in the alveolar septa of control rats challenged with *E. coli* LPS (Figure 6.2.4.A). In contrast, LPS treated BDL rats showed extensive staining for LPS in the alveolar septa as well as in mononuclear cells present in the blood vessels (Figure 6.2.4.B).

6.2.6. MCP-1 Expression

6.2.6.1. MCP-1 mRNA expression: Total RNA extracted from the lungs of rats was subjected to RT-PCR for MCP-1. PCR products separated on 1% agarose gel showed differential expression of MCP-1 transcripts. Gel densitometry showed that LPS treatment of BDL rats increased levels of MCP-1 mRNA compared to the control or LPS-treated control rats (*p=0.000 & 0.01* respectively). BDL rats treated with gadolinium chloride 6 hours or 48 hours prior to LPS challenge showed reduced MCP-1 expression compared to that in LPS challenged BDL rats (*p=0.017 & 0.000* respectively) (Figure 6.2.5.A).

6.2.6.2. MCP-1 protein expression: LPS treatment of BDL rats caused increase in lung concentrations of MCP-1 compared to the control or LPS treated control rats (*p=0.000* both). Gadolinium chloride treatment of BDL rats 6 hours or 48 hours before LPS

challenge reduced MCP-1 protein expression compared to LPS-treated BDL rats
($p=0.042$ & 0.000 respectively) (Figure 6.2.5.B).

6.2.7. IL-1β Expression

6.2.7.1. IL-1β mRNA expression: Densitometric analysis of gels showed higher levels
of IL-1β mRNA transcripts in LPS-challenged BDL rats compared to control rats
($p=0.012$) but not LPS-challenged control rats ($p=0.746$). Gadolinium chloride treatment
of BDL rats at 6 hours or 48 hours prior to LPS administration did not affect IL-1β
expression compared to LPS-challenged BDL rats ($p=0.712$ & 0.509 respectively)
(Figure 6.2.6.A).

6.2.7.2. IL-1β protein expression: BDL rats challenged with LPS contained increased
concentrations of IL-1β in their lungs in comparison to the control rats ($p=0.000$) and
LPS-challenged control rats ($p=0.000$). Although gadolinium chloride treatment of BDL
rats at 6 hours prior to LPS challenge did not affect IL-1β concentration ($p=0.455$)
compared to BDL+LPS rats but a treatment 48 hours prior to the LPS challenge reduced
IL-1β concentrations in lungs ($p=0.008$) (Figure 6.2.6.B).

6.2.7.3. Immuno-electron microscopy for IL-1β: Staining of lung sections with IL-1β
antibody and then gold conjugated secondary antibodies showed IL-1β labeling in PIM
(Figure 6.2.7).

6.2.8. TNF-α Expression

6.2.8.1. TNF-α mRNA expression: Gel densitometry revealed lower levels of the
mRNA transcripts in lungs of control rats compared to all the groups except the LPS-
challenged control rats ($p=0.193$). Higher levels of TNF-α mRNA in LPS challenged
BDL rats were detected compared to control rats ($p=0.006$) but not LPS-challenged
control rats ($p=0.062$). Compared to BDL+LPS rats, gadolinium chloride treatment of

BDL rats 6 hours or 48 hours prior to the LPS injection did not affect TNF-α mRNA expression (*p=0.499, p= 0.297* respectively) (Figure 6.2.8.A).

6.2.8.2. TNF-α protein expression: Lungs from BDL rats treated with LPS showed increased concentrations of TNF-α compared to control rats (*p=0.000*) and LPS-challenged control rats (*p=0.000*). Gadolinium chloride treatment of BDL rats 48 hours (*p=0.001*) but not 6 hours (*p=0.572*) prior to LPS challenge resulted in decreased lung concentrations of TNF-α compared to LPS challenged BDL rats (Figure 6.2.8.B).

6.2.8.3. Immuno-electron microscopy for TNF-α: Immunogold electron microscopy showed TNF-α labeling in PIMs (Figure 6.2.9).

6.2.9. IL-10 Expression

6.2.9.1. IL-10 mRNA expression: Densitometry showed increased expression of IL-10 mRNA in LPS-challenged BDL rats compared to control rats (*p=0.000*) and LPS-challenged control rats (*p=0.000*). Gadolinium chloride administration in BDL rats at 6 hours or 48 hours before LPS challenge suppressed IL-10 expression compared to LPS-treated BDL rats (*p=0.038 & 0.000* respectively) (Figure 6.2.10.A).

6.2.9.2. IL-10 protein expression: There was increase in IL-10 concentration in lungs of LPS-treated BDL rats compared to the control (*p=0.018*) or LPS-treated control rats (*p=0.002*). IL-10 concentration in lungs of control rats was similar to those of LPS-treated control rats (*p=0.353*). Gadolinium chloride treatment of BDL rats 48 hours (*p=0.030*) but not 6 hours (*p=0.420*) before the LPS challenge decreased concentration of IL-10 in lungs in comparison to LPS-challenged BDL rats (Figure 6.2.10.B).

6.2.10. TGF-β Expression

6.2.10.1. TGF-β mRNA expression: Compared to the control rats, TGF-β expression was increased in lungs of LPS-challenged BDL (*p=0.000*) or LPS-challenged control rats

(*p=0.001*). Gadolinium chloride treatment of BDL rats at 48 hours (*p=0.000*) but not at 6 hours (*p=0.182*) prior to the LPS challenge suppressed lung transcription of TGF-β compared to BDL+LPS rats (Figure 6.2.11.A).

6.2.10.2. TGF-β protein expression: Lung homogenates from LPS-treated control rats showed lower TGF-β concentrations compared to all other groups. Gadolinium chloride treatment of BDL rats prior to LPS challenge had no effect on TGF-β concentration compared to LPS treated BDL rats (Figure 6.2.11.B).

Table 6.1: Clinical response to LPS challenge

Treatment	Number of rats (N)	Number of rats died within 6 hours of LPS challenge	Mortality (%)
Control+LPS	5	0	0
BDL+LPS	6	6	100
BDL+GC(6H)+LPS	5	1	20
BDL+GC(48H)+LPS	5	0	0

Histopathology (LPS challenge):
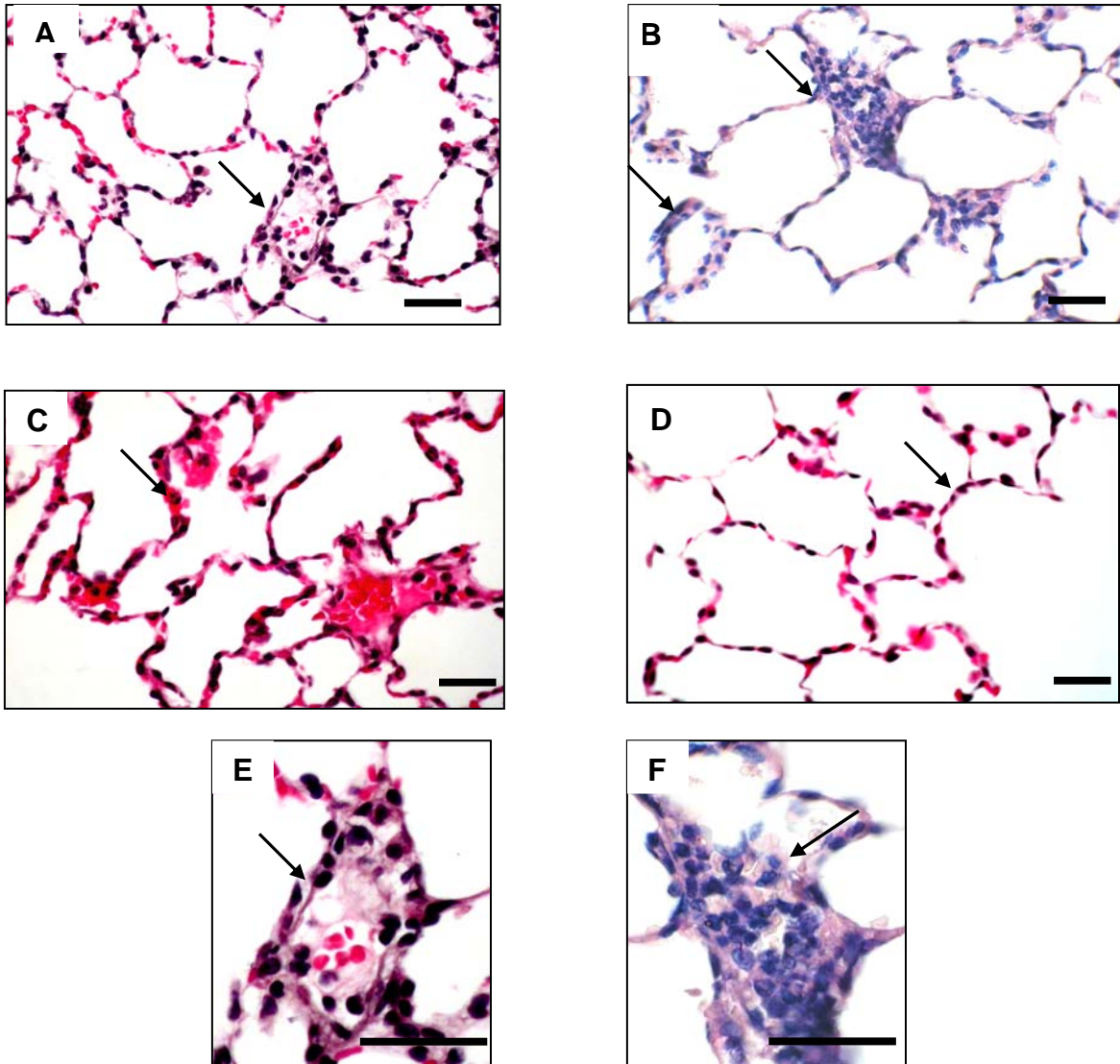
Figure 6.2.1: H & E staining: LPS challenge of BDL rats (B) resulted in more vascular congestion (arrows) compared to LPS-challenged control rats (A). Gadolinium chloride treatment of BDL rats 6 hours (C) or 48 hours (D) prior to LPS challenge resulted in less infiltration of mononuclear cells and near normal lung histology. High magnification views of lung sections from LPS-challenged control rats (E) and LPS-challenged BDL rats (F) show differences in vascular congestion (arrows) between the two treatments. Bar = 25 µm.

Immunohistology for ED-1 (LPS challenge):
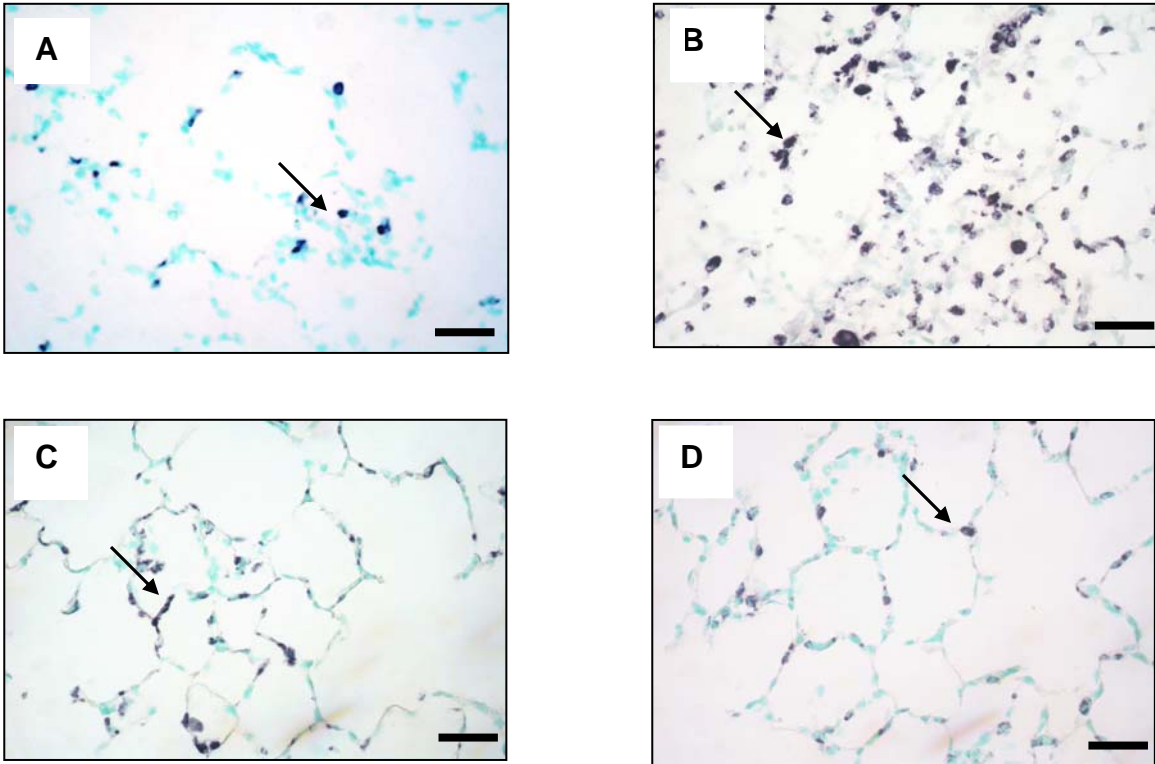
Figure 6.2.2: Immunohistology for macrophages (ED-1 staining): LPS challenge of BDL rats (B) resulted in dramatic increase in the number of ED-1 positive cells (arrows) compared to that in LPS challenged control rats (A) and BDL rats treated with gadolinium chloride at 6 hours (C) or 48 hours (D) before the LPS treatment. Bar = 25μm.

ED-1 Positive Cell Count:

Figure 6.2.3: ED-1 cell counts:. Groups bearing superscripts (for example 'a') differ from the groups carrying different superscripts, (for example 'b' or 'c') while the groups with similar superscripts did not differ. See "Results" for details.

Immunohistology for LPS:
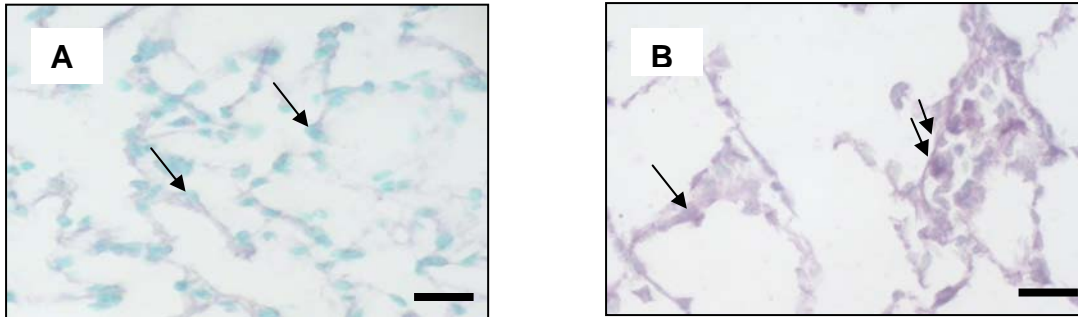
Figure 6.2.4: Lung sections stained with anti-LPS antibody show negligible staining for LPS (arrows) in the alveolar septa of control rats challenged with LPS (A). However, BDL rats challenged with LPS show extensive staining for LPS in the alveolar septa (arrows) as well as in mononuclear cells present in the blood vessel (double arrows) (B). Micrometer bar = 25µm.

MCP-1 Expression:

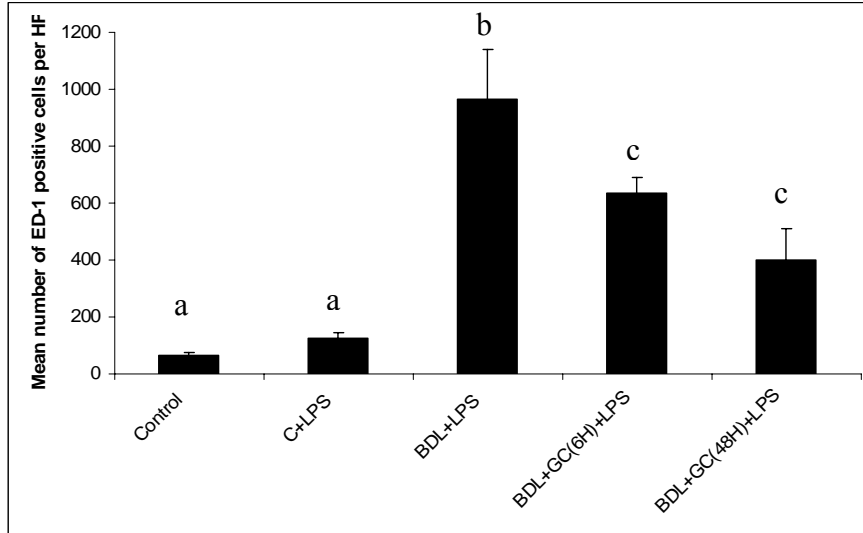**Figure 6.2.5: MCP-1 mRNA (A) and protein (B) expression:** Groups bearing superscripts (for example 'a') differ from the groups carrying different superscripts, (for example 'b' or 'c') while the groups with similar superscripts did not differ. For detail see "Results".

IL-1β Expression:

1 Control
2 Control+LPS
3 BDL+LPS
4 BDL+GC (6H) +LPS
5 BDL+GC (48H) +LPS

Figure 6.2.6: IL-1β mRNA (A) and protein (B) expression: Groups bearing superscripts (for example 'a') differ from the groups carrying different superscripts, (for example 'b' or 'c') while the groups with similar superscripts did not differ. For detail see "Results".

Immuno-electron microscopy for IL-1β:
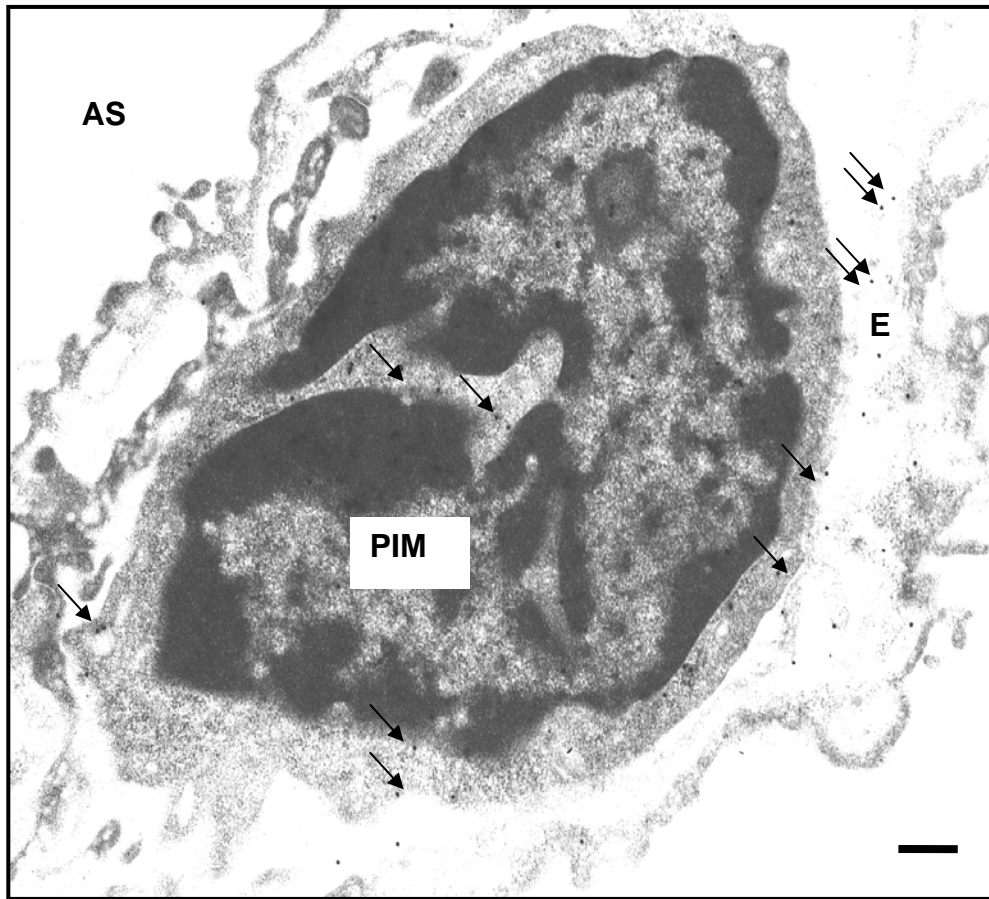
Figure 6.2.7: An immuno-electron micrograph showing IL-1β labeling (arrows) in a pulmonary intravascular macrophage (PIM) as well as capillary endothelium (E: double arrows). AS: alveolar space. Bar = 1 micron

TNF-α Expression:
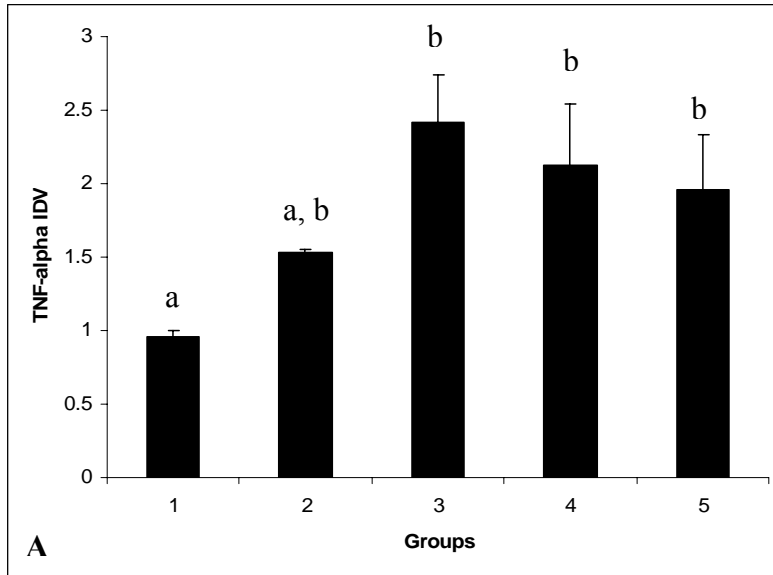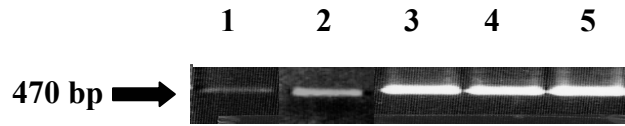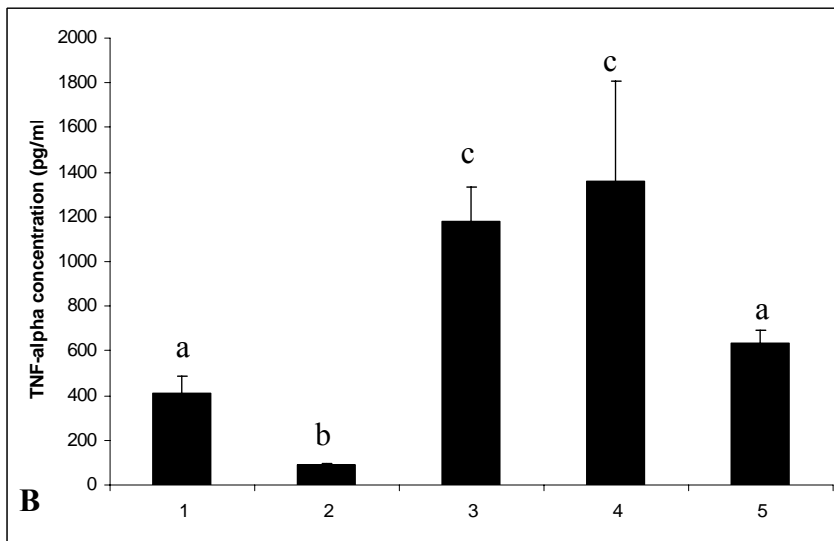
1 Control
2 Control+LPS
3 BDL+LPS
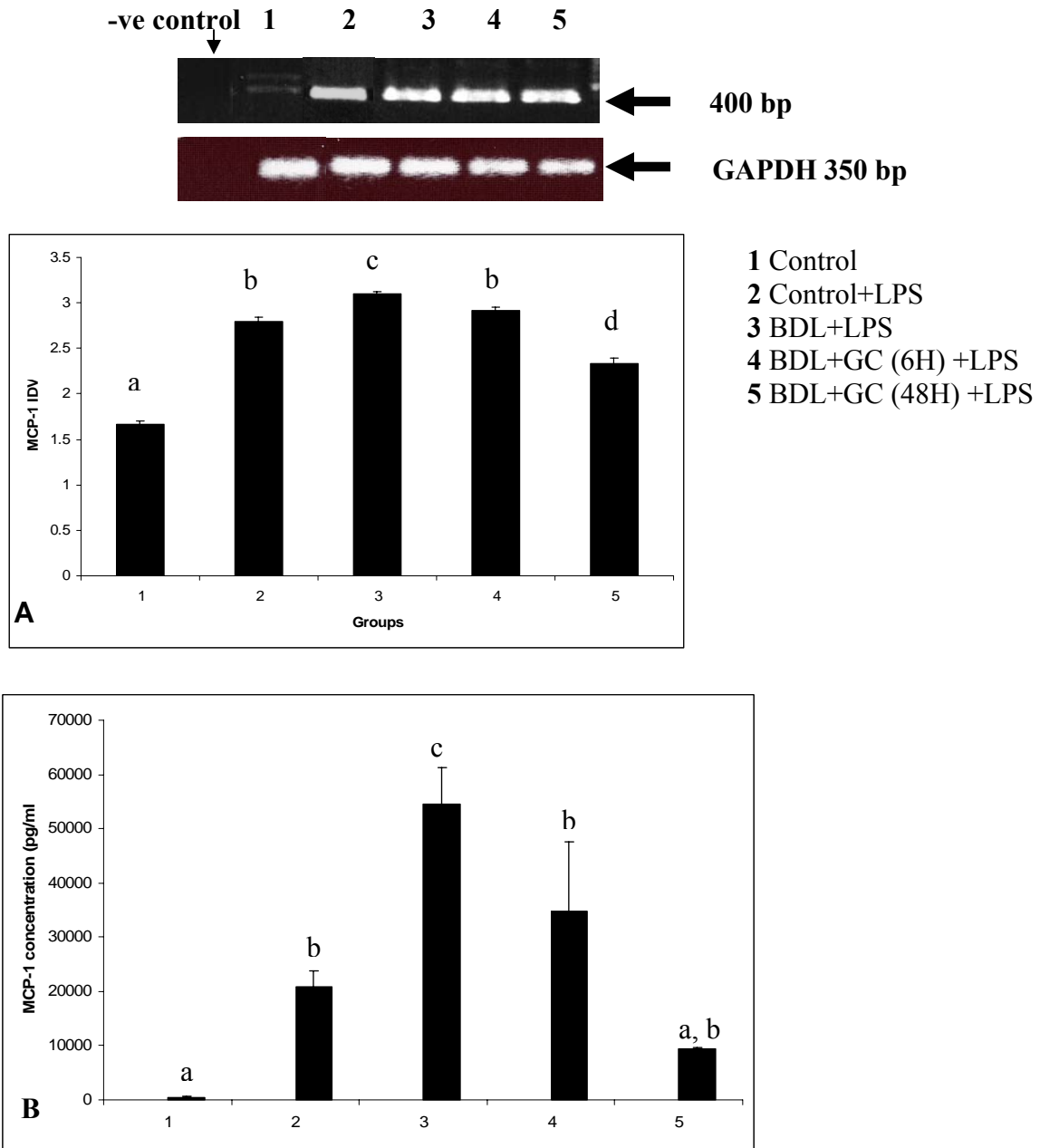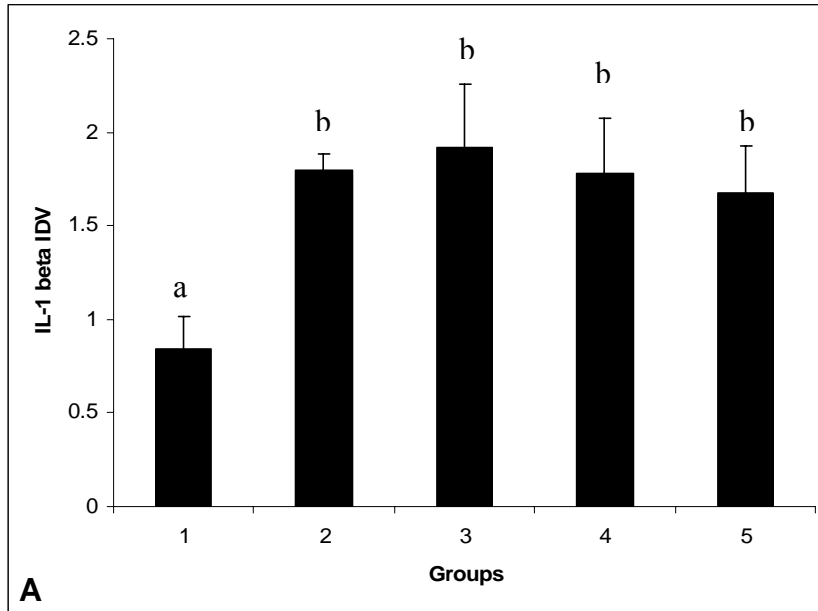**4** BDL+GC (6H) +LPS
5 BDL+GC (48H) +LPS

Figure 6.2.8: TNF-α mRNA (A) and protein (B) expression: Groups bearing superscripts (for example 'a') differ from the groups carrying different superscripts, (for example 'b' or 'c') while the groups with similar superscripts did not differ. For detail see "Results".

Immuno-electron microscopy for TNF-α:
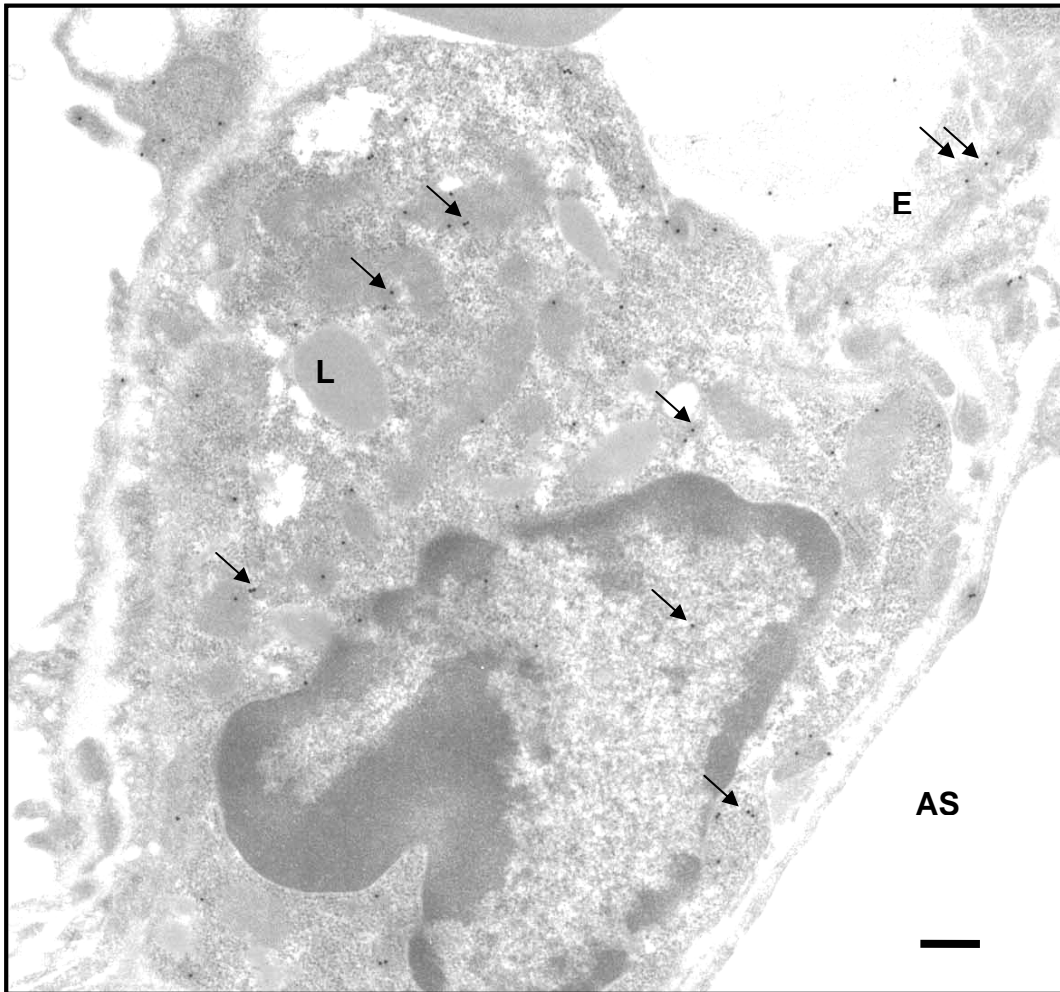
Figure 6.2.9: An immuno-electron micrograph showing TNF-α labeling (arrows) in a PIM as well as capillary endothelium (double arrows). AS: alveolar space; E: endothelium; L: lysosome; AS: alveolar space. Bar = 1 micron

IL-10 Expression:

1   2   3   4   5

680 bp ➡

A

1 Control
2 Control+LPS
3 BDL+LPS
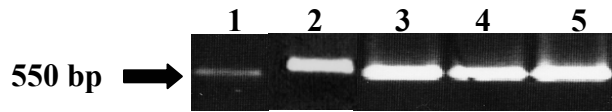**4** BDL+GC (6H) +LPS
5 BDL+GC (48H) +LPS

B
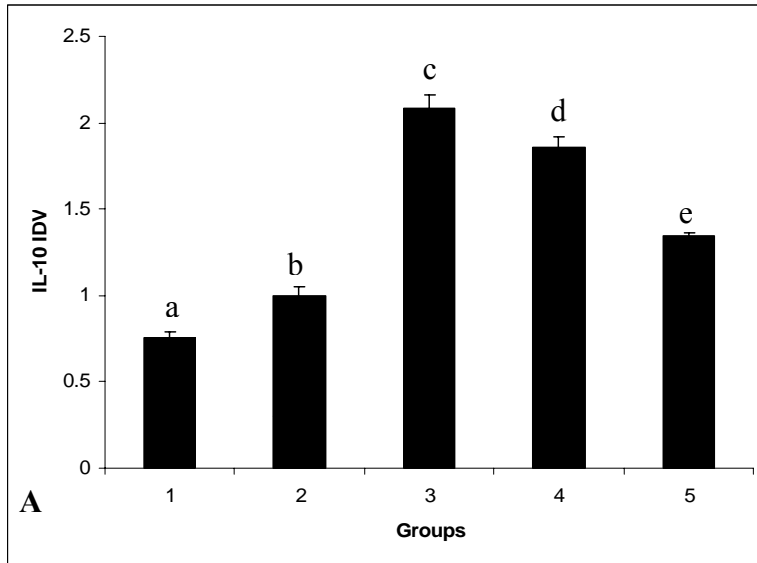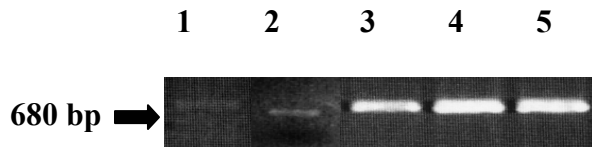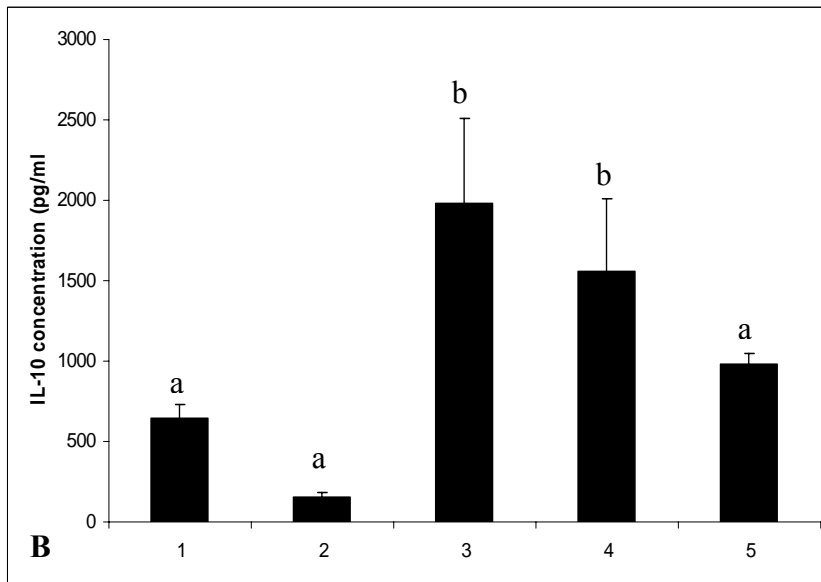
Figure 6.2.10: IL-10 mRNA (A) and protein (B) expression: Groups bearing superscripts (for example 'a') differ from the groups carrying different superscripts, (for example 'b' or 'c') while the groups with similar superscripts did not differ. For detail see "Results".
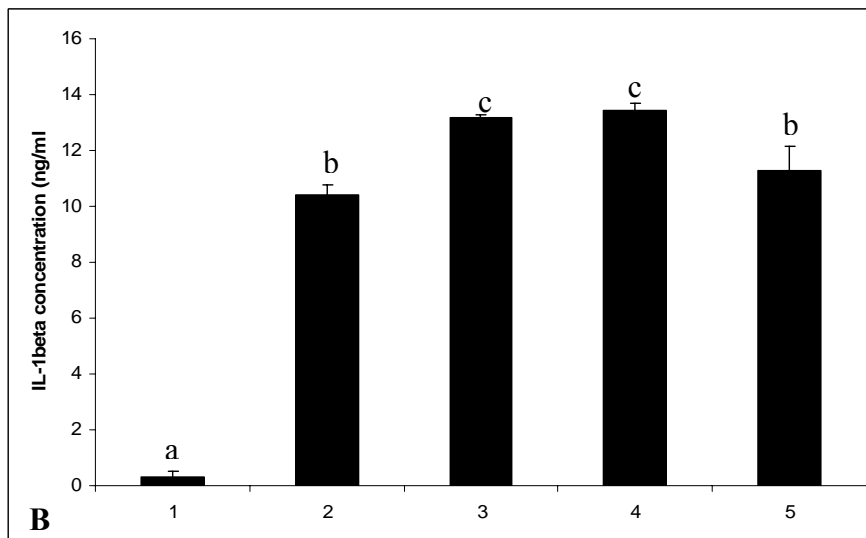
TGF-β Expression:
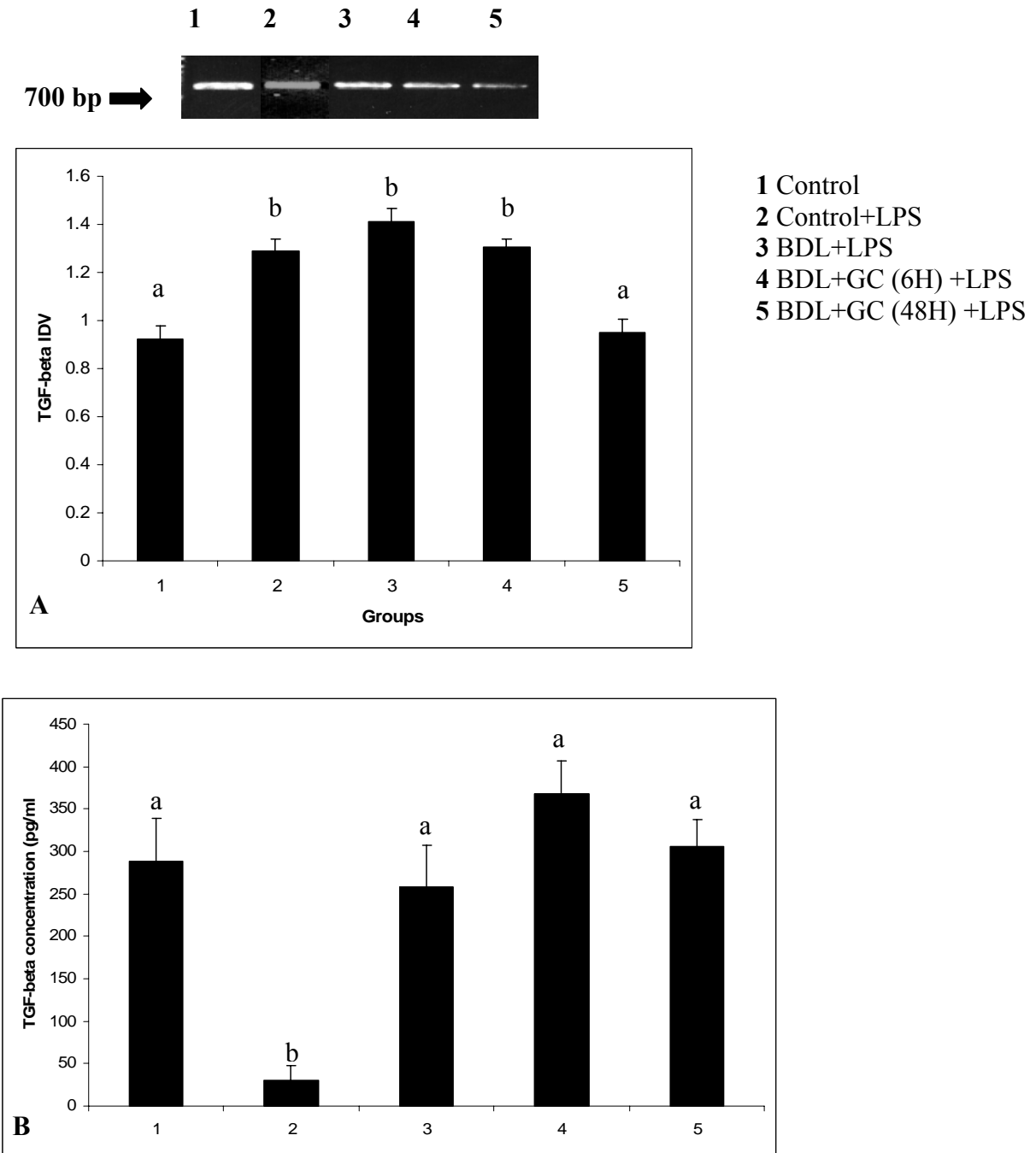
Figure 6.2.11: TGF-β mRNA (A) and protein (B) expression: Groups bearing superscripts (for example 'a') differ from the groups carrying different superscripts, (for example 'b' or 'c') while the groups with similar superscripts did not differ. For detail see "Results"

7. DISCUSSION:

7.1. BDL as a model of recruited PIMs

Biliary cirrhosis induced by bile duct ligation causes permanent recruitment of intravascular macrophages in rats (Chang and Ohara,1994). Bile duct ligation model in rats has been used to study the mechanisms of HPS in human patients with liver cirrhosis (Nunes *et al.*, 2001), (Sztrymf *et al.*, 2004), (Rabiller *et al.*, 2002) as well as to study biology of PIMs (Chang and Ohara,1994). I am interested in investigating the role of PIMs in the development of HPS as well as pulmonary diseases in other domestic species. Therefore, I used BDL model to study these aspects.

Initially, I encountered high mortality in rats following ligation of their bile ducts. However, with improvement of my surgical and post-operative skills, the mortality rates came below 20%, which are similar to those reported by other workers (Nunes et al., 2001). I found that recruited cells are monocytes/macrophages as indicated by ED-1 antibody. ED-1 antibody recognizes a single chain glycoprotein of 110 kD. This protein is normally expressed on the lysosomal membrane of monocytes/macrophages. Electron-microscopy further confirmed that recruited cells contained ED-1, which established recruited cells as macrophages. Therefore, BDL rats in my experiments showed recruitment of PIMs. Although intravascular recruitment of PIMs in BDL rats has been previously demonstrated by Chang and colleagues (Chang and Ohara, 1994), these cells have not been immunophenotyped. Thus, ED-1 staining in recruited PIMs confirms their identity as macrophages. It appears that recruited PIMs in BDL rats form much tighter adhesion with capillary endothelium compared to the transiently recruited PIMs following a single intraperitoneal *E. coli* injection (Singh and Atwal, 1997). Therefore, BDL rats may be a useful model to investigate PIM biology in liver dysfunction as well as in species in which these cells are normally present.

The mechanisms of recruitment of PIMs following bile duct ligation remain unknown. Macrophage recruitment is regulated by a variety of chemoattractants such as MCP-1. Because MCP-1 is one of the most potent chemokines for lung recruitment of monocytes and macrophages (Maus *et al.*, 2001), I examined its expression in normal and BDL rats.

MCP-1 exists in two isoforms (13kD & 9kD) (Jiang *et al.*, 1991). MCP-1 mRNA as well as protein expression was increased in lungs of BDL rats compared to the normal rats. MCP-1 was localized in septal cells including recruited PIMs. These data suggest that MCP-1 may play a role in PIM recruitment in BDL rats. However, this does not preclude the possibility that other chemokines such as MIP-2 or CINC-1 may also be promoting accumulation of PIMs in BDL rats. Furthermore, I will need to perform MCP-1 blocking experiments to demonstrate specific contributions of MCP-1 in PIM recruitment in BDL rats.

Because BDL rats are used as a model for HPS and they may also be useful in study of PIMs in domestic animal species, it is important to examine baseline cytokine expression in lungs and compare it to lungs from normal rats. I selected two pro-inflammatory (IL-1β & TNF-α) and two anti-inflammatory (IL-10 & TGF-β) cytokines. There were no significant differences in the mRNA and protein expression of IL-1β, TNF-α and TGF-β between control and BDL rats. Although IL-10 mRNA expression was altered, the protein concentrations were similar between the two groups. Because the protein is the functional product of gene transcription, I conclude that compared to the control rats PIM recruitment in BDL rats did not alter lung expression of various cytokines. Therefore, the BDL rats may be a valid model to investigate biology of PIMs in domestic animals.

7.2. PIM depletion with gadolinium chloride

To directly evaluate the role of recruited PIMs, we should be able to deplete these cells to examine lung inflammation in the presence or absence of PIMs. Therefore, I used gadolinium chloride (GC) to deplete PIMs. GC has previously been used to remove PIMs *in vivo* in sheep, cattle and horses and alveolar macrophages in mice and rat (Staub, 1994), (Singh *et al.*, 2004), (Parbhakar *et al.*, 2004). GC induces apoptosis in PIMs to significantly reduce their numbers within 48 hours of the treatment (Singh *et al.*, 2004). I found that GC reduced PIM numbers in BDL rats at 48 hours of the treatment to near significance levels (*p=0.06*). Histological evaluation of lung sections suggested that PIMs in GC-treated rats may be undergoing apoptosis. However, I did not confirm this observation with additional methods such as TUNEL or detailed electron microscopy. It is possible that PIM function may be more compromised than suggested by histological

numerical counts of ED-1 cells. Decline in ED-1 positive PIMs in lung sections directly quantifies the dead cells that have been physically removed from the capillaries. Although GC can block Kupffer cells as early as 6 hours of treatment (Kim and Choi, 1997), (Brown *et al.*, 1997), it takes much longer for the induction of apoptosis and removal of apoptotic cells. Dysfunctional or dead PIMs in GC-treated rats that are still to be removed from the capillaries may be recognized by ED-1 antibody. Therefore, GC may compromise PIM function to a higher degree than is captured by ED-1 staining of lung sections.

There were no effect of GC treatment on the lung expression of IL-1β and TNF-α. However, lungs of rats treated with GC compared to BDL rats had higher concentrations of TGF-β and IL-10 mRNA but not the protein. It is possible that the time point examined in my study may not be sufficient for translation of mRNA into the protein, However, if increased levels of mRNA are translated into the protein forms of TGF-β and IL-10, the data would fit with previously demonstrated increased expression of TGF- β in tissues that contain apoptotic cells (Lucas *et al.*, 2003), (Huynh *et al.*, 2002). IL-10 is also an anti-inflammatory cytokine and its increased expression may be related to GC-induced apoptosis in lungs. Furthermore, BDL rats treated with GC continued to express higher levels of MCP-1 mRNA and protein. Based on the established roles of MCP-1 in recruitment of monocytes/macrophages, higher expression of MCP-1 in GC-treated BDL or BDL rats would suggest ongoing recruitment of monocytes/macrophages. Increased MCP-1 expression may also be necessary to promote migration of monocytes/macrophages to remove apoptotic cells in GC-treated rats. However, these speculations need to be confirmed with additional studies to show recruitment of fresh monocytes/macrophages into the lungs. These studies may include experiments where MCP-1 is blocked with antibodies or the use of MCP-1 knockout rats.

I noticed discordance in the expression of TGF-β and IL-10 mRNA and proteins. Although I do not know the precise reasons but it could be due the following reasons. First, the RT-PCR followed by densitometry is semi-quantitative at best while the ELISA is a quantitative method. Second, there could be an interference in the translation of

mRNA into the protein product in these tissues. Lastly, because protein is the active and functional product of the gene, I decided to rely more on the protein data than the mRNA.

These data show that BDL causes substantial recruitment of ED-1 positive PIMs. However, recruitment of PIMs does not induce expression of inflammatory cytokines in the rats. Because GC effectively depletes PIMs, this provides us with a reliable model to study lung inflammation with or without PIMs to delineate their contributions.

7.3. Recruited PIMs in lung inflammation

I examined the role of recruited PIMs in LPS-induced lung inflammation. The data show that LPS treatment of BDL rats induced more severe inflammation and mortality compared to that of LPS-treated normal rats. It is interesting to note that BDL rats suffered 100% mortality within 3 hours of the LPS administration while the normal LPS-challenged rats were euthanized at 6 hours after the treatment. The mortality data in the current study are similar to those reported by Chang and O'Hara in their experiments on BDL rats challenged with LPS (Chang and Ohara, 1994). However, these authors did not examine the mechanisms of increased mortality in BDL rats challenged with LPS. To address this issue, I evaluated the expression of IL-1β and TNF-α which are central to LPS-induced lung inflammation and mortality. Although densitometry did not show increase in mRNA expression but ELISA detected increased IL-1β and TNF-α concentrations in lungs from LPS-treated BDL rats compared to the normal LPS-treated rats. Both of these cytokines are central to LPS-induced inflammation associated with mortality and morbidity (Zanotti *et al.*, 2002), (Strieter *et al.*, 1993), (Debets *et al.*, 1989), (Dinarello *et al.*, 1989), (Creasey *et al.*, 1991), (Shapira *et al.*, 1996). Specifically, IL-1β is central to endotoxin-induced cardiopulmonary shock (Dinarello *et al.*, 1989), (Fischer *et al.*, 1991). Immuno-electron microscopy localized both the cytokines in PIMs of LPS-challenged BDL rats. These data are similar to the expression of IL-1β and TNF-α in PIMs of LPS-challenged horses; a species which is highly susceptible to endotoxin-induced lung inflammation and mortality (Parbhakar *et al.,* 2005). Therefore, PIMs may orchestrate lung inflammation and mortality by producing proinflammatory cytokines to induce expression of adhesion molecules and activate other vascular cells.

The increased LPS-induced mortality in BDL rats compared to LPS-challenged normal rats suggests a causal link with recruited PIMs. However, to clearly establish the role of recruited PIMs, I investigated LPS-induced lung inflammation in BDL rats treated with GC. The data showed effectiveness of GC in removal of PIMs in BDL rats. Therefore, I treated rats with GC 6 hours or 48 hours before an intravenous challenge with LPS. The reason for selecting these two post-GC time points was based on previous evidence that GC can quickly deactivate macrophages within 6 hours before actually causing apoptosis in them by 24-48 hours (Kim and Choi, 1997), (Brown *et al*., 1997). Therefore, I thought to examine the two time points that capture the impact of functional deactivation as well as physical death of PIMs on LPS-induced lung inflammation and mortality.

I noticed striking reduction in mortality rates in GC and LPS treated rats. Rats that were treated with GC 6 hours before the LPS treatment showed 80% survival rates. The survival increased to 100% if the rats were given LPS at 48 hours post-GC treatment. This is in contrast to 100% mortality in BDL rats within 3 hours of LPS challenge. I conclude that both physiological deactivation as well as physical elimination of PIMs has beneficial effects on lung inflammation and host survival.

I found a significant reduction in the mRNA expression as well as protein concentration of MCP-1 in rats treated with GC at 6 hours and 48 hours before LPS administration. Because monocytes and macrophages produce as well as regulate production of MCP-1, the inactivation or depletion of PIMs may be responsible for reduced levels of MCP-1 mRNA and protein in lungs of LPS-challenged BDL rats. BDL rats treated with GC before LPS challenge showed a decline in numbers of ED-1 positive cells compared to BDL rats treated with LPS. Because MCP-1 is a critical regulator of monocyte recruitment (Maus *et al*., 2001) , its reduced expression in lungs of PIM-depleted rats may suppress further recruitment of ED-1 positive cells and result in less severe lung inflammation.

Although GC treatment of BDL rats at 6 hours prior to LPS challenge resulted in increased survival, it did not reduce mRNA expression and protein concentrations of IL-1β and TNF-α compared to LPS-treated BDL rats. One of the reasons could be that GC causes only partial inactivation and not death in macrophages at this early time point after the treatment. This would result in secretion of preformed or newly formed cytokines by the PIMs. Another possibility could be that GC interfered with release of cytokines and chemokines from recruited PIMs as well as other lung cells by blocking their chloride channels. This would create a scenario where lung homogenates would contain higher levels of cytokines but there will not be increased expression of adhesion molecules followed by recruitment of neutrophils resulting in the survival of LPS-challenged animals. Of more interest are the results on the expression of IL-1β and TNF-α in BDL rats treated with LPS at 48 hours post-GC treatment. Lungs of these rats showed reduced expression of both the cytokines compared to BDL+LPS rats. Immuno-electron microscopy confirmed IL-1β and TNF-α in PIMs in LPS-challenged BDL rats. Therefore, reduced levels of IL-1β and TNF-α protein may be a direct in addition to indirect result of GC-mediated PIM depletion. Because PIMs produce pro-inflammatory cytokines, their removal prior to LPS challenge will eliminate a key regulator of cell activation and lung inflammation to result increased host survival.

Because inflammation is an outcome of interplay between molecules that promote or suppress inflammation, I also focused on the expression of TGF-β and IL-10. TGF-β protein concentrations were higher in all the groups including the BDL groups compared to the LPS-challenged control rats. As discussed earlier, TGF-β is an anti-inflammatory cytokine and its expression increases in tissues undergoing apoptosis. Therefore, it is an expected result where GC has induced apoptosis in PIMs, which in turn contributes to increased expression of TGF-β. Furthermore, LPS treatment of rats has been shown to suppress expression of TGF-β. However, mRNA expression and protein concentrations of IL-10 in both 6 hour and 48 hour GC groups were lower as compared to LPS treated BDL group. IL-10 is produced by activated mononuclear cells on exposure to LPS, which in turn deactivates macrophages (Bogdan *et al*., 1991) and inhibits pro-inflammatory cytokine production by the activated macrophages (Strassmann *et al*., 1994), (Platzer *et*

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al., 1995). This suggests higher mRNA expression and protein concentrations of IL-10 in our LPS treated BDL rats. It has also been shown that IL-10 concentration increases with the number of recruited mononuclear phagocytes with in first 3 days after LPS challenge and then decreases to undetectable levels (Salez *et al*., 2001), (Chabot *et al*., 2003). Therefore, my data show GC treatments resulted in lower mRNA expression and protein concentration of IL-10. The effect on IL-10 expression could be due to direct inactivation and depletion of PIMs.

This study has some weaknesses. First and foremost it is critical to examine the recruitment of neutrophils in rat lungs in various groups. Although both PIM inactivation or depletion resulted in increased survival of BDL rats, there may be differences in the mechanisms between these two instances. For example PIM inactivation caused increased survival without reducing the expression of cytokines. Because activated neutrophils cause tissue damage and death, it will be interesting to see if there were quantitative differences in the migration of neutrophils in these two scenarios. I believe that it is important to undertake a detailed examination of morphology and cytokine expression in the liver of these animals. Although BDL stimulates recruitment of PIMs, it will be interesting to see if such phenomenon also occurs in the spleen as well.

The results presented in this thesis show that PIMs are recruited in a rat model of biliary cirrhosis and that recruited PIMs do not induce lung inflammation. However, upon an LPS challenge these PIMs become inflammatory and induce significant lung inflammation and mortality. The direct role of PIMs in inflammation and mortality in the rats is established by inhibition of these events upon deactivation or death of PIMs. These data are novel and have direct bearing on our understanding of PIMs' roles in HPS in humans and lung inflammation in domestic animals. Furthermore, the data underscore PIMs as potential targets for therapeutic interventions. These studies also provide a rat model to study PIMs contributions to lung inflammation in domestic animals at a much reduced rates.

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8. CONCLUSIONS

1. Bile duct ligation induces recruitment of PIMs in rats without activation of lung inflammation.

2. Recruited PIMs increases sensitivity to LPS induced lung inflammation and result in substantial mortality.

3. Depletion or inactivation of PIMs protects against the severity of LPS induced lung inflammation and mortality.

9. FUTURE RESEARCH

First, it is important to further characterize the expression of various inflammatory genes in lungs of BDL rats as well as directly in recruited PIMs. Second, there is a need to develop methods to cause more rapid and effective depletion or inactivation of PIMs. Third, we need better delivery methods to target depleting chemicals specifically to PIMs. Fourth, I think it will be useful to examine if PIMs are present in lung autopsy or biopsy materials from human patients suffering from various systemic or lung diseases.

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