MOLECULAR MECHANISMS

OF NEUTROPHIL AND MONOCYTE RECRUITMENT

IN ACUTE LUNG INFLAMMATION

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By

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ABSTRACT

Neutrophils are implicated in many inflammatory lung disorders. However, the mechanisms regulating neutrophil migration in acute lung inflammation are incompletely understood. Although, integrin β_2 mediates neutrophil migration in lungs in response to many stimuli such as *E. coli*, integrin involved in *S. pneumoniae* induced neutrophil migration is not known. Therefore, the role of integrin $\alpha_v\beta_3$ in neutrophil recruitment was tested. First, it was found that the number of neutrophils expressing the integrin subunits α_v and β_3 is reduced or remains in lung inflammation induced by *E. coli* or *S. pneumoniae*, respectively. Next, the role of integrin $\alpha_v\beta_3$ using β_3 knockout mice ($\beta_3^{-/-}$) and function blocking antibodies was addressed. Neutrophil recruitment did not vary between wild type and $\beta_3^{-/-}$ mice. Although β_3 antibodies reduced neutrophil recruitment, similar effect was observed with isotype antibodies. Therefore, one can conclude that integrin $\alpha_v\beta_3$ is not critical for neutrophil recruitment in *S. pneumoniae* induced pneumonia.

Apart from integrins, TLR4 also regulate neutrophil migration. Because, the pattern of TLR4 expression at various times of lung inflammation is not known, TLR4 expression during different phases of lung inflammation in a rat model of LPS-induced inflammation was studied. TLR4 expression in the septum increased and decreased at 6h and 12-36h of inflammation, respectively. Since these correlate with the time of increase and decline of neutrophil recruitment, the findings support previously observed requirement for TLR4 in neutrophil recruitment.

Neutrophils recruited into the lungs regulate the inflammatory process by controlling subsequent monocyte/macrophage recruitment. The mechanisms involved and the pattern of monocyte/macrophage recruitment in lungs are not completely understood. Therefore, the possible involvement of monocyte chemoattractant protein (MCP)-1, which is a premier chemokine in monocyte/macrophage migration and produced by neutrophils and other cells was tested. This was addressed by quantification of monocytes/macrophages at various times and using neutrophil depletion experiments in LPS-induced lung inflammation in rats. It was found that monocytes/macrophages migrate very early and before neutrophils in addition to their migration in the late phase of acute lung inflammation. Neutrophil depletion abrogated both early as well as the late monocyte/macrophage recruitment without altering the expression of MCP-1. Therefore, possibly other chemokines and not MCP-1 are involved in neutrophil dependent monocyte/macrophage recruitment.

To conclude, the experiments further the understanding on acute lung inflammation by ruling-out the involvement of integrin $\alpha_v\beta_3$ and MCP-1 in β_2-independent neutrophil migration and neutrophil dependent monocyte/macrophage recruitment, respectively. Further studies are essential to find the integrins and chemokines operating in the above situations. Equally important will be to understand the functional significance of early recruited monocytes/macrophages in the lung.

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LIST OF ABBREVIATIONS

ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BHI broth	Brain heart infusion broth
CFU	Colony forming unit
E. coli	*Escherichia coli*
E-selectin	Endothelial selectin
fMLP	formyl-methionyl-leucyl-phenylalanine
GRO	Growth related oncogene
ICAM	Intercellular adhesion molecule
IL	Interleukin
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
L-selectin	Leukocyte selectin
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
PMA	Phorbol myristate acetate
P-selectin	Platelet selectin
PSGL	Platelet selectin glycoprotein ligand
S. pneumoniae	*Streptococcus pneumoniae*
TLR	Toll-like receptor
TRALI	Transfusion related acute lung injury

CHAPTER 1:REVIEW OF LITERATURE

1.1. Introduction

Inflammation is tissue's response to a physical, chemical or biological stimulus, characterized by cellular and vascular changes (Allison *et al.* 1955). Vascular changes are characterized by congestion and increased permeability leading to edema formation. Cellular changes are characterized by accumulation of neutrophils followed by monocytes/macrophages, which would normally result in resolution of inflammatory process (Ryan and Majno. 1977). Inflammation is an essential process for the host defense. However, when dysregulated, the inflammatory process could result in tissue injury (Smith. 1994).

Lung has the largest surface area of the epithelium in the mammalian body that is constantly exposed to various agents such as particulate matters and microbes (Reynolds. 1987; Zhang *et al.* 2000). Normally these agents are cleared from the lungs by the mechanical barriers including mucociliary clearance and immune cells such as macrophages to maintain homeostasis (Reynolds. 1987). When the stimulus is not cleared completely from the lung, inflammatory process is initiated to clear the stimulus (Delclaux and Azoulay. 2003). Such a process will result in the recruitment of inflammatory cells in a tightly regulated fashion. In lung the majority of neutrophil migration occurs from the microvasculature (Lien *et al.* 1991). Lung microvasculature holds 20-40% of total neutrophils in the body (Reynolds. 1987; Zhang *et al.* 2000) and has a huge surface area to an extent of 60 m^2 (Hogg. 1987). Therefore, process of dysregulated neutrophil recruitment can result in massive influx and activation of neutrophils and other cells such as endothelium, which leads to altered microvascular permeability, edema formation, decreased oxygenation and lung injury. Neutrophil recruitment is an essential process and its absence or defective function results in overwhelming lung infections (Marlin *et al.* 1986; Moore *et al.* 2000; Tsai *et al.* 2000; Tsai *et al.* 1998). However, dysregulated recruitment results in several inflammatory lung disorders such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and transfusion related acute lung injury (TRALI)

(Matthay *et al.* 2003; Menezes *et al.* 2005; Piantadosi and Schwartz. 2004; Toy *et al.* 2005; Ware and Matthay. 2000). All these pathological conditions have significant morbidity and mortality rates. For example, ALI/ARDS affects approximately 20-50 per 100,000 persons annually in the United States of America with a mortality rate of 40% (Matthay *et al.* 2003; Rubenfeld. 2003). TRALI has a mortality rate of up to 25% (Silliman *et al.* 2003; Toy *et al.* 2005). In all these cases neutrophils play a critical role (Silliman *et al.* 2005; Silliman *et al.* 2003; Ware and Matthay. 2000). Apart from the conditions mentioned above, neutrophils play an important role in several bacterial pneumonias (Rijneveld *et al.* 2005; Tsai *et al.* 2000). Amongst various causes, *Streptococcus pneumoniae* (*S. pneumoniae*) is a major pathogen causing bacterial pneumonia and it accounts for 40-50% of community acquired pneumonia (Kadioglu and Andrew. 2004). World wide, over one million children suffer from *S. pneumoniae* induced pneumonia (Kadioglu and Andrew. 2004). Pneumonia is also considered to be one of the important causes for ALI/ARDS. Because dysregulated migration of neutrophils into lungs has profound implications, it is critical to have a precise understanding of the mechanisms that regulate their migration.

Neutrophil migration is followed by the entry of monocytes/macrophages into the inflamed lungs (Ryan and Majno. 1977). Apart from their direct effects on the tissues, neutrophils also influence the outcome of an inflammatory process by playing a role in the recruitment of monocytes/ macrophages (Doherty *et al.* 1988; Maus *et al.* 2002b). However, knowledge on the mechanisms of neutrophil mediated monocyte recruitment in the lung is incomplete. Understanding this mechanism is important as macrophages in the alveolar space play an important role in lung inflammation (Fels and Cohn. 1986; Maus *et al.* 2002c; Reynolds. 1987; Reynolds. 2005). Also, pulmonary intravascular macrophages are known to play a major role in lung inflammation in the host species such as equines and bovines (Parbhakar *et al.* 2005; Singh *et al.* 2004). In addition, species which lack intravascular pool of macrophages, such as rats can also recruit monocytes/macrophages into pulmonary microvasculature and play a role in inflammation (Singh *et al.* 1998; Sztrymf *et al.* 2004). All these indicate that monocytes/macrophages, irrespective of their location (alveolar or intravascular), can regulate lung inflammation. However, because of the accepted paradigm that monocytes are recruited after the neutrophils, the studies which have examined the recruitment of monocytes/macrophages in the lung have predominantly concentrated on those which are

recruited into the alveolar spaces in the post-neutrophilic phase (Fillion *et al.* 2001; Maus *et al.* 2002b; Ulich *et al.* 1991; Yamamoto *et al.* 1998). Despite evidences for the early monocyte recruitment either before or along with the neutrophils in inflammatory conditions of other organs (Henderson *et al.* 2003; Issekutz and Issekutz. 1993; Issekutz *et al.* 1981), the total accumulation of monocytes/macrophages in lung inflammation is not well addressed. Since monocytes/macrophages in the inflamed lungs, irrespective of their location, can significantly contribute to inflammation by producing cytokines and free oxygen radicals (Elias *et al.* 1985a; Elias *et al.* 1985b; Li *et al.* 1998; Maus *et al.* 2002a), it is important to assess the total monocyte/macrophage recruitment in the lung.

Because of the reasons mentioned above, in the following sections I will review the literature pertaining to neutrophil recruitment and monocyte/macrophage recruitment with specific emphasis on integrin and neutrophil dependent mechanisms respectively, in the lung. This will provide information on the known and unknown facts in the area of lung leukocyte recruitment and provides a logical basis for the experimental work presented in this thesis.

1.2. Neutrophil recruitment

The mechanism of neutrophil recruitment in the lung varies considerably from those observed in other locations (Burns *et al.* 2003; Wagner and Roth. 2000). In the following sections general mechanisms will be reviewed first and wherever appropriate, mechanisms specific to the lungs will be discussed.

Leukocyte migration from the vasculature is a multi-step process involving sequential activation of adhesive proteins and the ligands present on both leukocytes and endothelial cells (Albelda *et al.* 1994). The process of migration begins with rolling of neutrophils on the endothelium (Allison *et al.* 1955; Florey and Grant. 1961; Marchesi and Florey. 1960; Tonnesen *et al.* 1984). If there is a stimulus, the rolling neutrophils firmly adhere to the endothelium and is followed by transmigration, resulting in recruitment of neutrophils out of the blood vessels (Allison *et al.* 1955; Florey and Grant. 1961; Marchesi and Florey. 1960; Muller *et al.* 1993; Springer. 1994; Tonnesen *et al.* 1984; van Buul and Hordijk. 2004; Zimmerman and Hill. 1984).

1.2.1. Rolling

Rolling of neutrophils from the circulating blood on to the endothelium, the initial event in neutrophil recruitment, is due to the reversible binding of transmembrane adhesive glycoproteins called selectins found on both neutrophils and endothelial cells (Bevilacqua and Nelson. 1993; Lasky. 1992; von Andrian *et al.* 1991). Initially the name "selectin" was proposed to represent the selective expression and function of these molecules as well as the presence of lectin domain in these molecules (Bevilacqua and Nelson. 1993). There are three selectins, namely leukocyte selectin (L-selectin), platelet selectin (P-selectin) and endothelial selectin (E-selectin), involved in the neutrophil recruitment (Lasky. 1992). These selectins share a common structure, a lectin like domain at the amino terminal end which allows them to interact with specific carbohydrate ligands (Varki. 1997).

L-selectin (CD62L) is constitutively expressed on the cell surface of lymphocytes, monocytes and neutrophils (Lasky. 1992) and the expression is more on the neutrophils newly released from the bone marrow (Matsuba *et al.* 1997). L-selectin is shed from the neutrophils as they interact with the endothelium (Kishimoto *et al.* 1989; Matsuba *et al.* 1997). The endothelial ligand of neutrophil L-selectin is a member of a group of sialomucin oligosaccharides that share affinity for selectins expressed on platelets, lymphocytes and monocytes (Varki. 1997). CD34 is a well characterized ligand for L-selectin (Krause *et al.* 1996). In addition, P-selectin glycoprotein ligand (PSGL)-1 (CD162) is also a ligand for L-selectin and mediates leukocyte rolling through leukocyte-leukocyte interactions during inflammatory conditions (Sperandio *et al.* 2003).

P-selectin (CD62P) is constitutively stored intracellularly in Wiebel-Palade bodies of endothelial cells and in the α-granules of platelets (Bonfanti *et al.* 1989; Hsu-Lin *et al.* 1984; McEver *et al.* 1989; Stenberg *et al.* 1985). P-selectin is rapidly mobilized to the surface of endothelial cells following exposure to inflammatory mediators (Patel *et al.* 2002). PSGL-1 and CD24 present on neutrophils and other cells are the ligands for P-selectin (Moore *et al.* 1995; Patel *et al.* 2002; Sammar *et al.* 1994; Yang *et al.* 1999). PSGL-1 consists of a disulfide-bounded homodimer allowing its binding with two P-selectin ligands simultaneously and this could increase the avidity of P-selectin and PSGL-1 interactions (McEver and Cummings. 1997; Wagner and Roth. 2000) . Like L-selectin binding, P-selectin interaction is short lived and reversible if additional adhesive events are not involved (Albelda *et al.* 1994; von Andrian *et al.* 1991).

E-selectin (CD-62), a selectin molecule expressed on endothelial cells, is not constitutively stored in the cells but expressed in response to inflammatory stimulus (Bevilacqua *et al.* 1987; Cotran *et al.* 1986; Pober *et al.* 1986). Peak expression and activity in endothelial cells, *in vitro,* occurs after 4 hours of exposure to inflammatory cytokines (Bevilacqua *et al.* 1987). E-selectin ligand-1 is the primary ligand for E-selectin (Levinovitz *et al.* 1993) and it can also bind to other ligands such as PSGL-1 and L-selectin (Zollner *et al.* 1997). However, PSGL-1 is not critical for E-selectin mediated neutrophil rolling (Yang *et al.* 1999). E-selectin may play a role in maintaining neutrophil rolling after P-selectin down regulation (Wagner and Roth. 2000).

The initial events in the neutrophil recruitment in pulmonary circulation are different compared to the systemic circulation. Pulmonary microvasculature contains 35-100 times more neutrophils than those seen in the systemic circulation (Doyle *et al.* 1997; Gee and Albertine. 1993; Hogg *et al.* 1988; Wagner and Roth. 2000). The diameter of pulmonary microvasculature ranges from 1-10 μm (mean of 6 μm) (Doerschuk *et al.* 1987; Hogg *et al.* 1988) whereas that of a neutrophil ranges from 5-9 μm (Schmid-Schonbein *et al.* 1980). Therefore a significant portion of the microvasculature has a diameter which is smaller than the diameter of a neutrophil (Doerschuk *et al.* 1993) and neutrophils have to change their shape to pass through these microvasculature (Gebb *et al.* 1995). The requirement for a change in the shape results in the slower movement of the neutrophils through the lung microvasculature (Hogg *et al.* 1988; Lien *et al.* 1987). Because of these anatomical differences the capillaries in the lung may be too small to allow rolling of neutrophil (Wagner and Roth. 2000). Kuebler *et al.* and Yamaguchi *et al.* have reported a reduced rolling of neutrophils in the lung microvasculature on blocking L-selectin whereas Doyle *et al.* and Mizgerd *et al.* have, using L-selectin and E/P selectin deficient mice respectively, found no role for selectins in the neutrophil recruitment in the lung (Doyle *et al.* 1997; Kuebler *et al.* 1997; Mizgerd *et al.* 1996; Yamaguchi *et al.* 1997). Burns *et al.* found antibody blocking of L-, P- or E- selectins in lung did not inhibit neutrophil recruitment in response to LPS (Burns *et al.* 2001). Thus, the role of selectins in neutrophil recruitment in the lung is controversial (Doyle *et al.* 1997; Kuebler *et al.* 1997; Mizgerd *et al.* 1996; Yamaguchi *et al.* 1997).

1.2.2. Firm adhesion

Firm adhesion of neutrophils follows rolling, in the presence of an appropriate stimulus (von Andrian *et al.* 1991). Under physiological conditions the strength of leukocyte binding

during rolling may not be sufficient to induce a stronger adherence but, the affinity of selectin interaction increases after stimulation of neutrophils (Spertini *et al.* 1991). This is brought about by the interaction of chemokines with their receptors which results in phosphorylation of L-selectin leading to increased affinity of L-selectin binding (Haribabu *et al.* 1997). Engagement of selectins on neutrophils and presence of proinflammatory molecules results in increased expression and adhesive affinity of neutrophil integrins that are essential for the firm adhesion of neutrophils on the endothelium (Crockett-Torabi *et al.* 1995; Gopalan *et al.* 1997; Simon *et al.* 1995; Simon *et al.* 1999; Steeber *et al.* 1997).

1.2.2.1. Integrins

Integrins are a group of heterodimeric transmembrane glycoproteins found on neutrophils and other cells which mediate cell-cell and cell-matrix adhesions (Hynes. 1987). The heterodimers are formed from one α and one β sub-unit, and both are essential for ligand binding (Hynes. 1992). These molecules are named as "integrins" after their ability to integrate extracellular matrix with the cell's cytoskeleton (van der Flier and Sonnenberg. 2001). Integrins play a very important role in pathological processes such as tumor metastasis (Voura *et al.* 1998) and inflammation (Springer. 1994). To date there are 8 different β sub-units (β_1-β_8) that associate with one of the 16 α sub-units to form at least 24 known receptors in a variety of cells (Arnaout *et al.* 2005; van der Flier and Sonnenberg. 2001). The accepted model of regulation of integrin activation is that in a normal state the activity is inhibited by interaction of cytoplasmic tails of α and β subunits. Upon activation the subunits undergo conformational change which will allow interaction of integrin with the ligand and this further leads to clustering of integrins which increases the affinity of interaction (Laudanna and Alon. 2006; Springer. 1994; Vinogradova *et al.* 2000). The integrins are expressed on variety of cells including neutrophils and endothelial cells and they bind with various molecules such as vitronectin, fibronectin, laminin, fibrinogen, von Willibrand factor and other molecules, depending on the type of integrin and the cell in which they are expressed. The details of all the cell types and all the ligands are beyond the scope of this review and are detailed in several excellent reviews (Hynes. 1987; Hynes. 1992; Jones and Walker. 1999; Plow *et al.* 2000; Ruoslahti. 1991; van der Flier and Sonnenberg. 2001). In the context of this thesis I will focus only on the expression, interaction and function of those integrins which are present on neutrophils and endothelium. Although, the site of neutrophil

migration in the lung is predominantly through the capillaries, as apposed to the post capillary venules in the systemic circulation, much of the knowledge on neutrophil transmigration involving integrins are from experiments in systemic circulation (Burns *et al.* 2003). Wherever appropriate literature is available, the role of integrins specific to neutrophil migration in the lungs will be discussed.

1.2.2.2. Neutrophil integrins

Neutrophils express several integrins; 1) Integrins with β_2 subunit which include $\alpha_m\beta_2$, $\alpha_L\beta_2$, $\alpha_x\beta_2$ and $\alpha_d\beta_2$ (Springer. 1990; Springer *et al.* 1987; van der Flier and Sonnenberg. 2001; Walzog *et al.* 1999). 2) Integrins with β_1 subunit (Bohnsack. 1992; Bohnsack *et al.* 1990; Bohnsack *et al.* 1995; Gao and Issekutz. 1997; Issekutz *et al.* 1996; Kubes *et al.* 1995; Ridger *et al.* 2001; Shang and Issekutz. 1997; Springer. 1990; Taooka *et al.* 1999; Werr *et al.* 2000) which includes $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$and $\alpha_9\beta_1$. 3) Integrin with β_3 subunit which includes $\alpha_v\beta_3$ (Hendey *et al.* 1996; Horton. 1997; Lawson and Maxfield. 1995; Sixt *et al.* 2001; Taooka *et al.* 1999; van der Flier and Sonnenberg. 2001; Yauch *et al.* 1998).

1.2.2.3. Integrins with β_2 subunit

Neutrophil binding to activated endothelium is primarily mediated by integrins consisting of β_2 subunits. These integrins are specific to hematopoietic cells and are not present on other cells (Harris *et al.* 2000). The integrins in this category are;

1. CD11a/CD18 (LFA-1 / $\alpha_L\beta_2$),
2. CD11b/CD18 (Mac-1 / $\alpha_m\beta_2$ / MO-1 / CR3),
3. CD11c/CD18 (gp150; 95 / $\alpha_x\beta_2$), and
4. CD11d/CD18 ($\alpha d\beta_2$).

These integrins are preformed and stored in secretory vesicles, gelatinase granules and specific granules of neutrophils (Arnaout *et al.* 1984; Borregaard and Cowland. 1997; Borregaard *et al.* 1987; Buyon *et al.* 1997). These integrins stored in the granules are not functional because of the phosphates present in the granules, which prevent phosphorylation of integrin molecules (Buyon *et al.* 1997).Upon stimulation with a chemoattractant, these are rapidly mobilized onto the surface of the neutrophils (Arnaout *et al.* 1984; Borregaard *et al.* 1987; Buyon *et al.* 1997; de Haas *et al.* 1994). The surface translocation is substantial by 30 minutes after stimulation with agonists

such as phorbol myristate acetate (PMA) (Buyon *et al.* 1997). However, mere increase in the expression will not result in increased adhesiveness of neutrophils (Buyon *et al.* 1988; Buyon *et al.* 1997; Philips *et al.* 1988; Vedder and Harlan. 1988). Phosphorylation of these integrins after translocation on to the plasma membrane, specifically on the cytoplasmic part of the α-chain is important in inducing the conformational change which is essential for integrin's interaction with its ligands (Buyon *et al.* 1997).

The activation of integrin and transendothelial migration involves what is known as "inside-out signaling" and "outside-in signaling". The inside-out signaling is initiated by the interaction of chemokine receptors on the leukocyte and the chemokines on the endothelium which results in affinity modulation (Hughes and Pfaff. 1998; Williams. 1999). Chemokines are chemotactic cytokines produced by various cells including neutrophils and endothelial cells (Lustor. 1998). There are mainly 4 classes of chemokines; namely, CXC, CC, CX3C and C depending on the number of amino acids present between the first two cysteine residues present at the amino terminal end of these proteins (Zlotnik and Yoshie. 2000). Amongst these, CXC chemokines are mainly involved in neutrophil recruitment and CC chemokines are associated with monocyte recruitment (Olson and Ley. 2002). There are several chemokines in the CXC category and include interleukin (IL)-8, neutrophil activating peptide-2, GRO-α (Growth related oncogene/melanoma growth stimulating activity; MIP2/KC), GRO-β (MIP-2α), GRO-γ (MIP-2β), Neutrophil activating peptide-78 and platelet factor-4 (Olson and Ley. 2002). Corresponding to these chemokines there are several receptors named from CXCR1- CXCR6 (Rollins. 1997).

The interaction of chemokines with their receptors initiate "inside-out signaling" through G-proteins coupled to cytoplasmic tail of the receptors (Hughes and Pfaff. 1998; Murdoch and Finn. 2000). G-proteins in turn activate phospholipase-C which results in formation of secondary messengers such as phosphatidylinositol 1,4,5-triphosphate and diacyl-glycerol. These secondary messengers activate Protein Kinase C. In addition, several proteins that interact with the cytoplasmic tails of the integrins have been identified. Despite these findings, the exact mechanism by which these molecules bring about affinity modulation of integrins is poorly understood (Hughes and Pfaff. 1998). Nevertheless, it is accepted that the inside-out signaling brings about conformational change and clustering of integrins (Calderwood *et al.* 2000). This favors the interaction of integrins (for example β_2) with ligands such as intercellular adhesion molecule-1 (ICAM-1), ICAM-2, ICAM-3 and junctional adhesion molecule (JAM) present on

the endothelial cells, initiating the "outside in signaling" process. This results in cytoskeletal rearrangements and shape changes, which help neutrophil transmigration (van Buul and Hordijk. 2004; Wittchen *et al.* 2005). Apart from this, it also leads to increased gene expression for pro-inflammatory molecules such as IL-8, which augments the process of neutrophil recruitment (Walzog *et al.* 1999).

1.2.2.4. Role of β_2 integrins in pulmonary vasculature

As apposed to systemic circulation, migration of neutrophils in the pulmonary vasculature is of two types. One is integrin β_2-dependent and the other is integrin β_2-independent (Doerschuk *et al.* 2000; Doerschuk *et al.* 1990; Folkesson and Matthay. 1997; Yamamoto *et al.* 1998). Results of various studies involving different stimuli indicate that the pathway involved is a stimulus and organ specific phenomenon. Monoclonal antibodies against integrin β_2 resulted in defective lung migration of neutrophils in response to endotoxin but not to *S. pneumoniae* and hydrochloric acid (Doerschuk *et al.* 1990). However, the same stimuli induced neutrophil migration in integrin β_2-dependent manner in the abdominal wall of the rabbits (Doerschuk *et al.* 1990). Similarly, Ramamoorthy *et al*. observed integrin β_2-dependent and -independent migration of neutrophils in *E. coli* and *Staphylococcus aureus* induced pneumonia respectively, in rabbits (Ramamoorthy *et al.* 1997). Within the integrin β_2-dependent pathway, $\alpha_m\beta_2$, compared to other β_2-integrins play a major role in neutrophil migration into the lung (Moreland *et al.* 2002). Several other stimuli are known to induce integrin β_2- dependent and -independent neutrophil migration in the lung (Doerschuk *et al.* 1990; Folkesson and Matthay. 1997; Hellewell *et al.* 1994; Kumasaka *et al.* 1996) and are tabulated in Table 1.1.

In addition to the above observations, various other factors affect the type of neutrophil migration in the lung. In an *in vitro* experiment using human pulmonary arterial endothelial cells, Mackarel *et al.* have shown that the neutrophil migration to formyl-methionyl-leucyl-phenylalanine (fMLP) is integrin β_2-dependent, whereas it is integrin β_2-independent for IL-8 and leukotriene-B4 (Mackarel *et al.* 2000). Similarly, in response to KC, a murine homolog of human IL-8, mice showed integrin β_2-independent neutrophil migration in the lung (Ridger *et al.* 2001). In an experiment involving intraperitoneal administration of *E. coli* bacteria in mice, neutrophil migration involves both integrin β_2-dependent and –independent pathways in a time dependent manner (Gao *et al.* 2001); When β_2 integrin specific antibodies were used, Gao *et al.* observed a

75% reduction in neutrophil migration into the lungs at 1 hour post infection, while it was only 50% by 3-6 hours. In another study in rabbits, neutrophil migration in *Pseudomonas aeruginosa* induced pneumonia was integrin β_2-dependent on the first exposure and was predominantly integrin β_2-independent, when pneumonia was induced seven days later at the same site (Kumasaka *et al.* 1996). These experiments indicate that apart from the stimulus, the type of neutrophil migration is also dependent on the time and chemoattractant inducing the response.

In addition to the above complexities contradictory observations do exist, both *in vitro* (Moreland *et al.* 2004) and *in vivo* (Burns *et al.* 2001; Ong *et al.* 2003), indicating a β_2-independent mechanism in response to *E. coli* or *E. coli*-LPS. In an *in vitro* experiment *E. coli* initiated predominantly integrin β_2-independent neutrophil migration across pulmonary microvascular endothelium (Moreland *et al.* 2004). In mice lung, neutrophils infiltrate in a β_2-independent manner in response to *E. coli* when Neutrophil Inhibitory Factor, a specific inhibitor of integrin β_2, was made to express in the pulmonary vasculature using a liposome based gene delivery system (Ong *et al.* 2003). Similarly, neutrophil migration caused by *E. coli*-LPS infection was not blocked by anti-integrin β_2 antibody (Burns *et al.* 2001). Despite these few reports the existence of integrin β_2-independent pathway in lungs in response to certain stimuli is well accepted and the integrin involved in the integrin β_2-independent pathway is not known (Burns *et al.* 2003; Wagner and Roth. 2000).

1.2.2.5. Integrins with β_1 subunit

In an attempt to unravel the molecule involved in the integrin β_2-independent pathway several integrin molecules have been evaluated in the context of lung inflammation. Several integrins containing β_1 subunit such as $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$and $\alpha_9\beta_1$ have been evaluated as these integrins are expressed on the neutrophils (Bohnsack. 1992; Bohnsack *et al.* 1990; Bohnsack *et al.* 1995; Gao and Issekutz. 1997; Issekutz *et al.* 1996; Kubes *et al.* 1995; Ridger *et al.* 2001; Shang and Issekutz. 1997; Springer. 1990; Taooka *et al.* 1999; Werr *et al.* 2000). In an experiment involving intratracheal instillation of KC, a chemokine known to induce integrin β_2-independent neutrophil migration, the neutrophil recruitment into the lung was reduced in response to antibody treatments against integrin subunits β_1, α_2, α_4, α_5 and α_6 (Ridger *et al.* 2001). However, these molecules blocked the neutrophil migration at the level of extracellular matrix and not at the level of endothelium. In LPS-induced inflammation in which

20-40% of neutrophil migration is integrin β_2-independent (Tasaka *et al.* 2002), blocking α_4 and α_5, the α chains of integrin heterodimers $\alpha_4\beta_1$ and $\alpha_5\beta_1$, alone had no effect on the neutrophil migration (Burns *et al.* 2001). Integrin $\alpha_4\beta_1$ also did not have a role in neutrophil migration from the vasculature in *S. pneumoniae* infection (Tasaka *et al.* 2002). These studies were not successful in identifying the molecule involved in integrin β_2-independent pathway. It appears that β_1 integrins strengthen leukocyte adhesion by mediating neutrophil-extracellular matrix interaction and possibly by "inside-out signaling" which has been demonstrated for lymphocytes following ligation with their ligand vascular cellular adhesion molecule (Chan *et al.* 2000; Frieser *et al.* 1996; Lindbom and Werr. 2002; Shang and Issekutz. 1997; Werr *et al.* 2000; Werr *et al.* 1998).

1.2.2.6. Integrins with β_3 subunit

There are only two integrin heterodimeric combinations that are possible with the integrin subunit β_3; 1) $\alpha_v\beta_3$ and 2) $\alpha_{IIb}\beta_3$ (van der Flier and Sonnenberg. 2001). Of these, only $\alpha_v\beta_3$ is expressed on neutrophils while $\alpha_{IIb}\beta_3$ is expressed on platelets. Integrin $\alpha_v\beta_3$ consists of α_v and β_3 subunits which have a molecular weight of 125 kD and 105 kD respectively and both the subunits are essential for the ligand recognition (Buckley *et al.* 1996; Horton. 1997). The subunit α_v can form heterodimers with at least 4 other β subunits, β_1, β_5, β_6 and β_8 while subunit β_3 can form a heterodimer with only α_v on neutrophils, endothelial and other cells (Hynes. 2002; van der Flier and Sonnenberg. 2001). The heterodimer $\alpha_v\beta_3$ on the cell surface is normally present in a bent conformation which represents its inactive state and it changes to an extended conformation upon activation (Beglova *et al.* 2002). Compared to all other integrins, integrin $\alpha_v\beta_3$ is promiscuous in its binding to various plasma and extracellular matrix proteins including vitronectin, fibronectin, laminin, and tumstatin by ligating Arginine-Glycine-Aspartic acid (RGD) residues present on its ligands (Maeshima *et al.* 2002; Stupack and Cheresh. 2002). Interaction of integrin $\alpha_v\beta_3$ with its ligands mediate cell signaling, cell migration, cell proliferation and cell survival (Wilder. 2002).

1.2.2.7. Known functions of integrin $\alpha_v\beta_3$

Angiogenesis

The role of integrin $\alpha_v\beta_3$ in angiogenesis has been studied extensively (Brooks *et al.* 1994a; Brooks *et al.* 1994b; Carmeliet. 2002; Rupp and Little. 2001; Tsou and Isik. 2001). Its interaction with its ligand vitronectin increases the expression of various growth factor receptors

such as vascular endothelial growth factor receptor-1, fibroblast growth factor receptor-1 and -2 on the endothelium (Tsou and Isik. 2001). Engagement of integrin $\alpha_v\beta_3$ results in increased expression and activation of vascular endothelial growth factor-2 to promote angiogenesis and inhibition of this interaction blocks angiogenesis (Carmeliet. 2002). Similarly, the vascular lumen formation can be inhibited *in vitro* by using anti- $\alpha_v\beta_3$ antibodies in a 3 dimensional fibrin matrix (Bayless *et al.* 2000). Interaction of integrin $\alpha_v\beta_3$ with extracellular matrix decreases expression of membrane-type 1 matrix metalloproteinase (MT-1 MMP). MT-1 MMP processes pro-MMP-2 to active MMP-2 and is important in degradation of extracellular matrix, which promotes angiogenesis (Yan *et al.* 2000). Blocking the integrin $\alpha_v\beta_3$ using antibodies decreases the expression of MT-1 MMP and prevent angiogenesis (Yan *et al.* 2000). Thus, there is clear evidence for the role of integrin $\alpha_v\beta_3$ in angiogenesis and several of $\alpha_v\beta_3$ antagonists are in clinical trials for their use in preventing angiogenesis in cancer patients (Carmeliet and Jain. 2000; McNeel *et al.* 2005).

Regulation of endothelial cell survival and apoptosis

Integrin $\alpha_v\beta_3$ is known to regulate survival and apoptosis of cells in both adhesion-dependent and –independent manner through a variety of mechanisms (Brassard *et al.* 1999; Brooks *et al.* 1994b). Blocking integrin $\alpha_v\beta_3$ on endothelial cells of chick chorioallantoic membrane using antibodies induce apoptosis of endothelial cells by increasing expression of p53, a protein which regulates the cell cycle progression. On the contrary, when the integrin is made to ligate with stabilized ligand *in vitro,* it decreases expression of p53 protein and increases bcl/bax ratio by increasing expression of bcl protein (Stromblad *et al.* 1996). Thus, integrin $\alpha_v\beta_3$ mediates proliferation or apoptosis of endothelial cells by regulating p53 and bcl protein levels in the endothelial cells. In another experiment, function blocking anti-β_3 antibodies prevented osteopontin-induced NF-kB activation and induced apoptosis in rat aortic endothelial cells (Scatena *et al.* 1998). This describes a pathway through which ligation of $\alpha_v\beta_3$ regulates NF-κB activation to control apoptosis and survival of endothelial cells. Unligated integrins recruit caspase-8, an initiator caspase involved in apoptosis, to mediate apoptosis of endothelial cells (Stupack *et al.* 2001). Human brain endothelial cells undergo apoptosis if integrin $\alpha_v\beta_3$ is inhibited by blocking peptides which activates acid sphingomyelinase and increases production of ceramide (Erdreich-Epstein *et al.* 2005). Although all these angiogenic mechanisms are well characterized, many of them have been questioned in the light of observations made in integrin β_3

knockout mice (Hynes. 2002; Reynolds *et al.* 2002). These mice show increase- as apposed to expected reduction, in the angiogenesis process in the absence of integrin $\alpha_v\beta_3$'s ligation with its ligands (Reynolds *et al.* 2002). Since subunit β_3 can form heterodimer with only α_v on neutrophils and endothelial cells, it indirectly relates to the function of $\alpha_v\beta_3$ (Hynes. 2002; van der Flier and Sonnenberg. 2001). Absence of transdominant inhibition of other integrins that promote angiogenic process (Bouvard *et al.* 2001) has been proposed as a possible explanation for the increased pathological angiogenesis in mutant mice (Carmeliet. 2002). Whatever be the mechanisms, the involvement of integrin $\alpha_v\beta_3$ in regulating angiogenesis is indisputable.

Endothelial cell signaling and barrier maintenance

Integrin $\alpha_v\beta_3$ is expressed on both luminal and abluminal surfaces of cultured endothelial cells and normal microvasculature in rat lungs (Cheng *et al.* 1991; Conforti *et al.* 1992; Gawaz *et al.* 1997; Singh *et al.* 2000). Microvascular barrier is maintained and regulated by a complex interaction between integrins and extra cellular matrix components involving fibronectin and vitronectin (Wu *et al.* 2001). The luminal integrin $\alpha_v\beta_3$ initiates endothelial cell signaling to increase capillary permeability following ligation with multimeric vitronectin (Bhattacharya *et al.* 1995; Bhattacharya *et al.* 2001; Bhattacharya *et al.* 2000; Tsukada *et al.* 1995). This effect of the integrin involves phosphorylation of various cytoskeletal proteins following activation of tyrosine kinases because permeability increases can be blocked using tyrosine kinase inhibitors (Tsukada *et al.* 1995). It also enhances the production of arachidonate from the endothelial cells through phospholipase A2 activation, which is brought about by either activation of phospholipase-C or by activation of focal adhesion kinase/mitogen activated protein kinase pathways (Bhattacharya *et al.* 2001). These studies show that integrin $\alpha_v\beta_3$ on the luminal side of the endothelial cells play a different role and could be a major molecule in regulation of lung inflammation.

Leukocyte recruitment

Although there is no direct evidence for the role of integrin $\alpha_v\beta_3$ in neutrophil recruitment in inflamed lungs, many observations do suggest such a possibility. First, integrin $\alpha_v\beta_3$ is expressed on neutrophils (Hendey *et al.* 1996; Lawson and Maxfield. 1995; Rainger *et al.* 1999; Sixt *et al.* 2001) and endothelial cells (Cheng *et al.* 1991; Conforti *et al.* 1992; Gawaz *et al.* 1997; Singh *et al.* 2000). Second, its expression on endothelial cells increases in response to proinflammatory stimuli such as IL-1β (Gawaz *et al.* 1997). Third, neutrophils are capable of

binding to various proteins including vitronectin, which is present both in the circulation and the extracellular matrix (Hendey *et al.* 1996; Lawson and Maxfield. 1995; Preissner. 1991; Tsukada *et al.* 1995). Fourth, vitronectin is an acute phase protein and its level in plasma increases under inflammatory conditions (Langlois and Gawryl. 1988; Seiffert *et al.* 1994). Inspite of all these, the role of integrin $\alpha_v\beta_3$ in neutrophil recruitment is not well studied and only a few studies have explored the role of integrin $\alpha_v\beta_3$ in leukocyte recruitment. The importance of integrin $\alpha_v\beta_3$ in leukocyte migration across the endothelium has been shown *in vitro* using monocytes (Weerasinghe *et al.* 1998). Integrin subunit β_3 has been shown to be important for neutrophil migration on the extracellular matrix *in vitro* (Bruyninckx *et al.* 2001) and transmigration of leukocytes from the mesenteric venules in response to fMLP (Thompson *et al.* 2000). All these highlight the possible involvement of integrin $\alpha_v\beta_3$ in neutrophil migration.

1.2.3. Transendothelial migration

Transendothelial migration is a process that follows firm adhesion of neutrophils on the endothelium (Springer. 1994). The transmigration of neutrophils can happen either by a paracellular route, *i.e*., migration through the endothelial junctions, or by a transcellular route in which neutrophils pass through the cytoplasm of an endothelial cells (Britta Engelhardt. 2004; Feng *et al.* 1998; Muller. 2001; Muller *et al.* 1993). Compared to the extensive studies on the rolling and firm adhesion processes in the neutrophil migration, the information on transendothelial migration is limited. Further, there are very few studies which address this issue specifically in lungs (Burns *et al.* 2003; Wagner and Roth. 2000). However, roles of several molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), JAM, CD99 and vascular-endothelial (VE) – cadherins have been studied in various other tissues (Burns *et al.* 2003). The role of each molecule is very complex and is not discussed in this review. Some of the excellent reviews highlight the complexities of molecular interactions and signaling involved in neutrophil transendothelial migration (Britta Engelhardt. 2004; Muller. 2001; Nourshargh and Marelli-Berg. 2005; van Buul and Hordijk. 2004; Wittchen *et al.* 2005).

1.2.4. Other molecules regulating neutrophil recruitment

Apart from the adhesion molecules, other factors such as fibroblasts in the alveolar septum, neutrophil migration across the alveolar epithelium and various signaling molecules contribute to the complexity of neutrophil recruitment in the lung (Andonegui *et al.* 2002; Burns

et al. 2003). In addition to adhesion molecules on the endothelium and neutrophils, understanding the signaling molecules such as toll-like receptor (TLR) 4 involved in the recruitment of neutrophils is also critical for regulating the inflammatory process (Takeda and Akira. 2005). TLR4 belongs to a family of transmembrane receptors that were first described for their involvement in innate immunity in Drosophila (Takeda *et al.* 2003). TLR4 is expressed on variety of cells including neutrophils and endothelial cells and it recognizes bacterial LPS to initiate signaling and augment the inflammatory process (Andonegui *et al.* 2003; Medzhitov *et al.* 1997; Takeda *et al.* 2003). The critical role of TLR4 in regulating inflammation is evident from the experiments in mice that lack TLR4 receptors and hence show a poor response to LPS (Hoshino *et al.* 1999; Poltorak *et al.* 1998). Recently, TLR4 was shown to be important for neutrophil recruitment in the lung (Andonegui *et al.* 2003; Andonegui *et al.* 2002; Hollingsworth *et al.* 2005). Mice lacking TLR4 were resistant to LPS administration and did not show an increase in neutrophil recruitment in the lung (Andonegui *et al.* 2002). Further, Andonegui *et al*. showed that TLR4 on the endothelium, rather than the neutrophil, is critical for the recruitment of neutrophils into the lung (Andonegui *et al.* 2003). However, studies from Dr. David Schwartz's laboratory have contradicted these findings (Hollingsworth *et al.* 2005). They showed that the TLR4 expressed on the neutrophils and not the TLR4 on the endothelium is important for neutrophil recruitment and the inflammatory response in the lung. Although, it is hard to account for the differences between these two studies, for sure they highlight the important role of TLR4 in neutrophil recruitment in the lung. Because of this and the lack of complete information on the expression of TLR4 in the lungs, it is essential to understand the pattern of TLR4 expression in lung inflammation.

1.3. Neutrophil dependent monocyte recruitment in the lungs.

Along with neutrophils, monocytes/macrophages also play a major role in regulating the inflammatory process (Andrews and Sullivan. 2003; Reynolds. 1987; Ryan and Majno. 1977). Compared to extensive list of studies addressing the mechanism and effect of neutrophil recruitment into the lung, there are very few studies on monocyte recruitment (Abraham. 2003; Burns *et al.* 2003; Maus *et al.* 2002a; Wagner and Roth. 2000). This creates a need for studies aimed at understanding the mechanisms of monocyte recruitment in lung inflammation. One of the factors regulating the monocyte recruitment into the lung is neutrophil (Doherty *et al.* 1988; Maus *et al.* 2002b). An elegant study by Doherty and colleagues, by depleting neutrophils in the

rabbit, clearly demonstrated the dependency of monocyte recruitment on neutrophils in the lung (Doherty *et al.* 1988). Another study involving neutrophil depletion in mice reconfirmed the neutrophil dependent monocyte recruitment (Maus *et al.* 2002b). Although, this phenomenon has been known for several years and several studies have tried to address the mechanism(s), the understanding is incomplete (Doherty *et al.* 1990; Maus *et al.* 2002b; Yamamoto *et al.* 1998).

Monocyte chemoattractant protein (MCP)-1 is a chemokine capable of inducing monocyte recruitment into the lung (Maus *et al.* 2002a; Maus *et al.* 2002b). MCP-1, a CC chemokine, is a major chemoattractant for monocytes and is produced by monocytes, epithelium, endothelium, fibroblasts and smooth muscle cells (van Coillie *et al.* 1999). The neutrophil expression of MCP-1 has been documented both *in vitro* and *in vivo* (Burn *et al.* 1994; Ogata *et al.* 1997; Sakanashi *et al.* 1994; Yamashiro *et al.* 1999) and it has been proposed that neutrophil dependent monocyte recruitment could be mediated through MCP-1 (Yamamoto *et al.* 1998). However, there is no direct evidence to confirm neutrophils as a major source of MCP-1 and hence its involvement in the neutrophil dependent monocyte recruitment.

A recent study examining the recruitment of monocytes found an early recruitment of monocytes into the peritoneal cavity of mice, even before the recruitment of neutrophils (Henderson *et al.* 2003). Similar findings have been documented in the past in dermatitis (Issekutz and Issekutz. 1993; Issekutz *et al.* 1981). However, no such observations have been made in lung inflammation. This is because the earlier studies, based on the accepted paradigm that monocytes are recruited in the post-neutrophilic phase (Doherty *et al.* 1988; Fillion *et al.* 2001; Li *et al.* 1998; Maus *et al.* 2002b; Ulich *et al.* 1991), concentrated on the later part of the inflammation to understand the monocyte recruitment (Doherty *et al.* 1988; Li *et al.* 1998; Yamamoto *et al.* 1998). In addition, the understanding of monocyte recruitment comes from the studies using bronchoalveolar lavage of inflamed lungs (Fillion *et al.* 2001; Maus *et al.* 2002b; Ulich *et al.* 1991; Yamamoto *et al.* 1998). These kinds of studies give us only the information on the number of cells in alveolar space and not on the cells present in septum, which can also contribute to the inflammatory process (Elias *et al.* 1985a; Elias *et al.* 1985b; Li *et al.* 1998; Maus *et al.* 2002a). Therefore, a better understanding on the recruitment of monocytes/macrophages in the whole lung is essential.

1.4. Conclusions

From the literature reviewed in this section it is evident that both neutrophils and monocytes are important in lung inflammation. Lung injury resulting from a dysregulated inflammatory process necessitates a better understanding of the recruitment of inflammatory cells. Studies addressing integrin β_2-independent pathway of neutrophil recruitment, the expression of TLR4 in lung inflammation and mechanism of neutrophil-dependent monocyte recruitment will enhance our understanding on lung inflammation.

Integrin β_2-dependent neutrophil migration	Integrin β_2-independent neutrophil migration
E. coli (Ramamoorthy *et al.* 1997)	*Streptococcus pneumoniae* (Doerschuk *et al.* 1990)
E. coli-LPS (Doerschuk *et al.* 1990)	Group B *Streptococcus* (Sherman *et al.* 1992)
Pseudomonas aeruginosa (Kumasaka *et al.* 1996)	Hydrochloric acid (Doerschuk *et al.* 1990; Folkesson and Matthay. 1997)
Phorbol myristate acetate (Doerschuk *et al.* 1990)	Leukotriene-B4 (Mackarel *et al.* 2000)
Immunoglobulin G-immune complex (Mulligan *et al.* 1993)	IL-8 (Mackarel *et al.* 2000)
IL-1 (Hellewell *et al.* 1994)	KC (Ridger *et al.* 2001)
fMLP (Mackarel *et al.* 2000)	*Staphylococcus aureus* (Ramamoorthy *et al.* 1997)
	C5a (Hellewell *et al.* 1994)

Table 1.1. Various stimuli involved in the integrin β_2-dependent and -independent neutrophil migration in the lungs.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1. Hypotheses

1. Integrin $\alpha_v\beta_3$ mediates neutrophil recruitment into the lung in *S. pneumoniae* induced pneumonia.
2. Neutrophils are the major source of MCP-1 and therefore regulate monocyte/macrophage recruitment in acute lung inflammation.

2.2. Objectives

1. To evaluate the role of integrin $\alpha_v\beta_3$ in the recruitment of neutrophils into the lungs in response to *Streptococcus pneumoniae*.
2. To understand the expression of TLR4 in lungs at various time points of inflammation.
3. To understand the pattern of total monocyte/macrophage recruitment at various time points of acute lung inflammation.
4. To understand the mechanisms of neutrophil dependent monocyte recruitment.

The rationale and the questions being asked are depicted in Figure 2.1

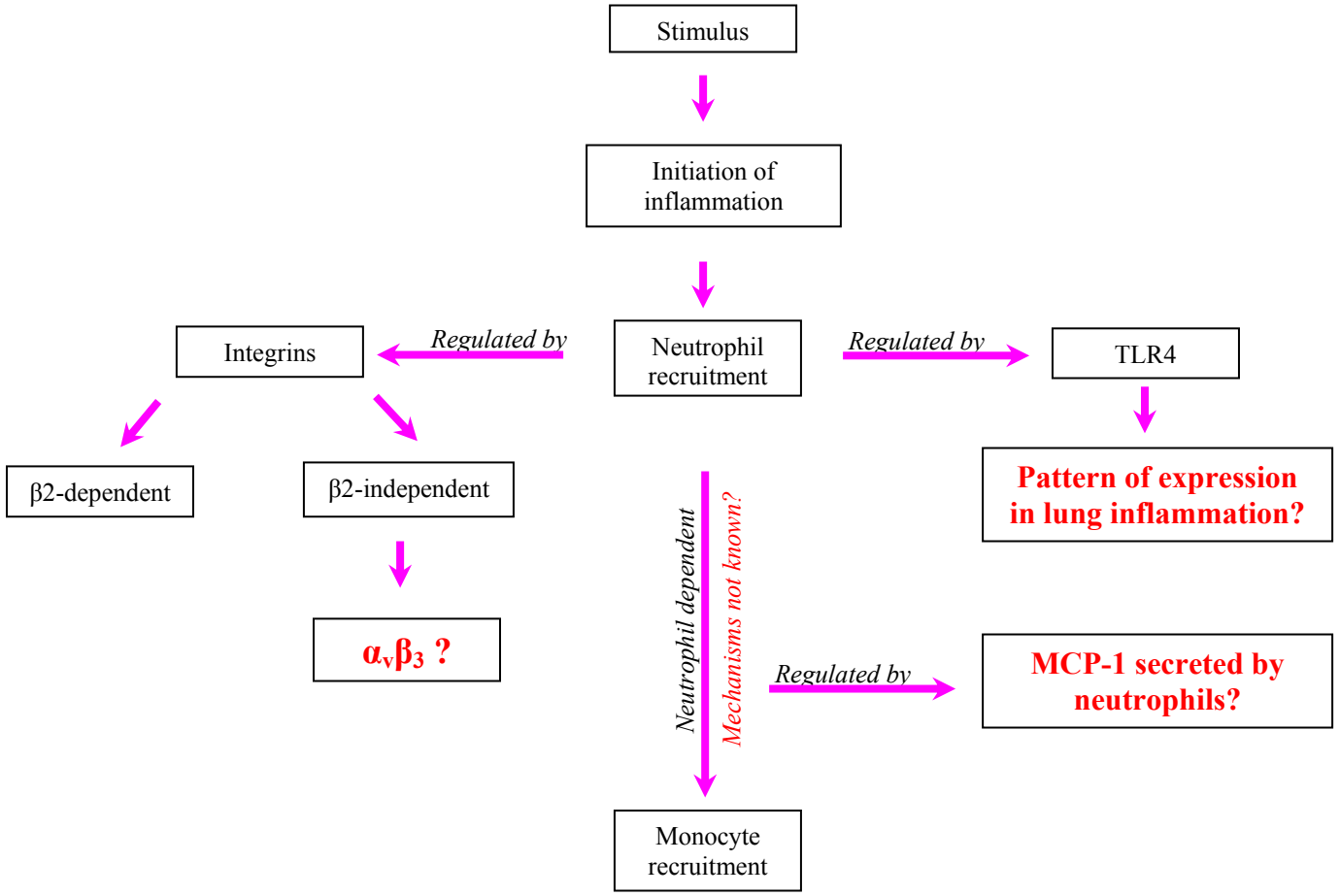

Figure 2. 1. Schematic representation of the rationale and the questions being asked

CHAPTER 3:EXPRESSION OF INTEGRIN SUBUNITS α_v AND β_3 IN ACUTE LUNG INFLAMMATION*

3.1. Abstract

Integrin subunits α_v and β_3 form a dimer $\alpha_v\beta_3$, which is expressed on normal neutrophils and endothelium. I investigated the expression of integrin subunits α_v and β_3 in acute lung inflammation in Sprague-Dawley rats (N = 5 each) following intratracheal challenge with *E. coli* or *S. pneumoniae*, which induce neutrophil recruitment through different mechanisms. Control rats (N = 5) were given endotoxin-free saline. Both bacterial challenges induced similar levels of recruitment of neutrophils in lungs. Western blots showed lower expression of integrin subunits α_v and β_3 in lungs challenged with *E. coli* compared to those given *S. pneumoniae*. Immunohistochemistry and immunogold electron microscopy localized both integrin subunits in neutrophils and endothelium in the control and treated rat lungs. Quantitative immunohistochemistry showed that *E. coli* -challenged rat lungs contained a lower percentage of neutrophils expressing integrin subunits α_v and β_3 compared to those challenged with *S. pneumoniae* ($P< 0.05$). I conclude that *E. coli* infection decreased the percentage of neutrophils expressing integrin subunits α_v and β_3 compared to *S. pneumoniae* infection. These data lay the foundation for further characterization of these integrin subunits in neutrophil migration specifically in *S. pneumoniae* infection that utilizes molecules other than β_2 integrins for neutrophil recruitment.

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"The original publication is available at www.springerlink.com".

3.2. Introduction

In the United States alone, approximately 75 out of every 100,000 people suffer from ARDS with a mortality rate of 50% (Ware and Matthay. 2000). Inflammation leading to diffuse alveolar damage is central to the pathophysiology of ARDS, and exuberant influx of neutrophils contributes to the damage (Weinacker and Vaszar. 2001). Neutrophil migration from the vasculature occurs by a multi-step process that is tightly regulated by the sequential activation of adhesive proteins and their ligands present on both leukocytes and endothelial cells (Hogg and Doerschuk. 1995; von Andrian *et al.* 1991). Neutrophil migration from lung microvessels is mediated mainly by β_2 integrins and partially by molecules other than β_2 integrins in response to *E. coli*, *E. coli*-LPS, *Pseudomonas aeruginosa*, immunoglobulin-G complexes and interleukin-1(Doerschuk *et al.* 1990; Mizgerd *et al.* 1997). However, β_2 integrin-independent pathways predominate in neutrophil migration incited by *S. pneumoniae, Staphylococcus aureus*, and hyperoxia (Doerschuk *et al.* 1990; Mizgerd. 2002). There is an extensive search underway to identify molecules other than β_2 integrins that mediate neutrophil migration in the lung and to date none has been identified (Burns *et al.* 2003). Because neutrophil migration is fundamental to acute lung inflammation, it is critical to identify and precisely understand the functions of molecules that impact the migration of neutrophils.

Integrin $\alpha_v\beta_3$, a receptor for vitronectin, is expressed on the luminal and abluminal surfaces of proliferating endothelial cells in culture and in resting microvessels of rat lungs (Conforti *et al.* 1992; Horton. 1997; Singh *et al.* 2000). The integrin occurs in large blood vessels and airway epithelium of human lungs (Damjanovich *et al.* 1992; Koukoulis *et al.* 1997). Integrin $\alpha_v\beta_3$ also occurs on neutrophils, monocytes and vascular smooth muscle cells(Horton. 1997; Lawson and Maxfield. 1995). The role of this integrin is well established in tumor angiogenesis and cell signaling (Bhattacharya *et al.* 2001; Brooks *et al.* 1994a; Bruyninckx *et al.* 2001). Ligation of luminal integrin $\alpha_v\beta_3$ by vitronectin initiates a signaling cascade and increases microvascular permeability (Bhattacharya *et al.* 1995; Bhattacharya *et al.* 2001; Bhattacharya *et al.* 2000). The integrin promotes monocyte migration and is involved in neutrophil mobility on extracellular matrix substrates *in vitro* and leukocyte migration across the mesenteric endothelium *in vivo* (Bruyninckx *et al.* 2001; Burns *et al.* 2003; Sixt *et al.* 2001; Thompson *et al.* 2000; Weerasinghe *et al.* 1998). Furthermore, neutrophils lacking β_2 integrins migrate normally on extracellular matrix and their migration is inhibited by blocking of β_3

integrin (Sixt *et al.* 2001). These data suggest a role for integrin β_3 in neutrophil mobility and recruitment.

There is considerable evidence on the expression and functions of β_2 and β_1 integrins in neutrophil migration in acute lung inflammation (Burns *et al.* 2003; Mizgerd. 2002). However, there are no data on the expression and functions of integrin subunits α_v or β_3 in acute lung inflammation. Because these integrins may play a fundamental role in neutrophil recruitment in inflamed lungs, it is logical and critical to study their expression to eventually determine their functions in neutrophil recruitment. Furthermore, it is also crucial to study the expression of integrin subunits α_v and β_3 in conditions where neutrophil recruitment is independent of β_2 integrins. Therefore, I investigated expression of integrin subunits α_v and β_3 in two different models of bacterial lung inflammation in which neutrophil migration is regulated by different molecular mechanisms.

3.3. Materials and methods

3.3.1. Rats and treatment groups

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and experiments were conducted according to the Canadian Council on Animal Care Guidelines. Fifteen specific pathogen-free, ten-week-old male Sprague-Dawley rats were procured from Charles River Laboratories, Canada. Animals were acclimatized for a period of one week in the animal care unit. Animals were randomly divided into three groups of five animals each.

3.3.2. Antibodies and reagents

Antibodies against integrin subunits α_v and β_3, and actin were purchased from Chemicon Int., USA, and Santa Cruz Biotechnology Inc., USA. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from DAKO Diagnostics Canada Inc., Canada and peroxidase substrate kit was from Vector Laboratories, USA. LR-white resin was purchased from London Resin Company, USA and gold-conjugated secondary antibody was from British BioCell International, UK. Endotoxin-free saline, protease inhibitor cocktail and bovine serum albumin were purchased from Sigma-Scientific, USA. ECL western blotting detection system and

nitrocellulose membrane (Hybond-ECL) were purchased from Amersham Pharmacia Biotech, UK.

3.3.3. Acute lung inflammation

Rats were anesthetized by intraperitoneal administration of xylazine (20 mg/kg) and ketamine (100 mg/kg). Trachea was exposed surgically and 300µL of endotoxin-free saline or *E. coli* (2 X 10^8 CFU) or *S. pneumoniae* (5 X 10^7 CFU) was instilled intratracheally. Animals were allowed to recover and euthanized 9 hours post-treatments.

3.3.4. Tissue collection and processing

From predetermined sites, three pieces from each lung were collected for light microscopy. Tissues for western blots were snap frozen using liquid nitrogen and stored at -80ºC until further use. Lung pieces for histology and immunohistology were fixed in 4% paraformaldehyde for 16 hours. These pieces were processed through ascending grades of alcohol and embedded in paraffin. Five µm sections were cut from all the 6 tissue blocks from each animal. Lung samples for immunoelectron microscopy were fixed in 2% paraformaldehyde containing 0.1% glutaraldehyde for 3 hours at 4°C. These samples were dehydrated in ascending grades of alcohol and embedded in LR-white resin followed by polymerization under UV light at -1^{o}C for 48 hours.

3.3.5. Western blots

Frozen lung samples were homogenized in lysis buffer [150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 5 mM EDTA and protease inhibitor cocktail (100 µl/10 ml)]. Homogenates were collected after centrifuging the samples at 25,000 *g* for 20 minutes. Equal amounts of proteins were resolved on 10% SDS-PAGE, transferred to a nitrocellulose membrane and blocked with 5% skim milk powder in PBS with 0.1% Tween20. The membrane was incubated with α_v (1:3,000) or β_3 (1:200) antibodies and appropriate HRP-conjugated secondary antibodies (1:30,000 or 1:50,000) followed by incubation with an enhanced chemiluminescence western blotting detection reagent. Incubation with normal serum was the negative control while probing for actin was the loading control. Spot densitometry was performed using Alpha Imager^{TM} (Alpha innotech corp., USA) and the results were interpreted using the average integrated density value.

3.3.6. Immunohistology

Tissue sections were deparaffinized in xylene and rehydrated in descending grades of alcohol followed by treatment with 5% hydrogen peroxide to quench endogenous peroxidase. Sections were treated with pepsin (2 mg/ml in 0.01N hydrochloric acid) for 45 minutes to unmask the antigens and with 1% bovine serum albumin to block non-specific binding. Sections were incubated with primary antibodies against α_v (1:100) or β_3 (1:100) followed by appropriate HRP-conjugated secondary antibodies (1:100). The antigen-antibody complex was visualized using a color development kit. Controls consisted of staining without primary antibody or with normal serum instead of primary antibody.

3.3.7. Quantification of immunohistology data

I wanted to precisely determine percentage of neutrophils positive for integrin subunits α_v or β_3 in inflamed lungs. Therefore, I used an objective lens graticule that covered an area of 0.0064 mm^2, to first count neutrophils in sections stained with hematoxylin and eosin. The counts were made in 10 randomly selected high-power fields (100×) in each section covering a total area of 0.064 mm^2/section (3 sections/animal; 30 fields/animal = total lung area 0.192 mm^2). Separate counts of neutrophils made in alveolar septa and alveolar space were added to obtain the total numbers. Total neutrophil counts were followed by numeration of neutrophils positive for integrin subunits α_v or β_3 in alveolar septa, alveolar space and the total. Neutrophil counts positive for the integrins were converted into percentages with the following equation. Percentage of α_v or β_3 positive neutrophils = average number of neutrophils positive for α_v or β_3 / average number of neutrophils in the hematoxylin and eosin stained section × 100.

3.3.8. Immunogold electron microscopy

Ultrathin sections (80-100 nm) were incubated with 1% bovine serum albumin to block non-specific sites. This was followed by incubations with α_v or β_3 (1:50) antibodies for 60 minutes and appropriate 15 nm gold-conjugated secondary antibodies (1:100; for 30 minutes). Sections were stained with uranyl acetate and lead citrate and examined in Philips 410LS transmission electron microscope. Control sections were labeled without primary antibody or with normal serum instead of primary antibody.

3.3.9. Statistical analyses

All values are presented as mean±SE. Analyses were performed using a statistical package (SPSS, release 10.05, SPSS Inc.). In *E. coli* and *S. pneumoniae*-treated groups, differences between the total neutrophil numbers and the percentage of neutrophils expressing integrins (in alveolar space and total) were tested using independent-samples *t*-test. The differences between the percentages of neutrophils expressing integrins, in the alveolar septa of the *E. coli* and *S. pneumoniae*-treated and control animals were compared using one-way analysis of variance. Fisher's LSD was used for post-hoc comparisons. Statistical significance was accepted at $p<0.05$.

3.4. Results

3.4.1. Acute lung inflammation

Lung sections from saline-treated rats had no histological signs of acute lung inflammation (Figure 3.1.A). However, the sections from lungs of rats challenged with either *E. coli* or *S. pneumoniae* showed edema, congestion, hemorrhage and infiltration of neutrophils into the alveolar spaces (Figures 3.1.B-C). Numerical counts of neutrophils in lung sections showed no differences between rats challenged with either *E. coli* or *S. pneumoniae* (Figure 3.1.D; P=0.067) to suggest similar degree of inflammation.

3.4.2. Expression of integrin α_v and β_3

Western blots on lung homogenates detected integrin subunits α_v and β_3 in all the groups. Densitometry showed that protein expression for the integrin subunits in *E. coli*-treated lung homogenates was lower compared to the control and *S. pneumoniae*-treated rats (Figures 3.2.A-C).

Immunohistochemistry showed similar patterns of septal staining for integrin subunits α_v (Figures 3.3.A, C and E) and β_3 (Figures 3.3.B, D and F) in lungs of the control and treated rats. However, numbers of neutrophils expressing integrin subunits α_v or β_3 appeared to be more in *S. pneumoniae* compared to *E. coli*-treated rat lungs (Figures 3.3.C and D; 3.3.E and F). These integrins were also localized in peribronchiolar blood vessels, large blood vessels and connective tissues (data not shown). Lung sections incubated with normal serum lacked any staining (Figure 3.3.G). Immunogold electron microscopy showed expression of integrin subunits α_v (Figure 3.4.)

and β_3 (data not shown) in the endothelium and the neutrophils but not the alveolar epithelium. The integrin subunits were localized on the membrane, cytoplasm and nucleus of neutrophils and endothelial cells.

3.4.3. Quantification of neutrophils stained for integrin α_v and β_3

Since control animals had no neutrophils in the alveolar space, these were excluded from group comparisons of neutrophils in the alveolar space and total neutrophils. The percentages of septal neutrophils expressing integrin subunit α_v were not different among the groups (Table 3.1; Figure 3.5.A). However, the percentages of alveolar and total neutrophils positive for integrin α_v were lower in rats given *E. coli* compared to those treated with *S. pneumoniae* (Table 3.1; Figure 3.5.C). Neutrophils positive for integrin subunit β_3, as a percentage of the total and those present in the alveolar septa and alveolar spaces, were lower in rats challenged with *E. coli* compared to those given *S. pneumoniae* (Table 1; Figures 3.5.B and D).

Table 3. 1. Percent neutrophils positive for integrin subunits α_v or β_3 in different groups.

Values are presented as Mean±SE. Since control animals had no neutrophils in the alveolar space, these were excluded from group comparisons of neutrophils in the alveolar space and total neutrophils.

Percent neutrophils positive for integrin subunit α_v	**Control**	***E. coli***	***S. pneumoniae***	**P value**
In the septa	52±14.60	49.90±17.33	85±8.00	> 0.05
In the alveolar space	-	14.469±6.70	51.77±12.50	0.046
Total	-	18.65±7.92	58.50±10.70	0.025
Percent neutrophils positive for integrin subunit β_3				
In the septa	92.44±4.00	42.49±13.01	86.48±9.20	< 0.05
In the alveolar space	-	22.33±4.00	69.99±8.27	0.002
Total	-	24.06±4.79	73.30±7.18	0.001

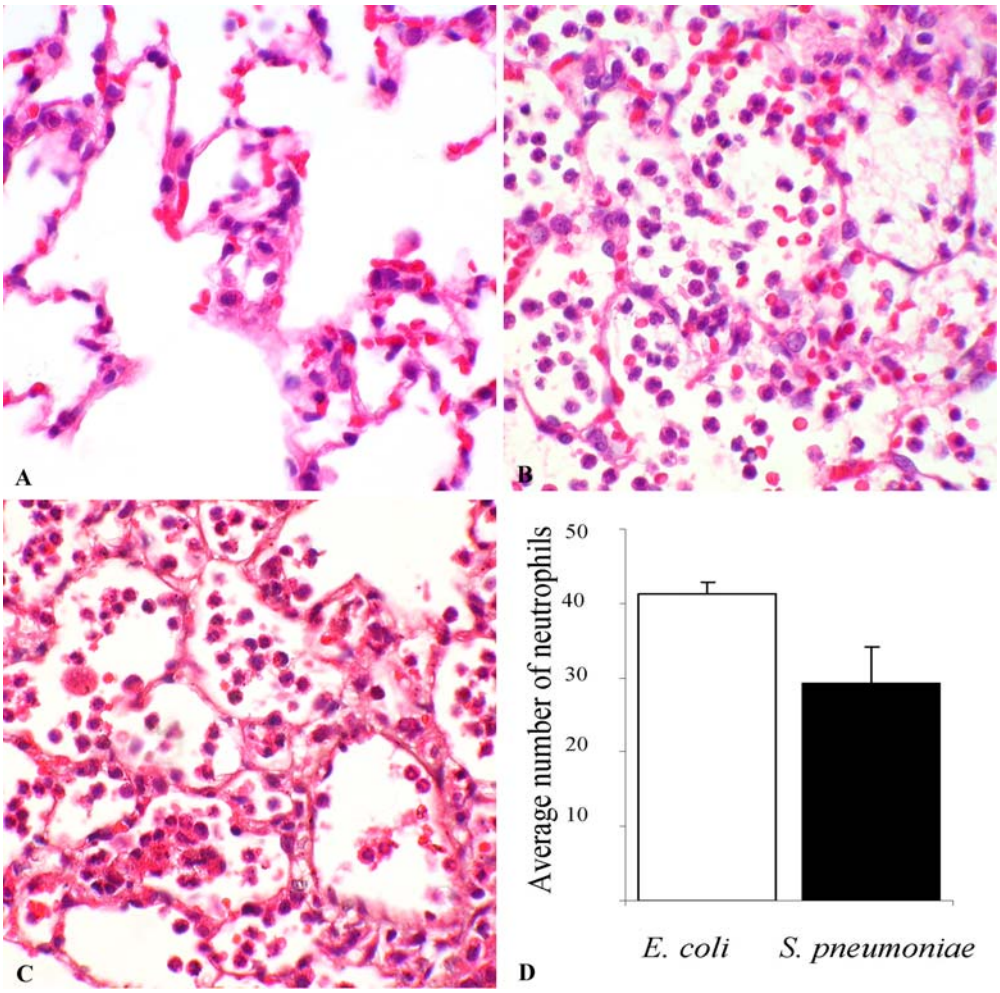

Figure 3. 1. Acute lung inflammation.

Lung sections from a saline treated rat (A), stained with hematoxylin and eosin show no signs of inflammation, while *E. coli* (B) and *S. pneumoniae* (C) treated rats show edema, congestion and neutrophil infiltration into the alveolar spaces. Both the treatments induced similar degree of acute lung inflammation as shown by the similar neutrophil recruitment (D; P=0.067). Magnification- A-C: ×400.

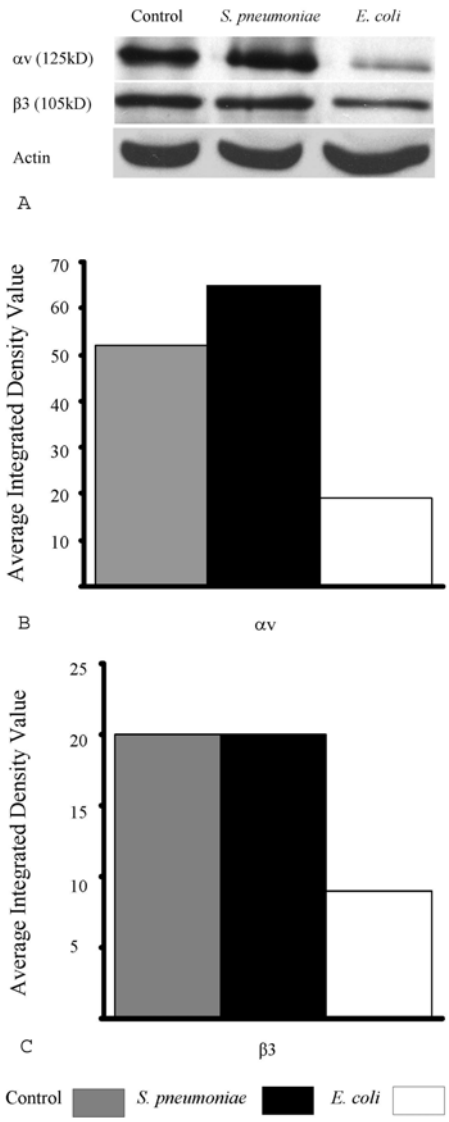

Figure 3. 2. Expression of integrin subunits α_v and β_3.

Western blot assay using lung homogenates show expression of integrin subunits in all the groups (A). Densitometric analysis for integrin subunits α_v (B) and β_3 (C) shows a decreased expression in *E. coli*-treated lungs compared to saline and *S. pneumoniae*- treated lungs. Actin was used as a loading control. Similar results were obtained from another set of rat lungs with similar treatments (two experiments, each in duplicates).

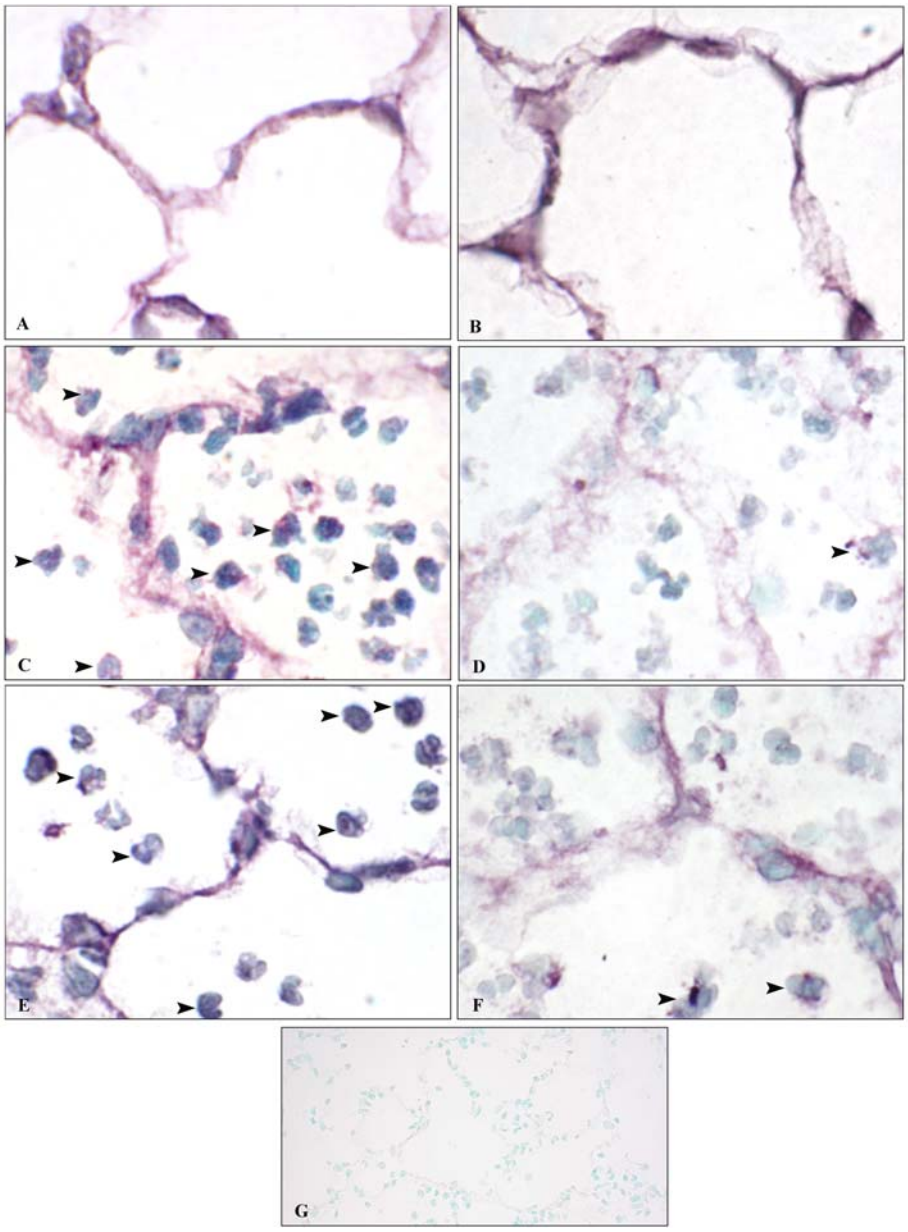

Figure 3. 3. Immunohistochemical expression of integrin subunits α_v and β_3.

Similar expression of integrin subunits α_v (A, C & D) and β_3 (B, E & F) is seen in lung sections of rats treated with saline (A & B), *E. coli* (D & F) or *S. pneumoniae* (C & E). The number of neutrophils (arrowheads) expressing these subunits appeared to be less in *E. coli*-treated rat lungs compared to those given *S. pneumoniae*. Lung sections incubated with normal serum in place of primary antibody showed no staining (G). Magnification- A-F: ×1,000; G: ×100.

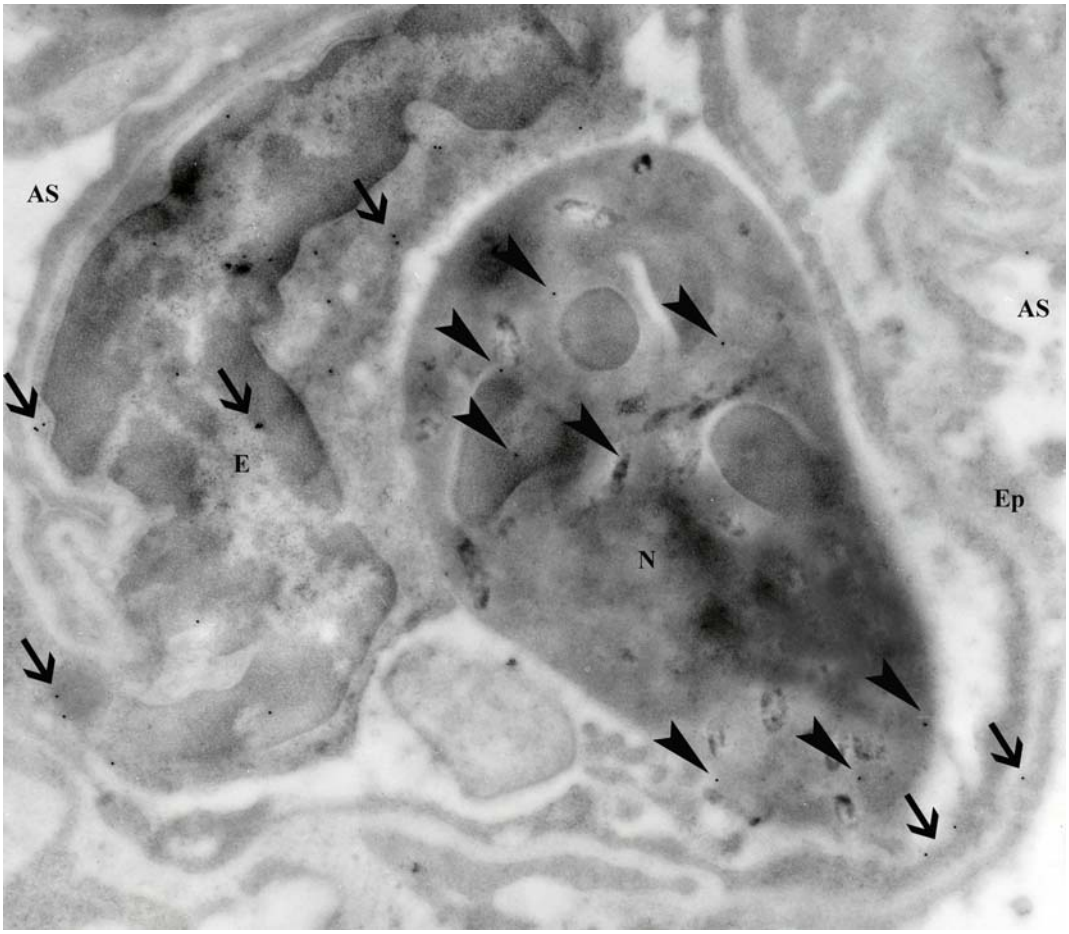

Figure 3. 4. Immunogold electronmicroscopy.

Integrin subunits α_v was localized in neutrophils in control and treated rat lungs. Representative picture from *S. pneumoniae* treated rat lung show gold particles (arrowheads) labeled for integrin subunit α_v on the surface, cytoplasm and nucleus of a neutrophil (N) in a lung microvessel. Endothelium (E) also shows labeling (arrows) for the integrin subunit. Ep: alveolar epithelium; AS: alveolar space; Magnification- × 20,000.

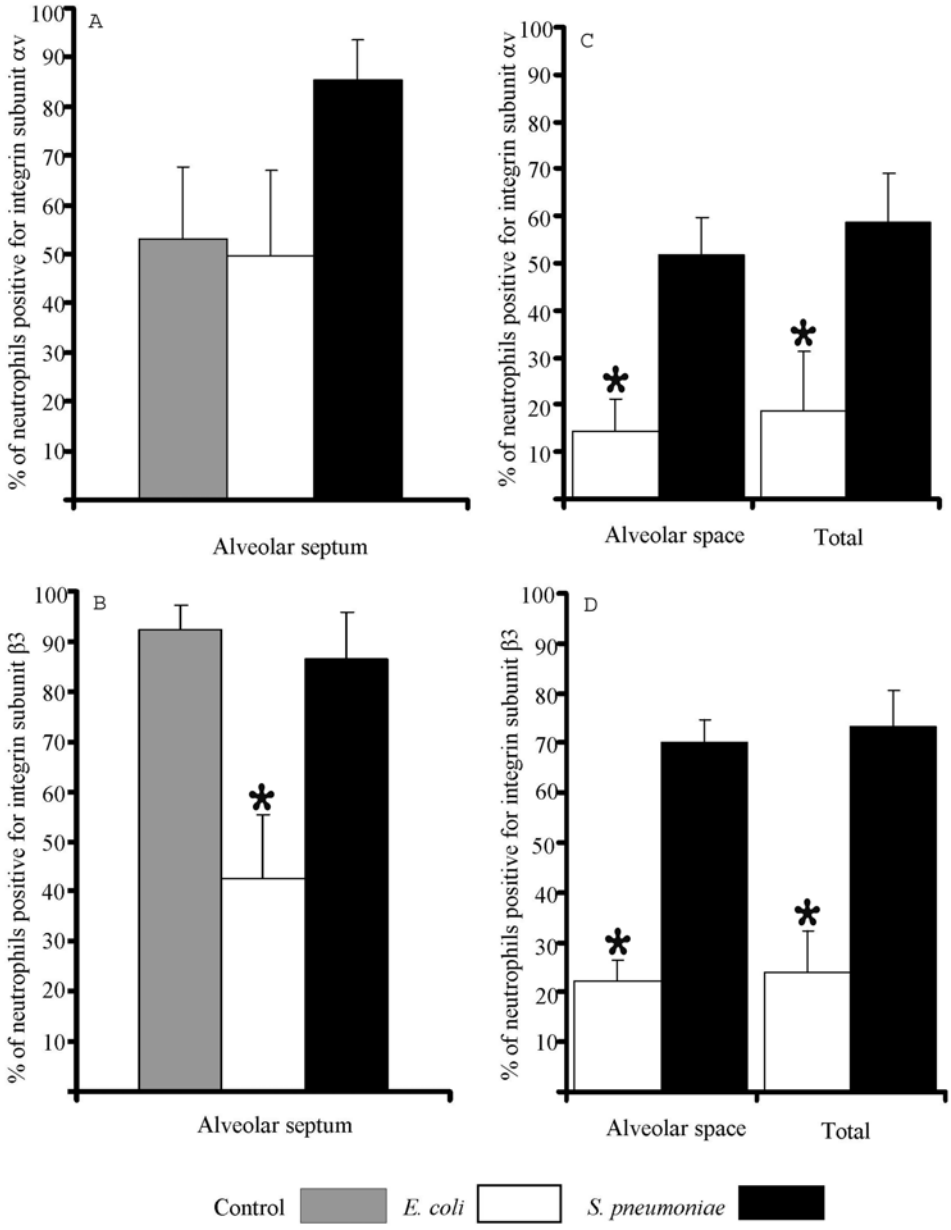

Figure 3. 5. Quantitative immunohistochemistry.

Percentage of neutrophils in the alveolar septa expressing integrin subunit α_v did not differ among the groups (A; $P>0.05$). However, percentages for β_3 were lower ($P< 0.05$) in *E. coli*-treated rats compared to those given saline or *S. pneumoniae* (B). Integrin subunit α_v or β_3-positive neutrophils as a percentage of the total and those present in the alveolar spaces were significantly lower ($P< 0.05$) in *E. coli*-treated rats compared to *S. pneumoniae* challenge (C-D). ∗ Indicates significant difference.

3.5. Discussion

There is considerable information on the expression and functions of β_2 integrins in neutrophils in acute lung inflammation (Mizgerd. 2002). However, neutrophil recruitment into lungs such as that provoked by *S. pneumoniae* is not mediated by β_2 integrins and there are yet no proven candidate molecules to account for it (Mizgerd. 2002). Many investigators have repeatedly stressed the need to evaluate the expression and functions of other adhesion molecules to explain neutrophil recruitment, which is independent of β_2 integrins (Burns *et al.* 2003). Therefore, as an initial attempt to address this question, I investigated the expression of integrin subunits α_v and β_3 in acute lung inflammation caused by *E. coli* or *S. pneumoniae*, which induce neutrophil recruitment into inflamed lungs through diverse mechanisms. Although integrin subunits α_v and β_3 and the $\alpha_v\beta_3$ heterodimer are constitutively expressed in normal human and rat lungs (Damjanovich *et al.* 1992; Singh *et al.* 2000), no such data exist for inflamed lungs.

Integrin subunits α_v or β_3 can form many distinct heterodimers such as $\alpha_v\beta1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ (van der Flier and Sonnenberg. 2001). Integrin $\alpha_v\beta_3$, however, is the only functional heterodimer of α_v and β_3 subunits, which is known to be expressed in neutrophils (Berton and Lowell. 1999). Moreover, studies on individual integrin subunits in addition to the heterodimers have yielded very useful information on their biology and cell signaling (Bruyninckx *et al.* 2001; Thompson *et al.* 2000). Therefore, I chose to investigate the expression of integrin subunits α_v and β_3, instead of their heterodimer $\alpha_v\beta_3$, in acute lung inflammation.

The constitutive expression of integrin subunits α_v and β_3 on neutrophils and endothelium in the lungs in our experiments is in agreement with data from previous *in vitro* and *in vivo* studies (Conforti *et al.* 1992; Hendey *et al.* 1996; Lawson and Maxfield. 1995; Rainger *et al.* 1999; Singh *et al.* 2000; Tsukada *et al.* 1995; Zanetti *et al.* 1994). In this study, immunohistology and immunogold electron microscopy showed both the integrin subunits α_v and β_3 in neutrophils and endothelial cells in control and inflamed lungs. It is interesting that neutrophils and endothelium showed considerable and novel nuclear labeling for both the integrin subunits. Although similar labeling was reported for β_2 integrins (Miller *et al.* 1987), the functions of nuclear integrins was not discussed and still remain unknown. However, a more interesting finding was the lower percentage of neutrophils expressing integrin subunits α_v or β_3 in *E. coli*-treated rat lungs compared to the lungs from *S. pneumoniae*-treated rats. These

numerical differences in neutrophils expressing the integrin subunits assume significance because total numbers of recruited neutrophils were similar in both the groups. This unique finding of differential expression of integrin subunits α_v or β_3 may have functional significance in neutrophil recruitment promoted by different bacteria through molecular tactics other than β_2 integrins.

The mechanisms that induce differential regulation of integrin subunits α_v and β_3 by *E. coli* and *S. pneumoniae* are neither known nor addressed by our experiments. One of the possible mechanisms of modulation of integrin expression is the cross-talk between various integrins. There is evidence that activation of β_2 integrins on lymphocytes decreases activity of $\alpha_4\beta_1$ integrins (Porter and Hogg. 1997). Similarly, the integrin β_1 increases decay of β_3 mRNA to inhibit expression of integrin subunit β_3 (Retta *et al.* 2001). It is well established that *E. coli* provokes neutrophil recruitment into inflamed lungs mainly via activation and changes in β_2 integrin avidity for its ligands (Mizgerd. 2002). Therefore, robust activation of β_2 integrins by *E. coli* may inhibit expression of integrin subunits α_v and β_3. On the other hand, sustained expression of α_v and β_3 subunits on neutrophils in rats challenged with *S. pneumoniae* may be important for their recruitment to sites of inflammation via β_2 integrin independent mechanisms.

The functions of integrin subunits α_v and β_3 in neutrophil migration in inflamed lungs remain unknown. However, differential neutrophil expression of integrin subunits in inflamed lungs and data from other organs suggest a functional role for these integrin subunits in neutrophil sequestration in the lungs. Blockade of integrin subunit β_3 inhibits fMLP-induced leukocyte migration from the mesenteric microvessels (Thompson *et al.* 2000). Monocytes that lack β_3 integrin subunit migrate defectively across an endothelial monolayer *in vitro* (Weerasinghe *et al.* 1998). Integrin $\alpha_v\beta_3$ may influence neutrophil migration by engaging multimeric vitronectin, which is one of its receptors and an acute phase protein (Seiffert and Smith. 1997; Zanetti *et al.* 1994). Vitronectin levels, including those associated with complement complexes, are increased in acute inflammation seen in various conditions such as ARDS (Langlois and Gawryl. 1988; Seiffert. 1997). Multimeric vitronectin may bridge the integrin $\alpha_v\beta_3$ expressed on vascular endothelium and neutrophils, as I observed for the subunits in inflamed lungs, to induce firm adhesion between them. While functional evaluation of integrin

subunits in neutrophil migration is awaited, I speculate that integrin subunits α_v and β_3 facilitate neutrophil recruitment in lung inflammation specifically that induced by *S. pneumoniae*.

In conclusion, the data show that integrin subunits α_v and β_3 are expressed on neutrophils and endothelium and their expression is differentially regulated in acute lung inflammation induced by *E. coli* or *S. pneumoniae*. The findings are significant because these bacteria elicit neutrophil recruitment into inflamed lungs through different molecular pathways. Further studies are needed to investigate functional significance of persistent expression of integrin subunits α_v and β_3 in neutrophils in acute lung inflammation induced by *S. pneumoniae*. Of equal importance is to examine the mechanisms by which *E. coli* reduces expression of these integrin subunits in neutrophils.

CHAPTER 4: INTEGRIN SUBUNIT β_3 IN NEUTROPHIL RECRUITMENT IN PNEUMOCOCCAL PNEUMONIA

4.1. Abstract

Streptococcus pneumoniae is one of the most common causes of bacterial pneumonias in humans. Neutrophil migration into *S. pneumoniae* infected lungs is central to host defense. But the mechanisms of *S. pneumoniae* mediated neutrophil recruitment into lungs are not completely understood. Therefore, I assessed the role of an adhesion molecule, integrin $\alpha_v\beta_3$, by evaluating the integrin's subunit β_3 in a mouse model of *S. pneumoniae* induced lung inflammation. Integrin subunit β_3 knockout ($\beta_3^{-/-}$) mice and the wild type (WT) mice were intratracheally instilled with either 50µl of *S. pneumoniae* (ATCC®6303; n=6/group) or saline (n= 4-7/group). Another group of WT mice were treated intraperitoneally with 25 µg or 50 µg of monoclonal antibody against integrin subunit β_3 (n=5) or with an isotype matched antibody (n=5), 1 hour before instillation of *S. pneumoniae*. Mice were euthanized 24 hours after the treatments. Flow cytometry confirmed absence and presence of integrin subunit β_3 on peripheral blood neutrophils in the $\beta_3^{-/-}$ and WT mice, respectively. Bronchoalveolar lavage (BAL) from $\beta_3^{-/-}$ and WT mice infected with *S. pneumoniae* showed no difference in the number of recruited neutrophils. The number of neutrophils in BAL was less in β_3-antibody+ *S. pneumoniae* mice compared to no-antibody+ *S. pneumoniae* mice. However, a similar effect was also evident in isotype-antibody+ *S. pneumoniae* mice. Further, there was no difference between the isotype-antibody+ *S. pneumoniae* mice and β_3-antibody+ *S. pneumoniae* mice. Therefore, I conclude that integrin $\alpha_v\beta_3$ is not critical for neutrophil migration into the *S. pneumoniae* infected and inflamed lungs.

4.2. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a major pathogen responsible for bacterial pneumonia in adults and children, and world wide over one million children suffer from *S. pneumoniae* induced pneumonia (Kadioglu and Andrew. 2004). The recruitment of neutrophils into the lung is critical for host defense in bacterial pneumonia (Rijneveld *et al.* 2005). Neutrophil recruitment from the blood vessel is a multi-step process involving rolling, firm adhesion and transmigration (Springer. 1994). The firm adhesion of neutrophils onto the endothelium, an essential step for transmigration, is mediated by integrins (Hynes. 1992). Integrins are heterodimeric proteins, consisting of α and β subunits and are involved in cell-cell and cell-matrix interactions (Hynes. 1987). While the process of neutrophil migration in the systemic vasculature is relatively well characterized, our knowledge on neutrophil migration in pulmonary microvasculature is incomplete (Burns *et al.* 2003; Wagner and Roth. 2000). In lung microvasculature, neutrophil recruitment into the alveolar space induced by gram negative bacteria such as *Escherichia coli* is integrin β_2-dependent, and the neutrophil recruitment induced by gram positive bacteria such as *S. pneumoniae* is integrin β_2-independent (Doerschuk *et al.* 2000). The molecule(s) mediating the integrin β_2-independent pathway of neutrophil recruitment in the lung are yet to be identified (Burns *et al.* 2003; Mizgerd. 2002).

Integrin $\alpha_v\beta_3$, a heterodimer formed by α_v and β_3 subunits, is expressed on endothelium and neutrophils (Conforti *et al.* 1992; Lawson and Maxfield. 1995; Singh *et al.* 2000). It can bind to various proteins including vitronectin in the circulation and in the extracellular matrix (Preissner. 1991; Tsukada *et al.* 1995). Although, the role of integrin $\alpha_v\beta_3$ in angiogenesis is extensively studied, its role in leukocyte involvement is not completely addressed (Brooks *et al.* 1994a; Hynes. 2002). The importance of integrin $\alpha_v\beta_3$ in monocyte migration across the endothelium has been shown *in vitro* (Weerasinghe *et al.* 1998). Integrin subunit β_3 is implicated in neutrophil migration on the extracellular matrix *in vitro* (Bruyninckx *et al.* 2001) and transmigration of leukocytes from the mesenteric venules in response to fMLP, *in vivo* (Thompson *et al.* 2000). Recently, I have shown that integrin subunits α_v and β_3 expression on neutrophils is reduced in acute lung inflammation induced with *E. coli* but not with *S. pneumoniae* (Janardhan *et al.* 2004). Therefore, it is logical to evaluate integrin $\alpha_v\beta_3$ as an adhesion molecule involved in *S. pneumoniae* induced neutrophil migration in the lungs.

Endothelial and neutrophil integrin subunit α_v can dimerize with many β subunits such as β_1, β_3, β_5, β_6 and β_8, where as integrin subunit β_3 can dimerize only with integrin subunit α_v (Hynes. 2002). Since both integrin subunits α_v and β_3 are essential for the ligand recognition, the specific role of integrin $\alpha_v\beta_3$ in neutrophil migration can be assessed by studying integrin subunit β_3 (10). Therefore I designed an experiment using integrin subunit β_3 knockout ($\beta_3^{-/-}$) mice and function blocking anti-integrin β_3 antibody to evaluate the role of $\alpha_v\beta_3$ in *S. pneumoniae* induced neutrophil recruitment in the lungs.

4.3. Materials and methods

4.3.1. Animal groups

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and experiments were conducted according to the Canadian Council on Animal Care Guidelines. $\beta_3^{-/-}$ mice were procured from the Jackson laboratory (B6;129S2-*Itgb3*^{tm1Hyn}/J; stock number 004669; The Jackson Laboratory, Bar Harbor, USA). B6129SF2/J mice (F2 hybrid of C57BL/6J and 129S1/SvImJ; Stock number 101045), the most appropriate control, were also procured from the Jackson laboratories. Mice were maintained in the animal care unit and were acclimatized at least for a period of one week. The treatment groups are depicted in Table 4.1.

4.3.2. Streptococcus pneumoniae for intratracheal instillation

S. pneumoniae ATCC® 6303 was procured from American Type Culture Collection (ATCC, VA, USA). Freeze dried bacterial culture was resuspended in 15 ml of Brain Heart Infusion (BHI) broth (3 tubes of 5 ml each) and grown for 24 hours at 37°C with 7% CO_2. After 24 hours the growth was mixed with an equal volume of BHI containing 20% glycerol. Aliquots of 1 ml each were prepared and stored at -80°C for future use in the experiments.

Time required for the bacteria to reach the mid logarithmic phase was determined in the initial experiments based on the findings of Branger *et al.* (Branger *et al.* 2004). One ml of frozen aliquot of bacterial culture was thawed and mixed with 9 ml of BHI broth. Bacteria were allowed to grow for 16 hours at 37°C with 7% CO_2. From this, 1 ml of bacterial culture was mixed with several tubes containing 9 ml each of BHI broth and incubated at the above mentioned conditions. Starting with 0 hour, OD at 600 nm (Tasaka *et al.* 2003) was recorded using a

spectrophotometer at regular intervals until no change in the OD was observed. At each time point (0, 2, 3, 4, 5, 6, 8 and 10 hours) serial dilutions of the culture were made and plated on blood agar plates to determine the number of colony forming units. These numbers were plotted against the time to determine the mid logarithmic phase. Based on the findings it was determined that if I mix 1 ml of fresh bacterial culture with 9 ml of BHI broth, it will take 5 hours to reach the mid logarithmic phase.

4.3.3. Induction of pneumococcal pneumonia

Mice were anaesthetized by intraperitoneal administration of xylazine (20 mg/kg) and ketamine (100 mg/kg). The treatments and the number of mice in each group are described in Table 4.1. Trachea was exposed surgically and 50 µl of endotoxin-free saline (Sigma, St.Louis MO, USA) or 50 µl of *S. pneumoniae* ($3.4 \pm 1.1 \times 10^7$ CFU/ml) was instilled intratracheally. An hour before instillation of *S. pneumoniae*, some of the wild type mice were intraperitoneally treated either with 25 µg or 50 µg of isotype matched immunoglobulins (purified hamster IgG) or anti-integrin β_3 monoclonal antibody (purified hamster anti-mouse CD61) purchased from BD-Biosciences, ON, Canada. This antibody has been used previously, both *in vitro* and *in vivo*, to block the function of integrin subunit β_3 (Illera *et al.* 2000; Piali *et al.* 1995; Schultz and Armant. 1995; Song *et al.* 2003). Animals were euthanized at 24 hours post-treatment. In the saline treated wild type mice group, five were euthanized 6 hours after the treatment and 2 were euthanized 24 hour after the treatment. Since there were no differences, in the number of cells in bronchoalveolar lavage, integrin β_3 expression on neutrophils and lung histology, these were considered together.

4.3.4. Flow cytometric analyses of integrin subunit β_3 expression on neutrophils

At the time of euthanizing the mice, blood was collected from the heart into a microtainer tube (BD Biosciences, ON, Canada) containing potassium EDTA. Blood (0.5-1 ml) was centrifuged at 400g for 20 minutes. After discarding the supernatant, 9 ml of ammonium chloride lysis buffer (8.7 g of Ammonium Chloride and 1.211 g of Tris in 1 l of distilled water; pH 7.2) was added and the solution was incubated in a water bath at 37°C for 5-6 minutes followed by centrifugation at 400g for 10 minutes. The cell pellet thus obtained was washed twice and resuspended in buffer [Calcium and magnesium free Dulbecco's PBS (Invitrogen, ON, Canada) containing 1% fetal calf serum and 1% sodium azide]. After determining the number of cells

using a hemocytometer, cells were mixed with either PE-conjugated anti-integrin β_3 (Sixt *et al.* 2001) or isotype matched antibody (BD Biosciences, ON, Canada; 5 $\mu g/10^6$ cells) and incubated on ice, in dark for 30 minutes. After staining, cells were washed twice using the buffer and resuspended in 1 ml of buffer. Cells were analyzed using Epics Elite ESP analyzer (Beckman Coulter). Neutrophils were gated using their side and forward scatter characteristics. From each sample 10,000 cells were analyzed for the expression of integrin β_3.

4.3.5. Bronchoalveolar lavage

To perform bronchoalveolar lavage (BAL), a small catheter was placed into the trachea and was held in the same position during BAL using a cotton thread which also prevented the backflow of BAL fluid. Lungs were lavaged by twice injecting and slowly aspirating the same 1 ml of Dulbecco's PBS containing 0.6 mM EDTA. This procedure was repeated three times and resulted in 2.8-2.9 ml of the BAL fluid. Total number of leukocytes were determined using a hemocytometer and cells were spun onto a slide using a cytocentrifuge (Shandon III). Cells were stained using Wright's stain and differential cell count was performed. In the experiments using blocking antibodies, the person counting the cells was blinded to the groups. The absolute number of neutrophils were calculated using the total number of leukocytes in the BAL.

4.3.6. Tissue collection and processing

After BAL, right lung was tied off at the level of the bronchus. All the lobes on the right side were snap frozen in liquid nitrogen and stored at -80°C for future use. Left lung was infused at 22 cm water pressure with a solution of 2% paraformaldehyde containing 0.1% glutaraldehyde in sodium cacodylate buffer. Lungs were collected *en bloc* into a jar containing the same fixative. After at least 60 minutes, left lung was cut into 3 pieces and fixed in 4% paraformaldehyde at 4°C for 16 hours followed by dehydration and embedding in paraffin.

4.3.7. Gram's staining of lung sections

To confirm the proper instillation of bacteria into the lungs, representative lung sections were stained with Gram's stain. Lung sections were deparaffinized, hydrated and stained with crystal violet for 3 minutes followed by a rinse using warm tap water (on and off for 3-4 times). Then the sections were treated with gram's iodine for 2 minutes and again rinsed with warm tap water. After blotting sections dry, they were differentiated in acetone (2-3 quick dips) until the

purple color ceased to run away and were rinsed in water. Sections were then incubated with Gram's iodine for 2 minutes and with 1% neutral red for 1 minute with a water rinse in between and after. The sections were dehydrated in alcohol and cleared in xylene.

4.3.8. Histopathology and immunohistochemistry

Lung sections were stained with hematoxylin and eosin to evaluate lung histology for induction of inflammation and neutrophil migration. Sections were stained for neutrophils using antibodies MCA771G (1:50) and goat anti-rat HRP-conjugated secondary antibody (1:100; STAR72; Serotec, NC, USA). Immunohistology protocol is explained under Section 3.3.6.

4.3.9. Quantification of septal neutrophils

To determine if there are any differences in the septal neutrophil accumulation, neutrophils in the control, WT- *S. pneumoniae*, 50 μg antibody + *S. pneumoniae* and 50 μg isotype antibody + *S. pneumoniae* mice were quantified using lung sections stained with anti-neutrophil antibody. The person counting the cells was blinded to the groups. Five mice in each group (4 in control) were used for evaluation. From each mouse, neutrophils in the septa were counted in 3 random fields and from 3 sections under 100× objective (0.025 mm^2 area per field; a total of 0.225 mm^2) and were expressed as number of neutrophils /0.025 mm^2.

4.3.10. Statistical analyses

Data were analyzed using Sigma stat software (Sigma stat version 3.1). Two groups were compared using student's *t*- test and more than two groups were compared using one-way analysis of variance. Fisher's LSD was used for post-hoc comparisons. Statistical significance was accepted at $P<0.05$.

4.4. Results

4.4.1. Induction of lung inflammation

Gram's staining revealed gram positive cocci in lungs of infected animals and indicated proper instillation of bacteria (Figure 4.1). Inflammation, as evidenced by leukocytes in BAL, perivascular and septal accumulation of leukocytes, perivascular edema and hemorrhage, was present in the lungs of all *S. pneumoniae* instilled mice (Figure 4.2).

4.4.2. Expression of integrin subunit β_3 on neutrophils

Flow cytometry confirmed the presence and absence of integrin subunit β_3 expression on the neutrophils from WT and $\beta_3^{-/-}$ mice respectively. The integrin expression on neutrophils from WT and $\beta_3^{-/-}$ mice was not altered by *S. pneumoniae* treatment (Figures 4.3A & B).

4.4.3. Neutrophil recruitment in $\beta_3^{-/-}$ mice

The neutrophil recruitment into the lung was evaluated using BAL. There were no differences between the alveolar leukocyte and neutrophil numbers in the WT and $\beta_3^{-/-}$ mice treated with saline (Figures 4.4 A & B). In *S. pneumoniae* infected WT and $\beta_3^{-/-}$ mice there were more neutrophils compared to respective saline controls (P=0.001). However, the leukocyte and neutrophil numbers did not vary between *S. pneumoniae* infected WT and $\beta_3^{-/-}$ mice (Figures 4.4 C & D) indicating recruitment of neutrophils in the absence of integrin subunit β_3.

4.4.4. Neutrophil recruitment after blocking integrin subunit β_3

I further confirmed the findings of $\beta_3^{-/-}$ mice, using a function blocking antibody against the integrin subunit β_3. The antibodies were given one hour before the intratracheal challenge with *S. pneumoniae*. Compared to no antibody treated *S. pneumoniae* infected mice, the number of recruited neutrophils were reduced in β_3-antibody treated mice. However, similar reduction in neutrophil numbers was also observed in the isotype-control antibody treated mice (Figure 4.5).

4.4.5. Total leukocyte numbers in blood

To ensure that the lack of difference in the leukocyte and neutrophil numbers in the lungs of *S. pneumoniae* infected WT and $\beta_3^{-/-}$ mice is not due to alterations in the circulating leukocyte numbers, I determined total leukocyte numbers in the blood. There was no difference in the leukocyte numbers between WT and $\beta_3^{-/-}$ mice either after saline or *S. pneumoniae* treatment (Figures 4.6A & B).

Among antibody treated mice, only 25 µg isotype antibody treated mice had lower number of leukocytes compared to mice given no antibody (Figures 4.6 C & D).

4.4.6. Quantification of neutrophils in the septum

To evaluate the possible mechanism of reduced leukocyte and neutrophil recruitment in the isotype and β_3-antibody treated mice, I quantified the number of neutrophils in the septum.

The number of neutrophils in the septum was significantly more in the no antibody and *S. pneumoniae* treated mice compared to saline treated mice lungs (Figure 4.7). The number of septal neutrophils in both the isotype and anti-β_3 antibody treated *S. pneumoniae* infected mice lungs was significantly lower compared to no antibody treated *S. pneumoniae* infected mice (Figure 4.7).

Table 4. 1. List of treatment groups

Mice	Treatment	'n' number
Wild type	Endotoxin free saline	7
Wild type	*S. pneumoniae*	6
Integrin β_3 knockout	Endotoxin free saline	4
Integrin β_3 knockout	*S. pneumoniae*	6
Wild type	Anti-integrin β_3 antibody (25 µg, intra peritoneal) + *S. pneumoniae*	5
Wild type	Isotype matched control antibody (25 µg, intra peritoneal) + *S. pneumoniae*	6
Wild type	Anti-integrin β_3 antibody (50 µg, intra peritoneal) + *S. pneumoniae*	5
Wild type	Isotype matched control antibody (50 µg, intra peritoneal) + *S. pneumoniae*	5

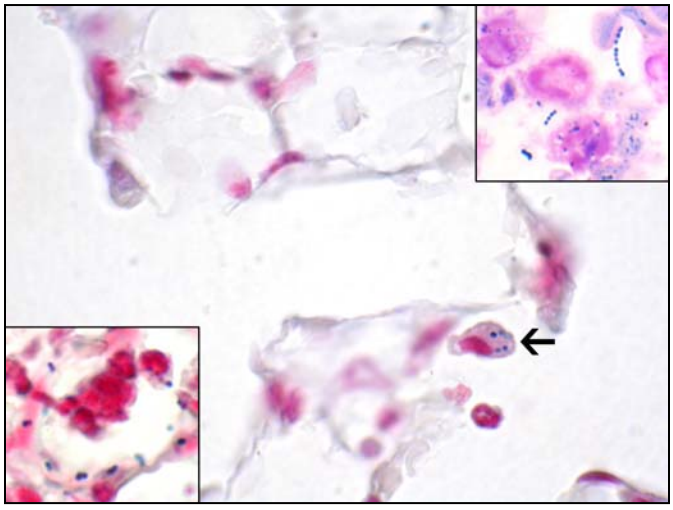

Figure 4. 1. Gram's staining of lung sections.

Lung section from a *S. pneumoniae* treated animal showing Gram positive cocci in an alveolar macrophage (arrow). Most of the bacteria were washed out during the bronchoalveolar lavage and were evident in the Gram stained cytospin preparations (Top inset). Lower inset shows another area of the lung containing many Gram positive organisms. Magnification- ×1,000

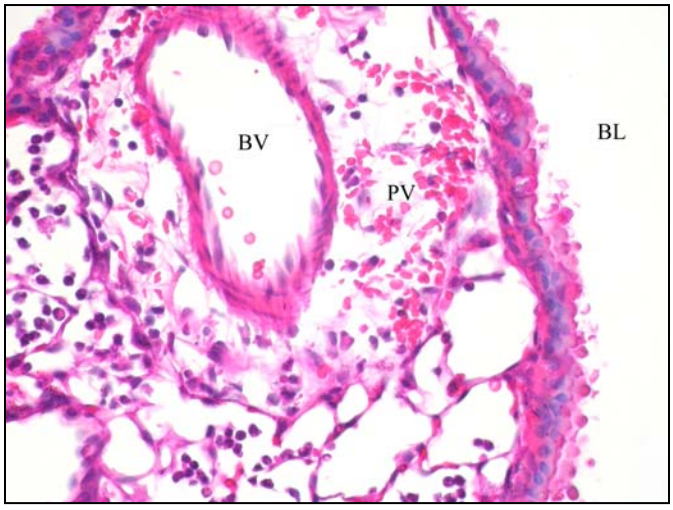

Figure 4. 2. Acute lung inflammation.

Representative, hematoxylin and eosin stained lung section from a *S. pneumoniae* treated mouse showing inflammation in the lung as evidenced by the presence of hemorrhage, edema and neutrophil recruitment in the perivascular area (PV). Neutrophils are also evident in the septum and alveolar space. BV: Blood vessel; BL: Bronchial lumen. Magnification- ×200

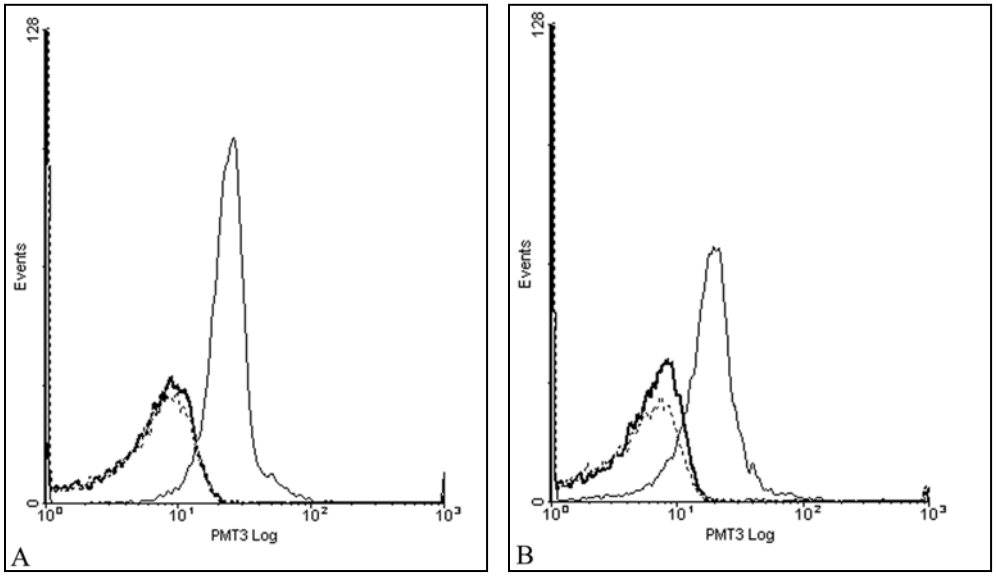

Figure 4. 3. Flow cytometric observations for expression of integrin subunit β_3 on neutrophils.

A: Neutrophils from saline treated (A) and *S. pneumoniae* treated (B), wild type (thin line) and knockout mice (dotted line) were stained with PE-conjugated anti-integrin subunit β3 antibody or an isotype matched antibody (thick line). Wild type mice showed β_3 expression, while knockout mice had no expression and was similar to the expression observed with the isotype matched antibody (thick line). The data presented is representative of observations from 4-5 mice in each group.

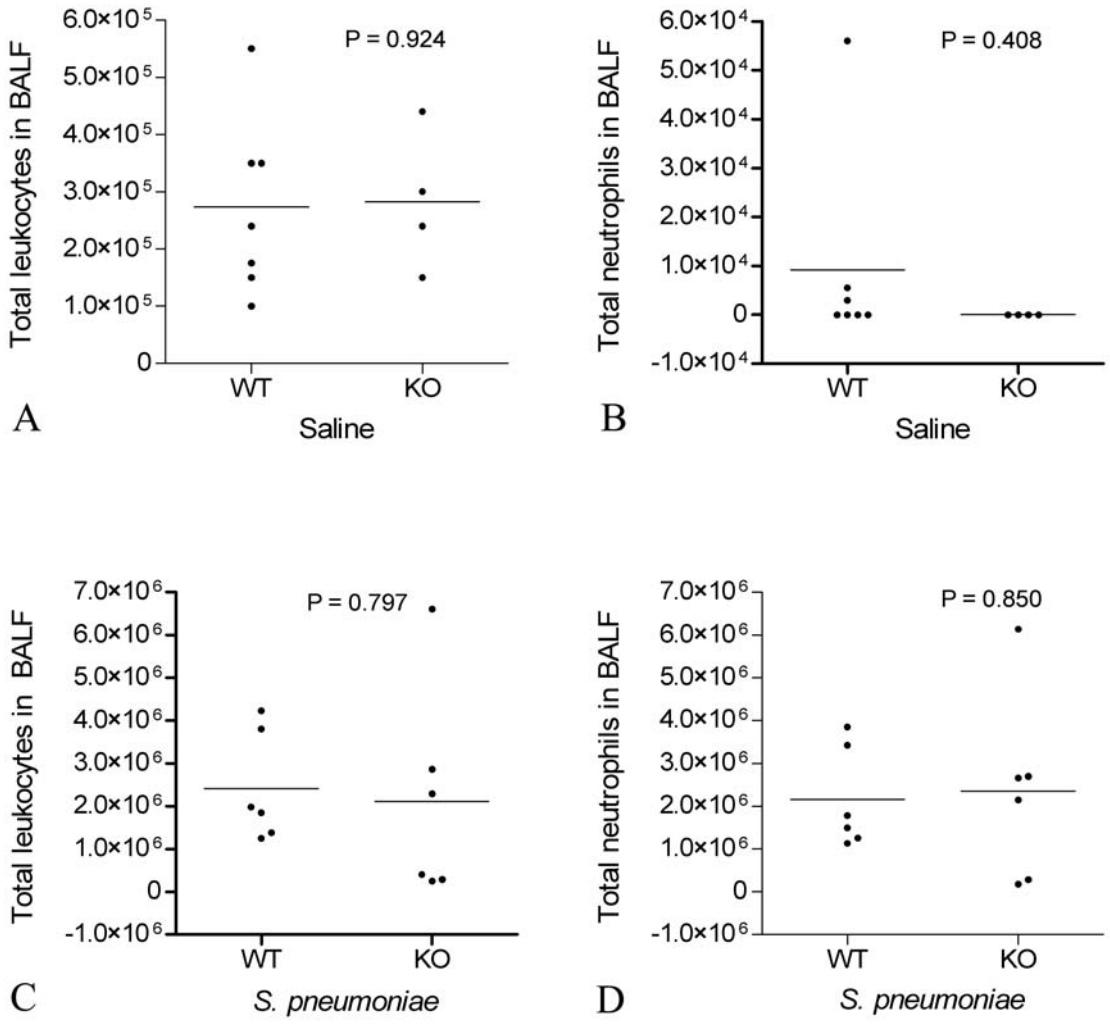

Figure 4. 4. Neutrophil recruitment in $\beta_3^{-/-}$ mice.

Total leukocytes and neutrophils were quantified in the bronchoalveolar lavage fluid (BALF) from wild type (WT) and $\beta_3^{-/-}$ (KO) mice treated with saline (A & B) or *S. pneumoniae* (C & D). There was no difference in the number of leukocytes or neutrophils in the bronchoalveolar lavage fluid of saline treated or *S. pneumoniae* infected mice. In each group, the solid bar represents the mean value.

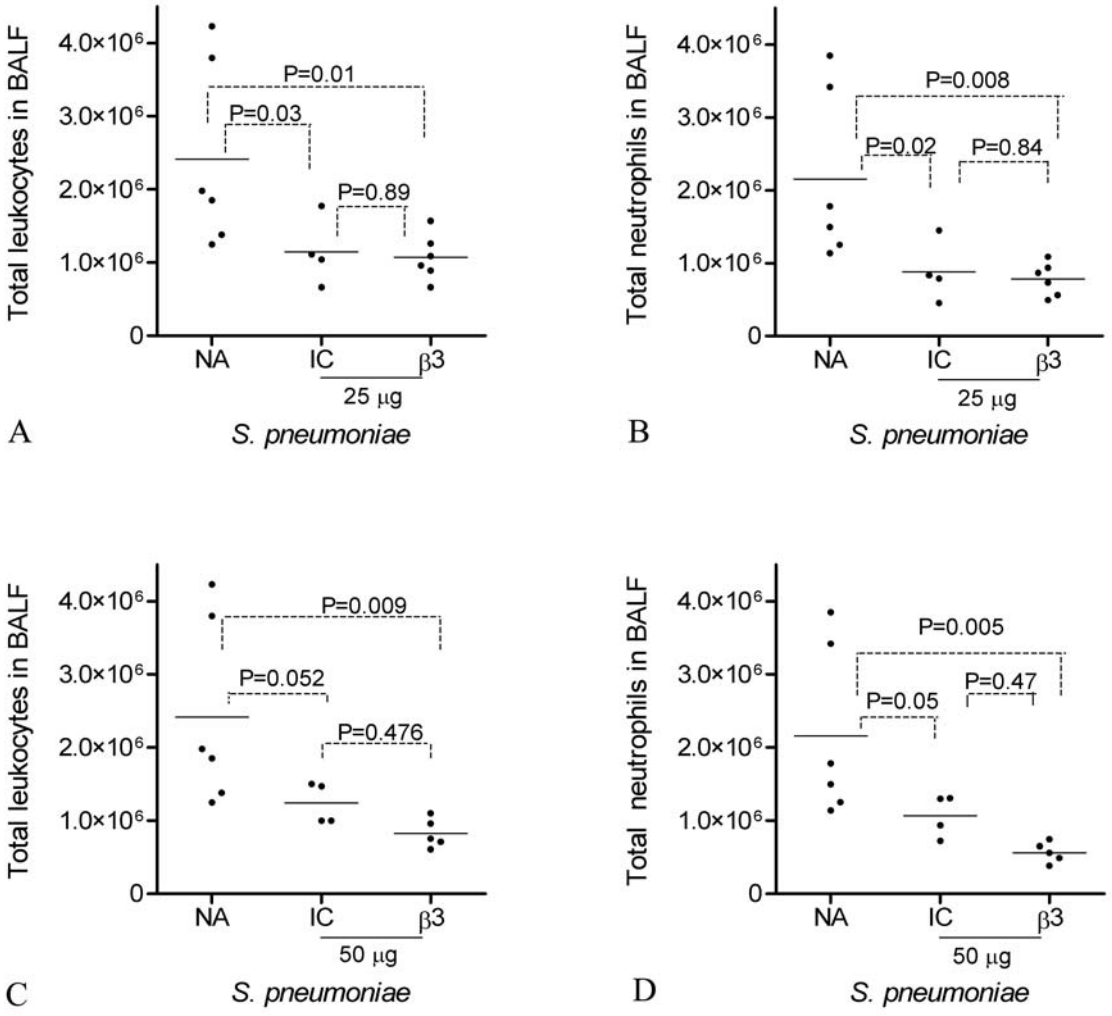

Figure 4. 5. Neutrophil recruitment in mice treated with anti-β_3 antibody.

One hour before intratracheal instillation of *S. pneumoniae*, mice were intraperitoneally treated with 25 or 50 μg of either integrin subunit β_3 antibody (β_3) or isotype matched antibody as a control (IC). Lungs were lavaged 24 hours post-treatment and the total number of leukocytes and neutrophils in the bronchoalveolar lavage fluid (BALF) were quantified. β_3 and IC treated mice showed reduced number of neutrophil recruitment into the lungs compared to *S. pneumoniae* instilled, no antibody (NA) treated mice. Further, there was no difference between the IC and β_3 treated mice. In each group, the solid bar represents the mean value. In 25 and 50 μg IC groups, using a statistical software (Grubb's test; Statagraphics Centurion XV, Version 15.0.10), one observation each was identified as an outlier and was excluded from the analyses.

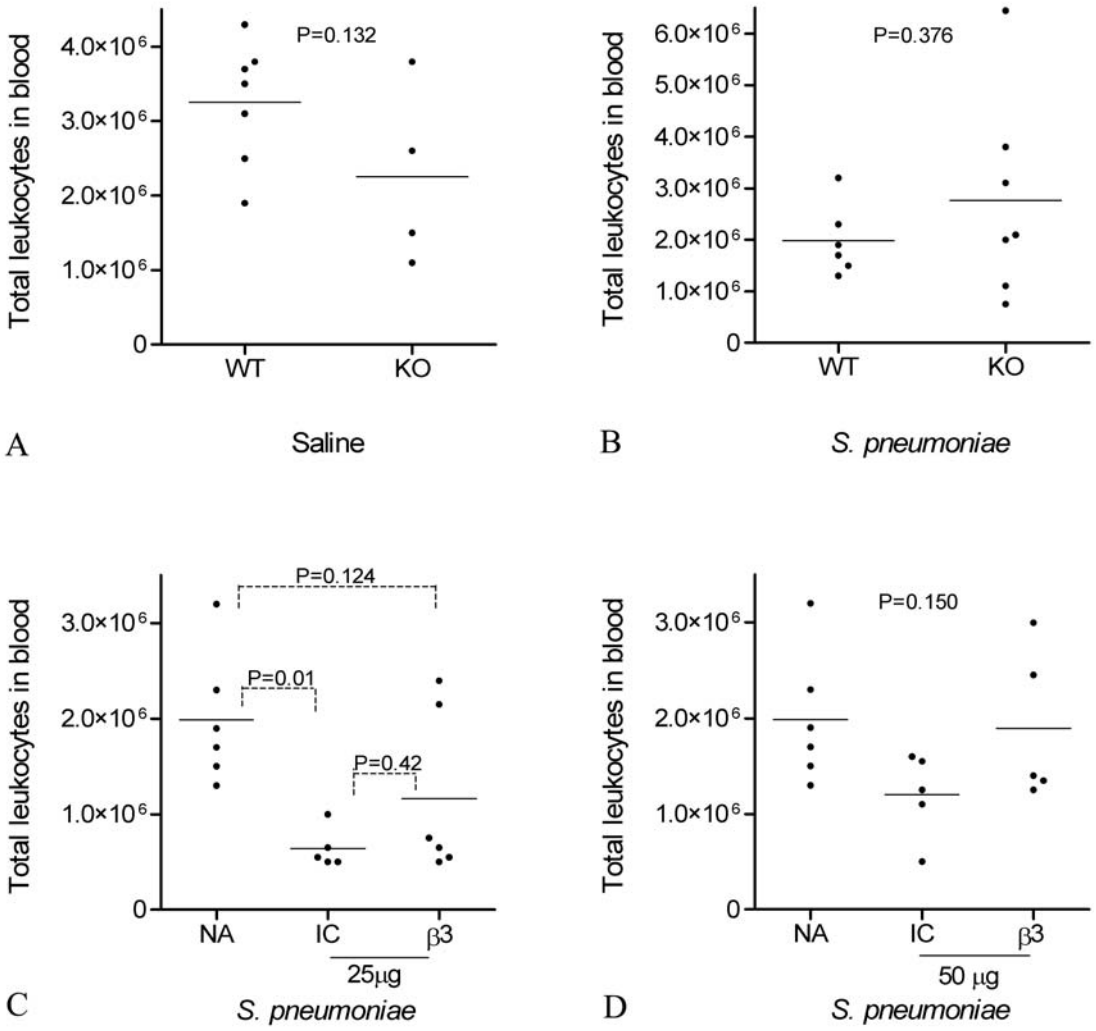

Figure 4. 6. Peripheral blood leukocyte count.

Total leukocyte counts between the wild type (WT) and $\beta_3^{-/-}$ (KO) did not vary either in saline treated (A) or in *S. pneumoniae* treated (B) mice. Among antibody treated mice, only 25 μg isotype antibody treated mice had lower number of leukocytes compared to mice given no antibody (C). NA: no antibody; IC: isotype control; β3: β3 antibody.

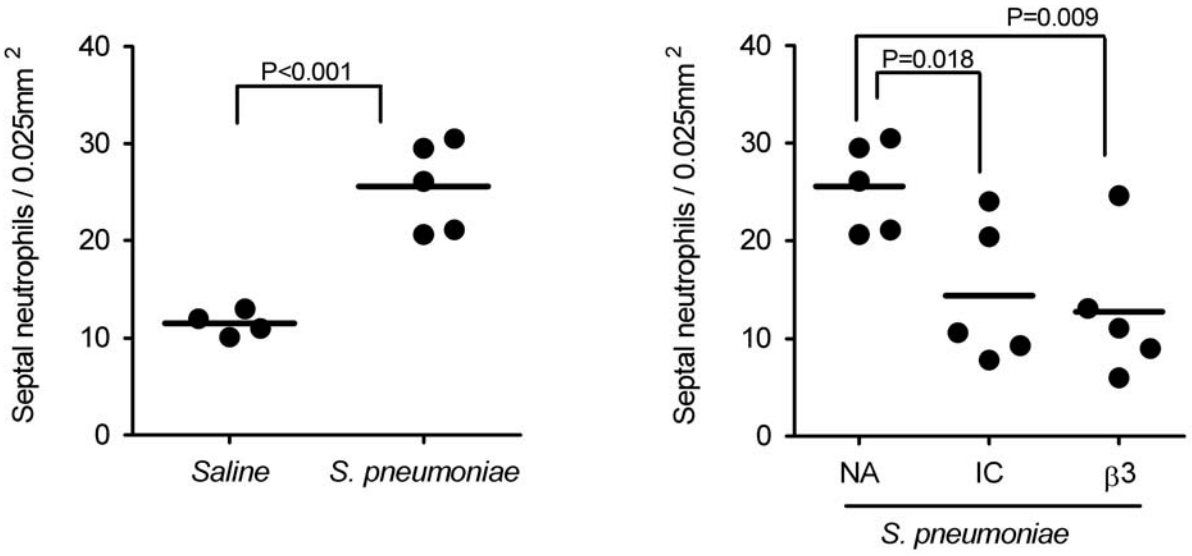

Figure 4. 7. Quantification of neutrophils in the septa.

To understand the mechanism of decreased neutrophil recruitment in antibody treated groups, I quantified neutrophils in the septa of saline, *S. pneumoniae* with no antibody(NA), *S. pneumoniae* with 50 μg isotype antibody (IC) and *S. pneumoniae* with 50 μg β_3 antibody (β3) treated mice. Compared to saline treated mice, there were more neutrophils in the alveolar septa of *S. pneumoniae* treated mice. In response to *S. pneumoniae*, both the isotype and β_3 antibody treated mice showed reduced number of neutrophils in the septa when compared with those given no antibody.

4.5. Discussion

My objective was to evaluate the role of integrin subunit β_3 in neutrophil recruitment in *S. pneumoniae* induced lung inflammation. I addressed this issue using $\beta_3^{-/-}$ mice and a function blocking monoclonal antibody. The data show that the absence of integrin subunit β_3 does not affect neutrophil recruitment in the lungs of mice infected with *S. pneumoniae*.

First, I confirmed the presence and absence of integrin subunit β_3 on neutrophils from the WT and $\beta_3^{-/-}$ mice, respectively, with flow cytometry. The $\beta_3^{-/-}$ mouse has been used by others to understand the role of integrin $\alpha_v\beta_3$ in various pathological conditions such as tumor angiogenesis and osteoporosis (Reynolds *et al.* 2002; Zhao *et al.* 2005). Neutrophils from the $\beta_3^{-/-}$ mice did not express β_3 while neutrophils from WT mice showed β_3 and the expression was unaltered after the challenge with *S. pneumoniae*. This is in accordance with the previous reports of integrin subunit β_3 expression on unstimulated and phorbol myristate acetate stimulated neutrophils (Janardhan *et al.* 2004; Lawson and Maxfield. 1995; Sixt *et al.* 2001).

Next, I evaluated BAL to quantify the neutrophil recruitment into the lung. Compared to saline treated mice, significant recruitment of neutrophils was observed in the lungs of both WT and $\beta_3^{-/-}$ mice infected with *S. pneumoniae,* and there were no differences between these two types of mice. Previous *in vitro* reports on monocyte migration across the endothelium and neutrophil migration in the extracellular matrix, and an *in vivo* report on leukocyte migration across the mesenteric vessels indicated a role for integrin subunit β_3 (Bruyninckx *et al.* 2001; Thompson *et al.* 2000; Weerasinghe *et al.* 1998). The lack of effect of β_3 integrin on neutrophil recruitment in lung inflammation in our experiments could be due to several reasons. One, there could be upregulation of other known and unknown molecules in the absence of integrin β_3 subunit. This process is known as "transdominance" where presence of one integrin could prevent the function and expression of other integrins, and in the absence of that particular integrin the control is lost, leading to expression or function of the suppressed integrin (Blystone *et al.* 1995; Blystone *et al.* 1999; Carmeliet. 2002; Diaz-Gonzalez *et al.* 1996). The best example is the role of integrin $\alpha_v\beta_3$ in angiogenesis. Its ligation using antibodies helps to control pathological angiogenesis. But in $\beta_3^{-/-}$ mice, increased angiogenesis is observed because of lack of transdominance and resulting increased expression of other angiogenic integrins such as $\alpha_v\beta_1$, $\alpha_2\beta_1$ and $\alpha_5\beta_1$ (Hynes. 2002). Second possibility could be that the absence of integrin subunit β_3 could lead to pronounced neutrophilia, which could result in relative increase in the neutrophils

recruited into the lung, as observed in integrin β_2 deficient mice (Mizgerd *et al.* 1997). But this is not the case in my experiments, as absence of integrin subunit β_3 did not alter the number of circulating neutrophils. The third possibility could be that integrin subunit β_3 may be functionally redundant and hence is not critical for the neutrophil recruitment. To further confirm this possibility I did another set of experiments using a function blocking antibody.

The antibody used in this experiment has previously been shown to block the function of integrin subunit β_3 both *in vitro* and *in vivo* (Illera *et al.* 2000; Piali *et al.* 1995; Schultz and Armant. 1995; Song *et al.* 2003) and it did not affect the neutrophil numbers in the blood. I tested two doses of β_3 antibody in our experiments and at both the doses there was a decrease in the number of neutrophils recruited into the lungs of infected mice. However, treatment with isotype antibody also induced similar reduction in neutrophil recruitment and there was no difference in number of recruited neutrophils between the mice treated with integrin β_3 antibody or isotype antibody. This implies that the observed effect is a general effect of treatment with immunoglobulins and not specific to integrin β_3 antibody. The lack of effect of β_3 antibody on neutrophil recruitment is in agreement with data from the $\beta_3^{-/-}$ mice and show that the integrin subunit β_3 is not critical for neutrophil recruitment into the lung in response to *S. pneumoniae*. Thus, my experiments rule out the role of one more integrin in the β_2-independent neutrophil migration but do not reveal the identity of molecule(s) that may mediate integrin β_2-independent neutrophil migration. Probably multiple integrins, rather than one single molecule, are involved in the β_2-independent neutrophil migration and experiments involving blocking of several integrins could help us to solve the puzzle.

The observed reduction in neutrophil recruitment due to immunoglobulin (both isotype and β_3 antibody) treatment was an unexpected finding in our study and is difficult to explain. However, it can be compared to the effects of intravenous immunoglobulin therapy (IVIg) observed in humans and animal models. In humans, IVIg is used to treat various inflammatory and autoimmune conditions and the mechanism is not completely understood (Bayry *et al.* 2003). A recent study in a feline model of ischemia reperfusion showed that intravenous immunoglobulin therapy interferes with selectins and β_2 integrins on the leukocytes to reduce the rolling and adhesion in the mesenteric microvessels (Gill *et al.* 2005). Similarly, interference with the leukocyte-endothelial interactions in our study seems to be the most likely possibility as I observed a reduced number of neutrophils in the septum of mice treated with both β_3 and isotype

antibodies compared to those which were treated with *S. pneumoniae* alone (Figure 4.7). However, there are certain caveats to comparison of our observation with IVIg treatments. First, neither the dose nor the method of preparation of antibodies used in our experiments are comparable to the ones which are used in IVIg treatment (Bayry *et al.* 2003; Gill *et al.* 2005). Immunoglobulin for IVIg treatment is obtained by pooling plasma from 3,000-10,000 healthy donors (Bayry *et al.* 2003) while the antibody used in the experiment is hamster IgGκ, purified from tissue culture supernatant. Second, the selectins and most of the integrins expressed by neutrophils have been shown not to play a role in *S. pneumoniae* induced pneumonia and other stimuli (Doerschuk *et al.* 1990; Mizgerd *et al.* 1996; Ridger *et al.* 2001; Tasaka *et al.* 2002) and hence one has to presume that treatment with immunoglobulins interferes with a yet-to-be identified molecule on neutrophils. Finally, I have no explanation as to why the previous studies using antibodies to understand leukocyte recruitment have failed to show reduced neutrophil recruitment with isotype matched immunoglobulins (Ridger *et al.* 2001; Tasaka *et al.* 2002).

To summarize, the experiments show that integrin subunit β_3, and therefore $\alpha_v\beta_3$, is not required for neutrophil recruitment in pneumococcal pneumonia in mice. Thus, this current study and previous studies rule out the requirement of all known integrins expressed on the neutrophils in their recruitment into the lung in *S. pneumoniae* induced pneumonia. This study also shows that pretreatment with immunoglobulins significantly reduce the neutrophils recruited into the lung in response to *S. pneumoniae* and the mechanisms mediating this need to be addressed.

CHAPTER 5: TOLL-LIKE RECEPTOR 4 EXPRESSION IN LIPOPOLYSACCHARIDE INDUCED LUNG INFLAMMATION*

5.1. Abstract

Bacterial lipopolysaccharides (LPS) initiate immune response through Toll-like receptor 4 (TLR4). More often host is confronted with secondary bacterial challenges; therefore it is critical to understand TLR4 expression following initial provocation. I studied TLR4 expression in rats at various times after intra-tracheal instillation of LPS. Although TLR4 mRNA was undetectable in normal lungs, it increased at 6 hours and 12 hours and declined at 36 hours post-LPS treatment. Western blots showed TLR4 protein at all time points. Immunohistochemistry localized TLR4 in alveolar septal cells, bronchial epithelium, macrophages and endothelium of large and peribronchial blood vessels. Dual label immunoelectron microscopy showed co-localization of TLR4 and LPS in the cytoplasm and nucleus of various lung and inflammatory cells. Nuclear localization of TLR4 was confirmed with Western blots on lung nuclear extracts. I conclude that TLR4 expression in lung is sustained up to 36 hours and that TLR4 and LPS are localized in the cytoplasm and nuclei.

* Histology Histopathology (2006) **21:**687-96.
http://www.hh.um.es/2006/HH_21_7_2006.htm
"The original publication is available at www.hh.um.es".

5.2. Introduction

Lung diseases characterized by acute inflammation arise from various causes including Gram-negative bacterial infections (Davidson *et al.* 1999; Matthay *et al.* 2003). The outer cell wall of Gram-negative bacteria contains lipopolysaccharide (LPS), that activates cells such as macrophages and endothelial cells to produce pro-inflammatory mediators, cytokines and chemokines leading to expression of adhesion molecules and recruitment of inflammatory cells (Aderem. 2001; Andonegui *et al.* 2003; Dayer *et al.* 1993). The inflammation that results is a necessary response to protect the body from pathogens.

Inflammatory response to endotoxins is largely mediated through Toll-like receptor 4 (TLR4) (Takeda *et al.* 2003). TLR4 belongs to a transmembrane family of receptors that were first described for their involvement in innate immunity in Drosophila (Takeda *et al.* 2003). Normal cellular expression of TLR4 is well characterized (Andonegui *et al.* 2003; Armstrong *et al.* 2004; Muir *et al.* 2004; Muzio *et al.* 2000; Zarember and Godowski. 2002). However, there is a lack of consensus on the effect of LPS on the expression of TLR4 in various organs including the lung. For example, TLR4 expression on the surface of peritoneal macrophages was decreased within a few hours of LPS stimulation and remained suppressed for more than 24 hours even though mRNA expression returned to normal by 24 hours (Nomura *et al.* 2000). In contrast, LPS exposures increased TLR4 expression in human monocytes and polymorphonuclear leukocytes (Muzio *et al.* 2000). Recently, I reported a reduced immunohistochemical expression of TLR4 on epithelium and large blood vessels, but not macrophages, in lungs of calves infected with *Mannheimia hemolytica* (Wassef *et al.* 2004). Otte and colleagues found that even though TLR4 mRNA and protein levels were unaffected in LPS exposed intestinal epithelial cells, the cell surface expression of TLR4 was significantly decreased suggesting internalization at 24 hours post-LPS treatment (Otte *et al.* 2004). Moreover, there is conflicting evidence of unaltered expression of TLR4 protein in normal or chronically inflamed intestinal epithelial cells (Abreu *et al.* 2001; Cario *et al.* 2000; Hausmann *et al.* 2002). These studies highlight the controversy regarding TLR4 expression in inflamed organs including the lung.

Since TLR4 expression is central to host's ability to respond to bacterial challenges, it is important to understand the impact of an initial challenge on subsequent expression of TLR4. Therefore, I determined the expression of TLR4 mRNA and protein at various times following a single intratracheal challenge with *E. coli* LPS. Our data show that LPS treatment induces TLR4

mRNA followed by its return to minimal levels while the protein expression is sustained up to 36 hours with few cell specific variations. I also show co-localization of TLR4 and LPS in cytoplasm and nucleus of various cells in the treated lungs and believe it to be the first report of nuclear localization of TLR4.

5.3. Materials and methods:

5.3.1. Animal groups

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and experiments were conducted according to the Canadian Council on Animal Care Guidelines. Specific pathogen-free, ten-week-old, male Sprague-Dawley rats were procured from Charles River laboratories, Canada. Rats were maintained in the animal care unit and were acclimatized for a period of one week. Rats were randomly divided into five groups (N = 5 each).

5.3.2. Acute lung inflammation

Rats were anaesthetized by intraperitoneal administration of xylazine (20 mg/kg) and ketamine (100 mg/kg). Trachea was exposed surgically and endotoxin-free saline (Sigma, St.Louis MO, USA) or *E. coli* LPS (250 μg; serotype 0128:B12; Sigma, St.Louis MO, USA) was instilled intratracheally. Animals were euthanized at 6, 12, 18, and 36 hours (n = 5 each) post-treatment. Control animals (n = 5) were euthanized at 6 hours post saline treatment.

5.3.3. Tissue collection and processing

Tissues for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blots were snap frozen and stored at -80ºC. Processing of lung pieces for histology and immunohistology and immunoelectron microscopy is explained under Section 3.3.4.

5.3.4. Preparation of whole lung homogenates and nuclear fractionation

Lung homogenization is explained in Section 3.3.5. Nuclear fractionation of lung was carried out using a previously described method (Spector *et al.* 1998) with slight modifications. Lung (0.3 g) was cut into small pieces and homogenized using a lysis buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 3 mM magnesium chloride, 2 mM PMSF, 2 mM benzamidine and 1 μM leupeptin. The homogenate was passed through glass wool to get rid

of the cell debris. The filtrate without cell debris was overlaid on the lysis buffer containing 1.5 M sucrose and spun for 15 minutes at 35,000 rpm in Beckman L8-55 ultracentrifuge using Type-45 T1 rotor. Even though, method described by Spector et al., (Spector *et al.* 1998) suggests using 2 M sucrose for rat liver nuclei, I was not able to precipitate nuclei either at 2 M or at 1.75 M. The pellet containing nuclei was washed twice with the lysis buffer and resuspended in 300 µl of RIPA lysis buffer.

5.3.5. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen Inc., ON, Canada) following manufacturer's protocol. Using a glass-homogenizer, 30 mg of frozen lung tissue was homogenized in 600 µl of buffer followed by the optimal on-column DNase digestion with the RNase-free DNase to eliminate DNA contamination. TLR4 and GAPDH primers were purchased from Invitrogen (Burlington, ON). For rat TLR4 (458bp) forward primer was - 5'-CATGAAGGCCTCCCTGGTGTT and the reverse primer was 5'-TGCCAGAGCGGCTACTCAGAA. For GADPH (298bp), forward primer was 5'-TGAAGGTCGGTGTGAACGGATTTGG and the reverse primer was 5'-ACGACATACTCAGCACCAGCATCAC. All other reagents were purchased from Fermentas Inc. (Burlington, ON, Canada)

The first step in a two step RT-PCR involved mixing of 5 µl of RNA with 1.5 µl of reverse primer, 4.5 µl of water and incubation at 70ºC for 10 minutes followed by addition of 9 µl of reaction mixture (5 µl of 5X-revert aid buffer, 0.5 µl 10 mM dNTP, 1.5 µl water, 1 µl RNase out and 1 µl Revert Aid enzyme). The samples were held at 42ºC for 30 minutes followed by incubation at 85ºC for 5 minutes. Second step involved amplification of 2 µl of RT product in a PCR mixture (34.25 µl water, 5 µl 10X-PCR buffer, 4 µl of $MgCl_2$, 0.5 µl of 25 mM dNTP mixture, 2 µl each of 25 pmol forward and reverse primers and 0.25 µl *Taq* DNA polymerase). After the initial denaturation at 94ºC for 3 minutes, 40 cycles were carried out as follows (TLR4: 94ºC for 30 sec, 57ºC for 30 sec and 72ºC for 45 sec; GAPDH: 94ºC for 1minute, 65ºC for 2 minutes and 72ºC for 3 minutes). This was followed by a final extension step at 72ºC for 10 minutes. One of the controls was direct PCR of lung RNA to rule out the DNA contamination. Second control was substitution of RNA extract with water.

The RT-PCR products from three rats from each treatment group were electrophoresed on a 1.5% agarose gel and were stained with ethidium bromide. Images were captured using Alpha

Imager^{TM} (Alpha innotech corp., USA). Spot densitometry was performed and the results were interpreted using the average pixel value. The values were normalized to the expression of GAPDH and are presented as ratio of TLR4:GAPDH.

5.3.6. Western blots

Equal amounts of protein from whole lung homogenate and nuclear extracts were resolved on 12% Precise protein gels (Biolynx Inc. Ontario, Canada) using SDS-PAGE. The method is described in Section 3.3.5. The blots were probed with anti- rat TLR4 (1:100), -actin (1:250), -Lamin (1:200) and -goat horseradish peroxidase (HRP)-conjugated secondary antibody (1: 7500), which were purchased from Santa Cruz Biotechnology Inc., USA. Use of isotype matched goat immunoglobulins instead of primary antibodies served as a negative control.

5.3.7. Histopathology

Lung sections were stained with hematoxylin and eosin to evaluate lung histology for induction of inflammation.

5.3.8. Immunohistochemistry

Immunohistology protocol is described in Section 3.3.6 and lung sections were stained using, TLR4 (1:50), *E. coli* LPS (1:300, Cedarlane Laboratories Limited, ON, Canada) and anti-goat HRP-conjugated antibodies (1:100; DAKO Diagnostics Canada Inc., ON, Canada).

5.3.9. Immunoelectron microscopy

Thin sections (80-100 nm) were incubated with 1% bovine serum albumin to block non-specific antigen sites. This was followed by incubations with primary (TLR4; 1:25; 60 minutes) antibodies and anti-goat 15 nm gold-conjugated secondary antibodies (1:100; for 30 minutes). This procedure was repeated using anti-LPS antibody (1:175) and anti-rabbit 10nm gold-conjugated antibodies (1:100; for 30 minutes) on the same grids followed by staining with uranyl acetate and lead citrate. The sections were examined in Philips 410LS transmission electron microscope. Controls consisted of using isotype matched immunoglobulins and labeling without primary antibody. The number of gold particles seen on these controls was subtracted while interpreting the results of labeled lung sections. Secondary antibodies used in this experiment were from British Bio Cell International, UK.

5.4. Results

5.4.1. Lung inflammation

Lung sections from control rats instilled intratracheally with endotoxin-free saline showed normal lung histology (Figure 5.1.A) while from those treated with *E. coli* LPS showed perivascular, peribronchiolar and alveolar infiltration of neutrophils and mononuclear phagocytes (Figure 5.1.B). The deposition of LPS into the lung was confirmed using an anti-LPS antibody. The LPS was localized in the epithelium, endothelium, smooth muscle cells and macrophages (Figure 5.1.C).

5.4.2. TLR4 mRNA expression

TLR4 mRNA expression was assessed in lungs from the normal and LPS-challenged rats with semi-quantitative RT-PCR. The absence of DNA was confirmed with direct PCR, without the RT step on RNA extracted from lungs (data not shown). Lungs from control rats lacked mRNA for TLR4 (Figure 5.2). The expression of TLR4 mRNA was increased at 6 hours followed by a further increase at 12 hours post-LPS treatment. However, the mRNA values were reduced by 36 hours post-treatment.

5.4.3. TLR4 protein expression

I examined TLR4 protein expression with Western blots on lung homogenates (Figure 5.3). Lungs from the control and all of the treated rats showed TLR4 protein (Figure 5.3). However, I observed two bands for TLR4; one at ~87 kD and another at ~69 kD. Spot densitometry for TLR4 at ~87 kD showed a decrease in TLR4 at 6 hours followed by an increase at later time points. Spot densitometry for TLR4 at ~69 kD showed unaltered TLR4 expression at 6 hours followed by an increase at later time points compared to the controls.

5.4.4. TLR4 immunohistochemistry

I used immunohistochemistry to precisely identify the cells expressing TLR4. Lung sections stained only with secondary antibody (Figure 5.4A) or isotype-matched antibody (Figure 5.4B) lacked any staining and ruled out non-specific binding of antibodies. Previously, I have used a TLR4 blocking peptide to determine specificity of the TLR4 antibody used in this experiment (Wassef *et al.* 2004). The immunohistochemical data are summarized in Table 5.1.

Lung sections from control rats showed a minimal expression of TLR4 in few of the septal cells and bronchial epithelium; however, alveolar macrophages and endothelium of large and peribronchial blood vessels were intensely positive (Figure 5.4C). Compared to the controls, the 6 hour post-LPS treated lungs showed intense TLR4 staining in the septum and infiltrating neutrophils (Figure 5.4D). Interestingly, the septa were negative for TLR4 while bronchiolar epithelium, endothelium of large blood vessels, macrophages and a few neutrophils were positive at 12 and 36 hours post-LPS treatment (Figure 5.4E).

5.4.5. TLR4 immunoelectron microscopy

I performed dual immunogold labeling for TLR4 and LPS on thin lung sections from the control and LPS-treated rats. Labeling with isotype matched immunoglobulins showed negligible labeling (Figure 5.5). Lung sections from the control and the treated rats contained TLR4 staining in the macrophages (Figure 5.6), Type I and II alveolar epithelial cells, microvascular and macrovascular endothelium, monocytes, neutrophils (Figure 5.7-5.10) and eosinophils (not shown). In both control and LPS-treated lung sections, TLR4 was mainly detected in the cytoplasm and the nucleus. LPS was also predominantly localized in the cytoplasm and nucleus of macrophages, monocytes, neutrophils and endothelium in the treated lungs (Figure 5.6-5.9). Although LPS and TLR4 were colocalized in the cytoplasm and nucleus of endothelial cells in capillaries as well as large blood vessels, Type I epithelial cells, neutrophils, monocytes in the inflamed lungs, colocalization was detected in only a few cells (Figures 5.6-5.9). Saline-treated control lungs showed a negligible labeling for LPS.

5.4.6. TLR4 in lung nuclear extracts

I confirmed immunoelectron microscopic observation on TLR4 localization in nucleus, with Western blots on nuclear extracts. The nuclear extracts were positive for lamin-B, which is a nuclear protein (Figure 5.11). The blots probed with TLR4 antibody revealed two bands, approximately 87 kD and 69 kD, similar to the proteins observed in the crude lung extracts.

Table 5. 1. Expression of TLR4 in various cells of control and LPS treated lungs.

+: minimal; ++: intense; -: not detected.

	Bronchioles	Septum	Endothelium-large blood vessels	Endothelium-peribronchiolar blood vessels	Macrophages	Neutrophils
Control	+	+	++	++	++	+
6h post-treatment	+	++	++	++	++	++
12h post-treatment	+	-	++	++	++	+
36h post-treatment	+	-	++	++	++	+

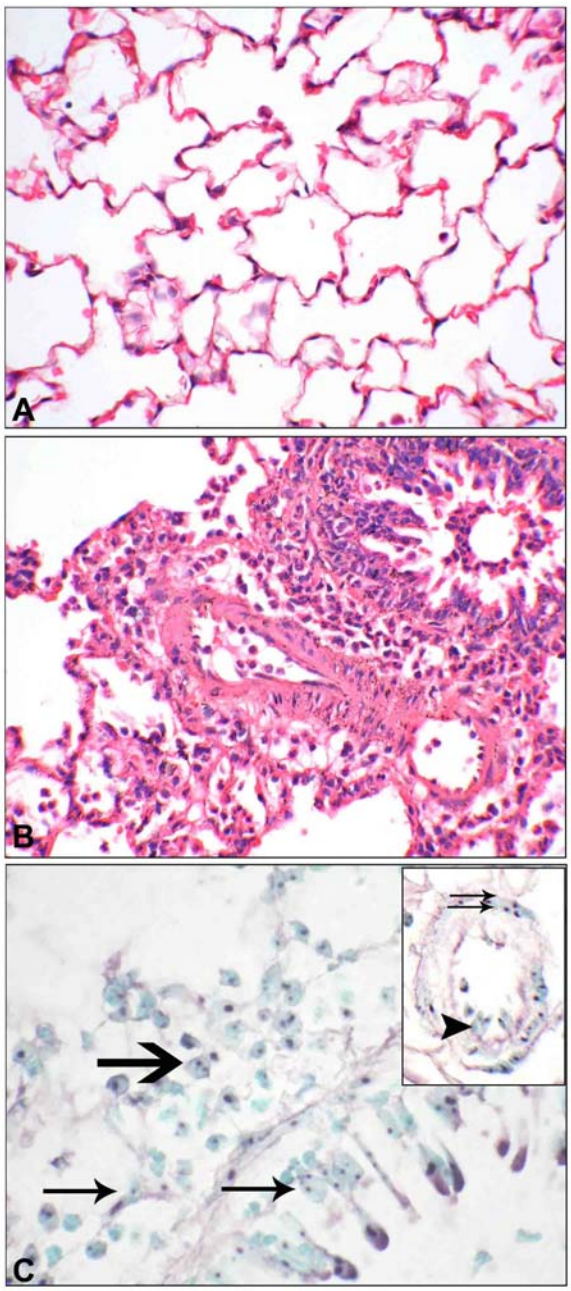

Figure 5. 1. *E. coli*-LPS induced lung inflammation.

Saline treated lungs showed normal histology (A). Instillation of LPS resulted in inflammation characterized by infiltration of inflammatory cells into the perivascular, peribronchiolar and alveolar spaces (B). Instillation of LPS was confirmed using an anti-LPS antibody (C). LPS was localized in the epithelium (arrows), macrophages (large arrow), endothelium (arrowhead) and smooth muscle cells (double arrow). Magnification A-B: ×400 C: ×1,000

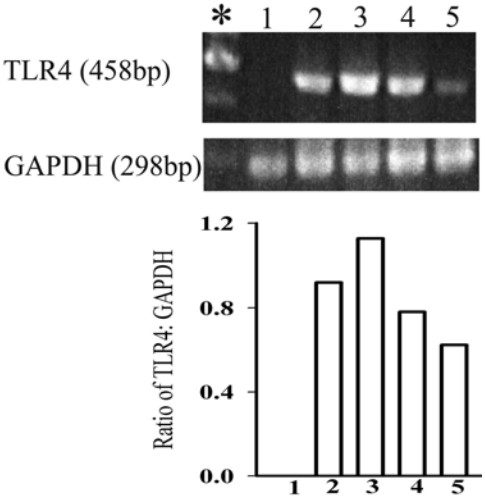

Figure 5. 2. Expression of TLR4 mRNA in the lung.

RT-PCR on RNA extracted from the saline- (1) and LPS-treated [6 hours (2), 12 hours (3), 18 hours (4) and 36 hours (5)] rat lungs showed expression of TLR4 only in LPS-treated lungs. Densitometric evaluation (average pixel value expressed as ratio of TLR4:GAPDH) showed maximal expression at 12 hours and lowest expression at 36 hours post LPS-treatment. *: lane for DNA ladder. For each time point, n = 3; densitometric values represent mean of 3 samples for each time point.

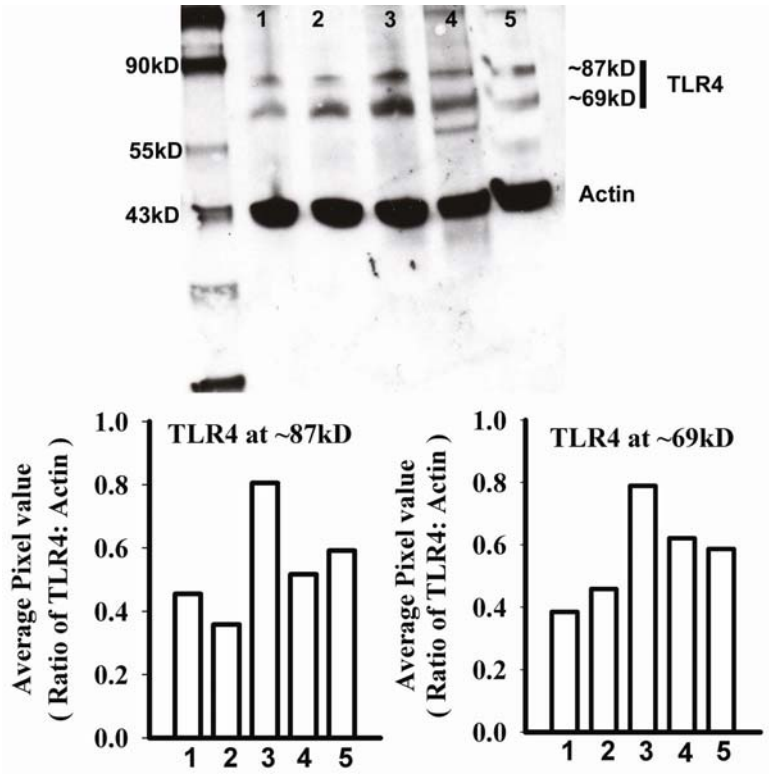

Figure 5. 3. Expression of TLR4 protein in the lung.

Western blots on lung homogenates from the saline (1) - and LPS-treated [6 hours (2), 12 hours (3), 18 hours (4) and 36 hours (5)] rat lungs showed expression of TLR4 at all the time points. There were two bands for TLR4; one at ~87 kD and another at ~69 kD. The results were interpreted using spot densitometry. For each time point, n=2 and the experiment was repeated twice.

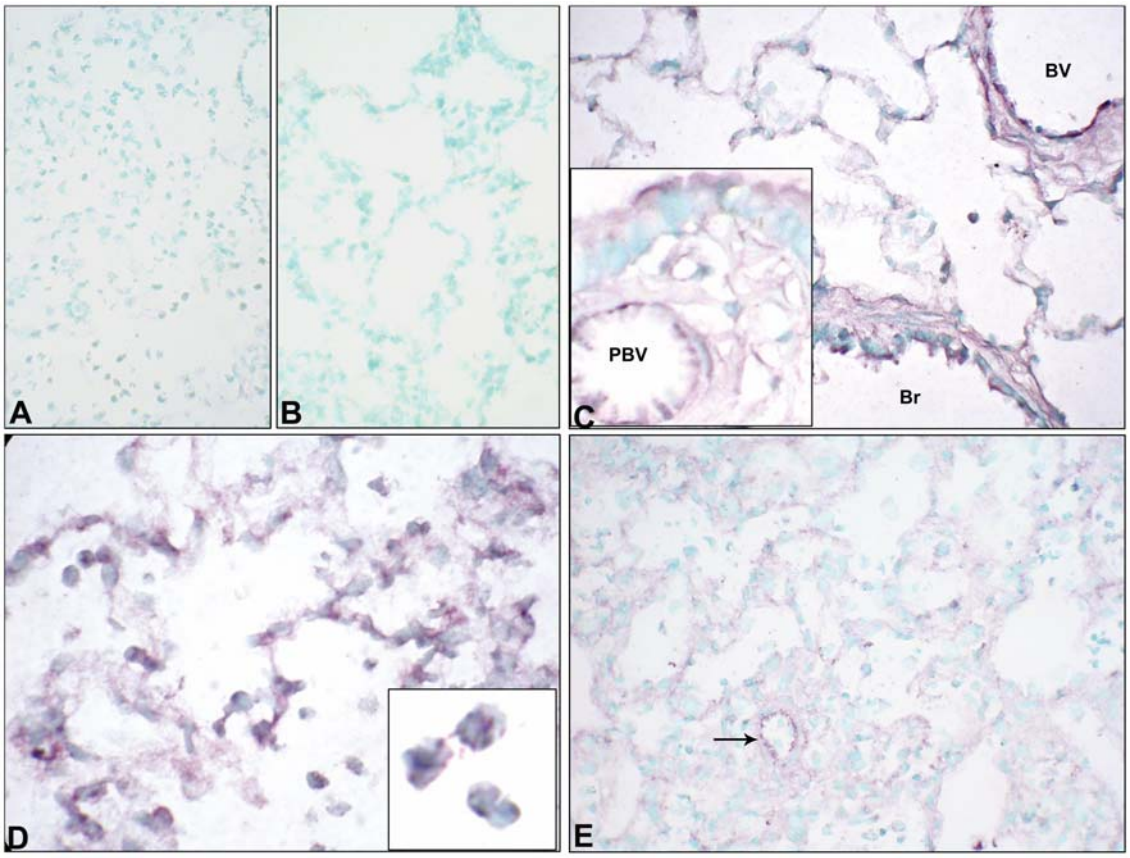

Figure 5. 4. TLR4 immunohistochemistry.

No reaction in the TLR4 antibody omitted (A) and isotype matched immunoglobulin treated (B) lung sections ruled out non-specific reactions. In saline treated lung sections (C), minimal expression was observed in few of the septal cells and in the bronchiolar epithelium (Br). Expression was more intense in the endothelium of large blood vessels (BV) and peribronchiolar blood vessels (PBV). Increase in the expression of TLR4 was observed in the septum and neutrophils (inset) after 6 hours of LPS treatment (D). At 36 hours post-LPS treatment (E), no expression was observed in the septum. However, expression was present in the large blood vessels (arrow) and few of the neutrophils. Magnification- A-C and E: ×400; D: ×1,000; Insets: ×2,000

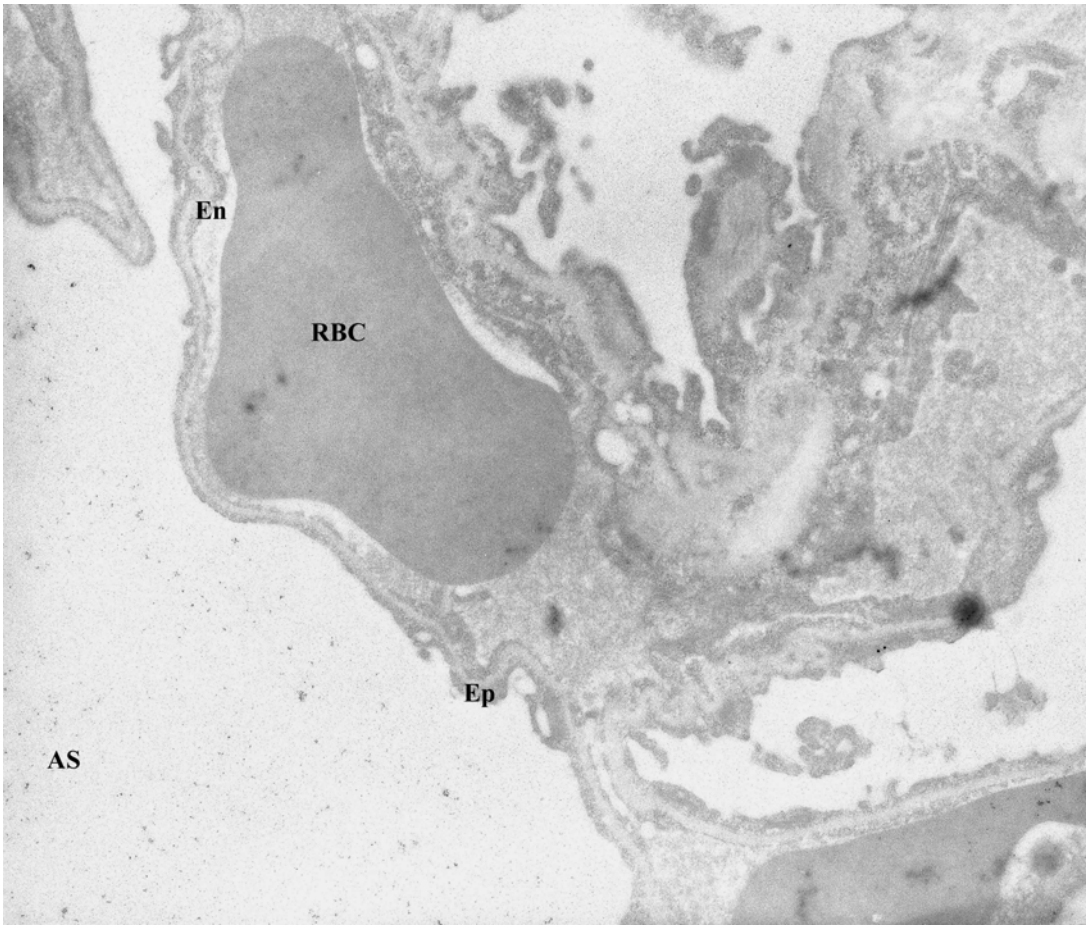

Figure 5. 5. Negative control.

Dual immunogold labeling on a lung section with isotype matched rabbit and goat immunoglobulins showed negligible labeling. AS: alveolar space; RBC: Red blood cell; En: endothelium; Ep: epithelium. Magnification- ×17,500.

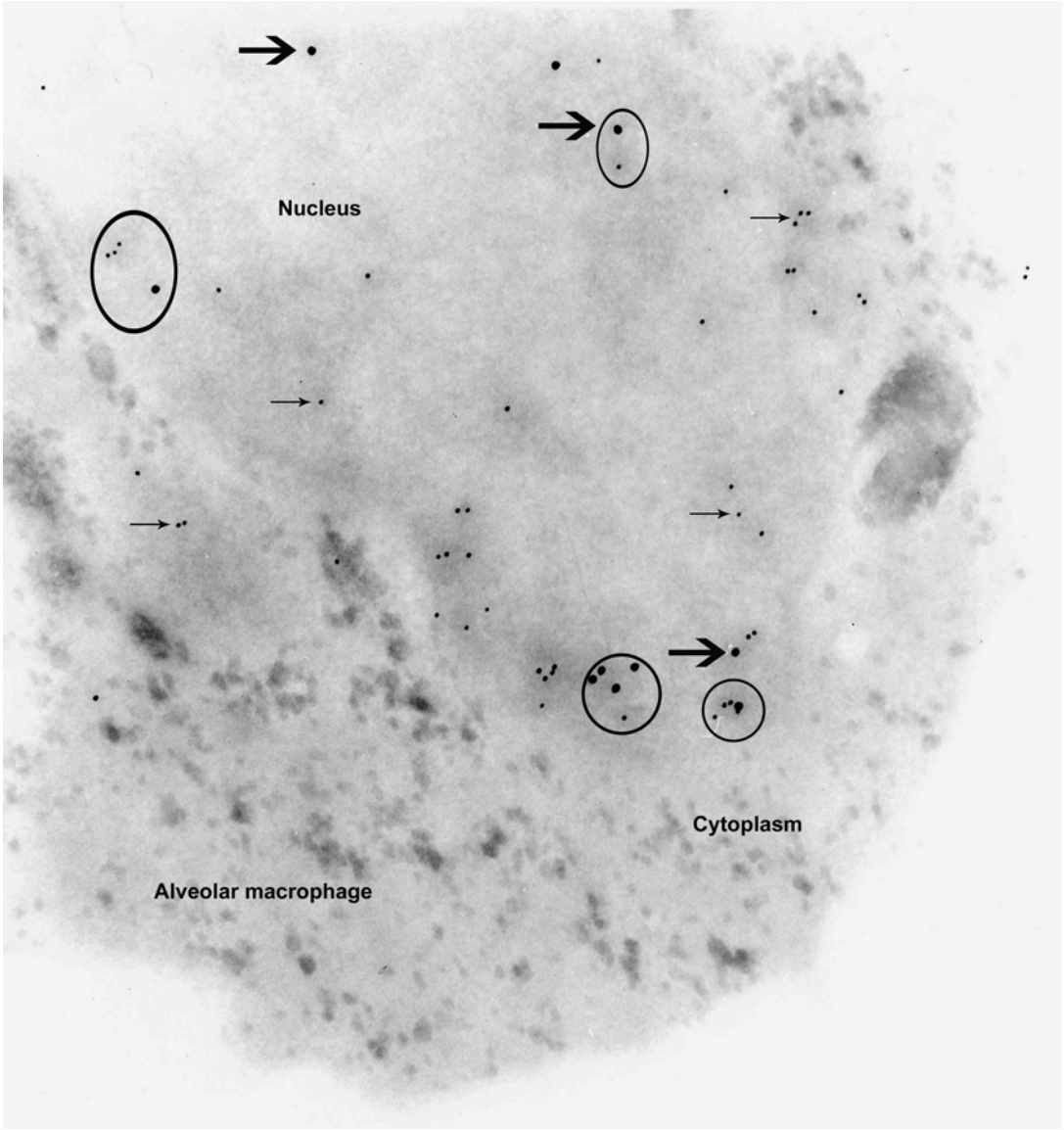

Figure 5. 6. Dual labeling for TLR4 and LPS in an alveolar macrophage.

Electron micrograph of an alveolar macrophage from an LPS-treated lung shows predominantly nuclear colocalization (circles) of TLR4 and LPS. TLR4: large particles (large arrows), LPS: small particles (small arrows). Magnification- ×56,000.

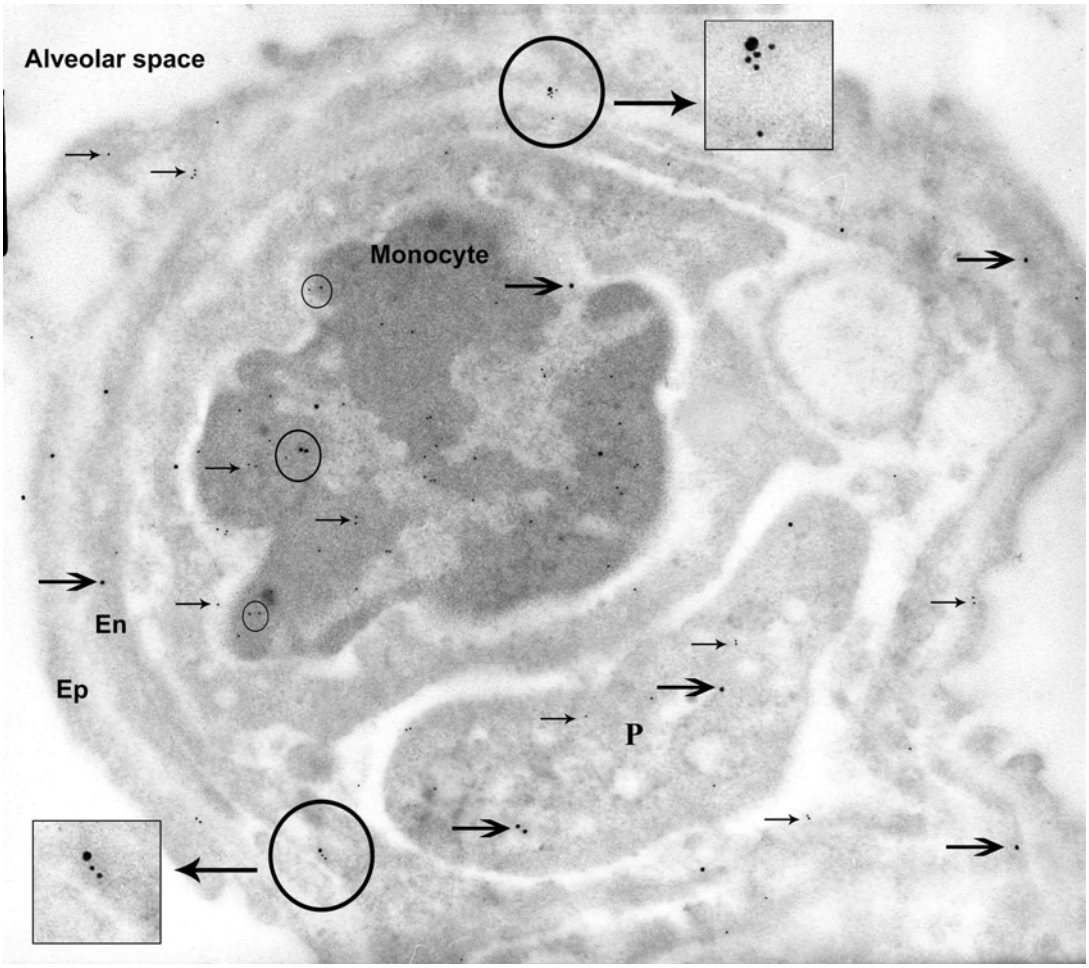

Figure 5. 7. Dual labeling for TLR4 and LPS in a monocyte and a platelet.

Electron micrograph from an LPS-treated lung shows localization of TLR4 and LPS in the epithelium (Ep), endothelium (En), platelet (P) and a monocyte. Colocalization of TLR4 and LPS (circles and insets) can be observed in both epithelium and endothelium. Monocyte shows predominantly nuclear localization of LPS and TLR4. TLR4: large particles (large arrows), LPS: small particles (small arrows). Magnification- ×36,000. Insets- ×72,000.

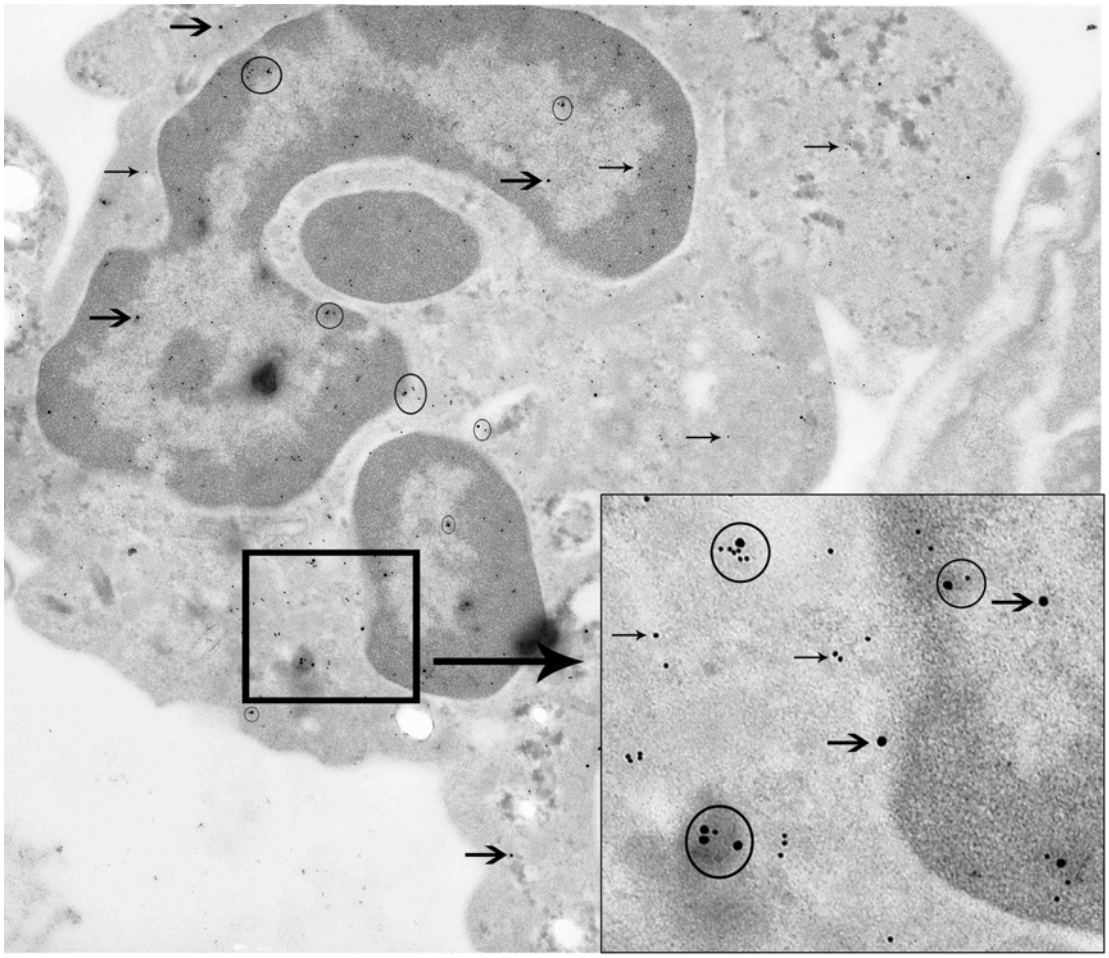

Figure 5. 8. Dual labeling for TLR4 and LPS in a neutrophil.

Neutrophil in the alveolar space of an LPS-treated lung shows colocalization of TLR4 and LPS in the cytoplasm and the nucleus (circles). TLR4: large particles (large arrows), LPS: small particles (small arrows). Magnification- ×30,000. Inset- ×52,000

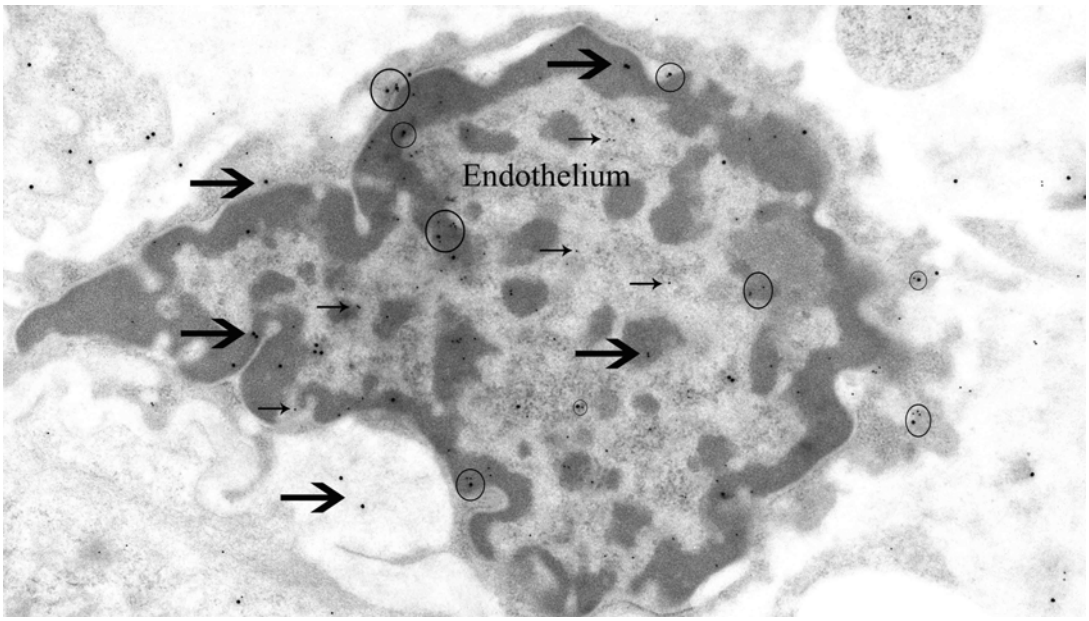

Figure 5. 9. Dual labeling for TLR4 and LPS in an endothelium.

Endothelium of a large blood vessel from a LPS-treated rat lung shows colocalization of TLR4 and LPS in the cytoplasm and the nucleus (circles). TLR4: large particles (large arrows), LPS: small particles (small arrows). Magnification- ×30,400

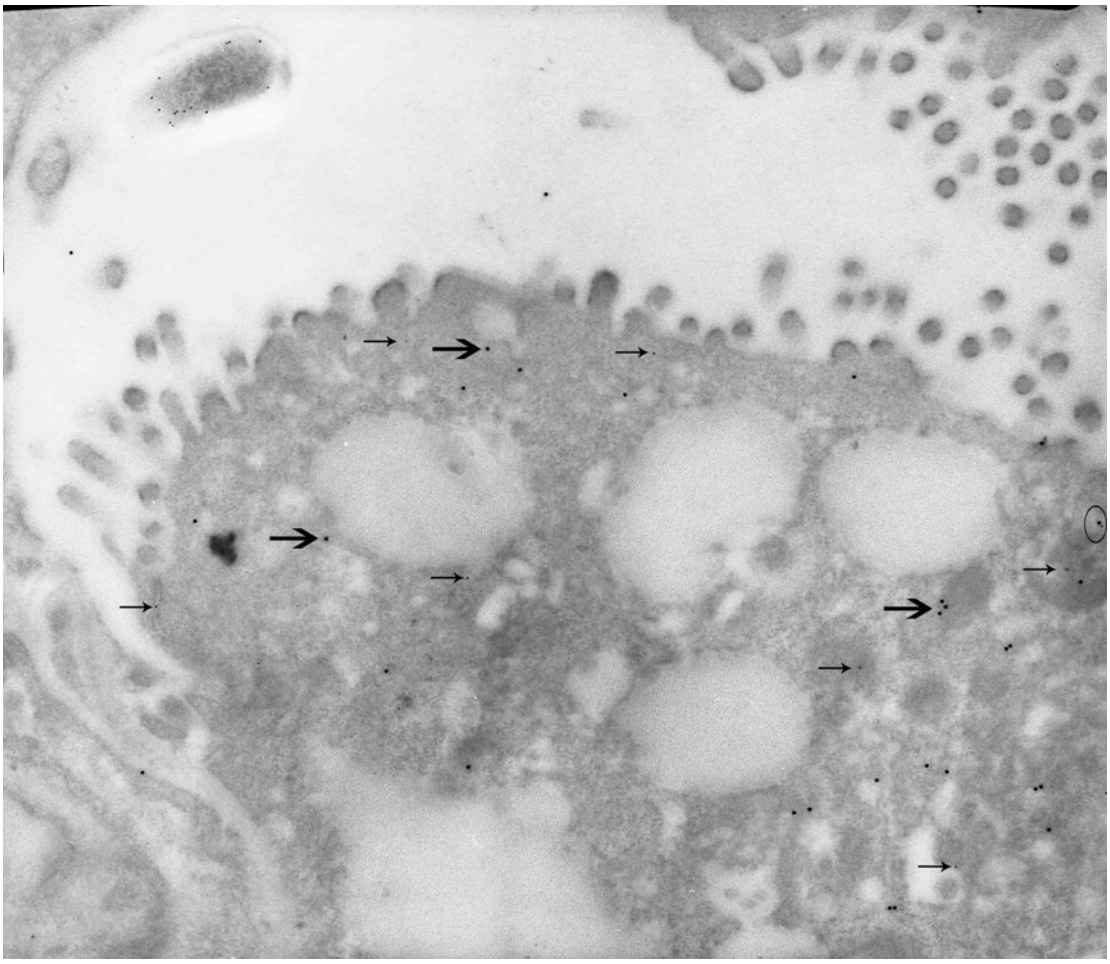

Figure 5. 10. Dual labeling for TLR4 and LPS in a type II alveolar epithelium.

Electron micrograph of a Type II alveolar epithelium shows labeling mainly for TLR4. Compared to other cells in the lung, relatively less LPS was observed in these epithelial cells. TLR4: large particles (large arrows), LPS: small particles (small arrows). Magnification - ×30,400.

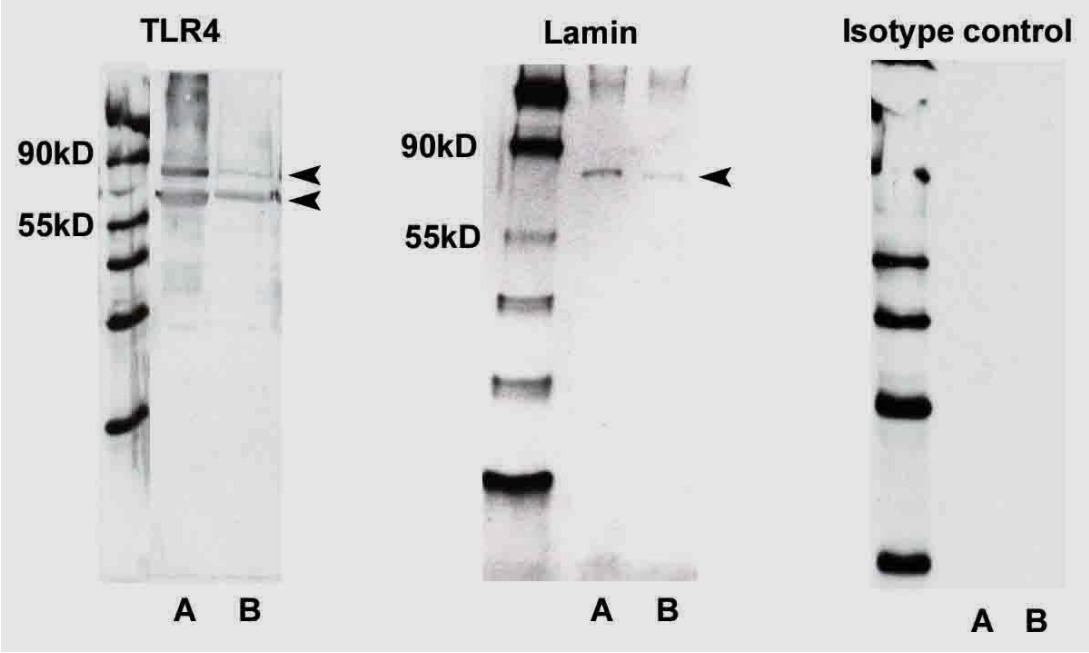

Figure 5. 11. TLR4 in lung nuclear extracts.

Western blots on lung nuclear extracts showed presence of TLR4 in the nucleus. Similar to the crude lung extracts, I observed two bands; one at ~87 kD and another at 69 kD. Presence of a nuclear protein, lamin confirmed that it is a nuclear fraction and probing with an isotype matched immunoglobulin ruled out possible non-specific reaction. A: Saline treated and B: LPS-treated rat lung nuclear extracts. The first lane on all the blots is a molecular weight marker. Experiment was performed on one sample from each treatment and was repeated twice.

5.5. Discussion

Because TLR4 is a molecule with well-established role in handling of Gram negative pathogens and LPS and there is a paucity of data on its temporal expression in inflamed lungs, I examined expression of TLR4 mRNA and protein in a rat model of acute lung inflammation. The expression of TLR4 was determined in lung homogenates with RT-PCR and Western blots, and *in situ* with immunohistology and immunoelectron microscopy. The data demonstrate that TLR4 gene transcription was increased at 6 hours followed by a decrease at 36 hours post-LPS challenge. There were cell-specific differences in TLR4 protein expression in control and LPS-treated rat lungs. I also report co-localization of TLR4 and LPS in the cytoplasm and nucleus of various lung cells.

I observed absence and presence of TLR4 mRNA in lungs from the control and LPS-treated rats, respectively. The absence of TLR4 mRNA in normal rat lungs is in disagreement with previous reports of its occurrence in normal mouse lungs (Fan *et al.* 2002). The reasons for this disagreement between the data are not apparent but there are some possibilities. First possibility that it could be due to technical reasons is discounted by the detection of mRNA in the treated rat lungs with the same protocol. Second possibility could be that our technique is not sensitive enough to detect minimal constitutive TLR4 gene expression in the lungs. Lastly, the absence of TLR4 mRNA in the lungs of control rats could be a species-specific phenomenon. Interestingly, the LPS treatment induced expression of TLR4 mRNA at 6 hours of the challenge and a return to barely detectable levels at 36 hour post-challenge. These data show that a single challenge with LPS induces TLR4 gene transcription.

The protein expression of TLR4 was detected with Western blots on lung homogenates and immunocytochemistry on lung sections of normal and all the LPS-treated rats. Western blots revealed two bands of ~87 kD and at ~69 kD and the first band is closer to the reported molecular weight of regular TLR4. I have not addressed the origin or the function of 69 kD band. Although this band could result from proteolysis of TLR4 molecule, I did include sufficient amounts of protease inhibitors in the lysis buffer and I did not observe proteolysis with few other proteins probed in our laboratory (B. Singh and K. Janardhan, unpublished observations). The other possibility could be the presence of a variant form of TLR4 protein. There are previous reports on the possibility of alternative splicing of TLR4 mRNA which could result in lower molecular

weight proteins (Iwami *et al.* 2000). There is evidence that recombinant soluble TLR4 has a molecular weight of approximately 80 kD (Hyakushima *et al.* 2004). Nevertheless, the data show expression of TLR4 protein with two different molecular weights in lungs from the control and LPS-treated rats.

Immunohistology showed cell specific differences in the expression of TLR4 in the lungs from control and the treated rats. The most interesting observation was the increase in TLR4 staining in alveolar septa and infiltrating neutrophils at 6 hours post-LPS treatment which was reduced at 12 and 36 hours of the treatment. In the light of recent evidence, the septal and neutrophil expression of TLR4 may have significant implications for leukocyte recruitment in inflamed lungs. Kubes and colleagues have reported that lung microvascular endothelial TLR4 is involved in the recruitment of neutrophils in inflamed lungs (Andonegui *et al.* 2003). There is also evidence that TLR4 on neutrophils is critical for maximal neutrophil recruitment into the inflamed lungs (Hollingsworth *et al.* 2005). Therefore, increased expression in the septum and infiltrating neutrophils may facilitate neutrophil migration into inflamed lungs. The decline in TLR4 expression in the septa and infiltrating neutrophils at 12 - 36 hours in our experiments coincides with well established decline in migration of neutrophils at this time point in LPS-induced lung inflammation (Ulich *et al.* 1991). Therefore, reduced expression of TLR4 in septal microvessels at 12 hours may be one of the molecular mechanisms to inhibit neutrophil recruitment in inflamed lungs. Sustained expression of TLR4 in large and peribronchiolar blood vessels at all the time points may also promote leukocyte migration into the peribronchial and perivascular spaces of lung; such perivascular and peribronchial leukocyte migration has been reported in various models of lung injury (Curtis *et al.* 1990; Ichikawa *et al.* 1996; Pabst and Tschernig. 2002). The minimal expression of TLR4 in the bronchial epithelium in both control and LPS-treated rats is in agreement with the previous *in vitro* observations on bronchial epithelial cells (Guillot *et al.* 2004; Sha *et al.* 2004). Taken together, septal and leukocyte expression of TLR4 may be critical for neutrophil trafficking into inflamed lungs.

The immunoelectron microscopy revealed predominantly cytoplasmic and nuclear staining for TLR4. Similar intracellular expression of TLR4 has been reported in the bronchial and alveolar epithelial cells, and intestinal epithelial cells grown *in vitro* (Guillot *et al.* 2004; Hornef *et al.* 2003). However, surface expression of TLR4 is also well established in macrophages and alveolar epithelium (Akashi *et al.* 2000; Armstrong *et al.* 2004; Punturieri *et*

al. 2004). One of the reasons for not detecting the surface expression of TLR4 in our tissue samples could be that the antibody used was raised against the carboxy terminus of the TLR4 protein. Furthermore, predominant cytoplasmic localization of TLR4 may result from rapid internalization of TLR4 (Guillot *et al.* 2004; Hornef *et al.* 2003). However, intracellular versus surface localization of TLR4 may have no bearing on LPS responsiveness, because even intracellular interactions of LPS with TLR4 can result in potent signaling (Espevik *et al.* 2003).

An intriguing finding was ultrastructural localization of TLR4 in the nuclei of monocytes, macrophages, neutrophils and endothelial cells in the lungs from control and LPS-treated rats. I further strengthened this observation by demonstrating TLR4 in nuclear extracts prepared from the normal and LPS-treated lungs. Previously, nuclear localization of TLR2 has been shown *in vitro* in monocytes and macrophages (Flo *et al.* 2001). However, to my knowledge this is the first report to demonstrate nuclear localization of TLR4. Even more interestingly, my data show colocalization of TLR4 with LPS in the nuclei of various lung cells. Although others have reported rapid trafficking of LPS into the nuclei of macrophages (Kang *et al.* 1990; Risco *et al.* 1991; Singh and Atwal. 1997), this study provides first evidence of nuclear localization of an LPS signaling receptor. My data do not clarify whether the TLR4-LPS complex is formed on the cell surface or in the cytoplasm prior to its migration into the nuclei or whether LPS complexes with pre-existing TLR4 in the nucleus of these cells. Because TLR4 is a signaling molecule, it is critical to address the mechanisms as well as implications of its nuclear colocalization with LPS in future studies.

To summarize, this study shows that TLR4 expression is sustained, with few cell specific variations, at least up to 36 hours in *E. coli*-LPS induced lung inflammation. The sustained TLR4 expression in the inflamed lungs suggests lung's ability to respond to a secondary challenge. Predominant localization of TLR4 in the cytoplasm and nucleus in my study creates a need to explore the mechanisms involved in trafficking of TLR4 from intracellular compartments to and from the cell surface, and the functional consequences of TLR4 and LPS localization in the nucleus.

CHAPTER 6: NEUTROPHIL DEPLETION INHIBITS EARLY AND LATE MONOCYTE/MACROPHAGE INCREASE IN LUNG INFLAMMATION[*]

6.1. Abstract

Monocytes/macrophages have critical impact on outcomes of lung inflammation. Kinetics and mechanisms of monocyte/macrophage increase in lungs are not completely understood. Therefore, I examined the pattern and mechanisms of monocyte/macrophage increase in acute lung inflammation. Sprague-Dawley rats were instilled intratracheally with *E. coli*-LPS (250 μg; N = 35) or endotoxin-free saline (N = 5). Increase in monocytes/macrophages, neutrophils and monocyte chemotactic protein-1 (MCP-1) were quantified at various time points after LPS treatment. The role of neutrophils in monocyte/macrophage increase was addressed in LPS-challenged neutropenic rats (N=8). In contrast to typical pattern of neutrophil influx between 6 and 24 hours, monocytes/macrophages increased in two distinct phases, early at 3 hours and late at 24 hours. Neutrophil depletion before LPS-instillation abrogated the early as well as late monocyte/macrophage increases in the lung. I quantified MCP-1, which is one of the major chemoattractants for monocytes, in lung homogenates and found similar concentrations of MCP-1 in neutropenic and non-neutropenic LPS-challenged rats. I conclude that monocyte/macrophage increase in lung occurs in two phases, a novel early phase and a well-established late phase, in LPS-induced acute lung inflammation and both phases of monocyte/macrophage increase are dependent on neutrophils.

[*] Frontiers in Bioscience **11**, 1569-1576, May 1, 2006
"The original publication is available at www.bioscience.org".

6.2. Introduction

Acute inflammation in the lung and other organs is characterized by early influx of neutrophils followed by monocytes and macrophages (Kaplanski *et al.* 2003; Larsen and Holt. 2000). It is well established that increase in monocyte numbers occurs in late, post-neutrophilic, phase of inflammation in the lung (Doherty *et al.* 1988; Fillion *et al.* 2001; Li *et al.* 1998; Maus *et al.* 2002b; Ulich *et al.* 1991). However, in animal models of inflammation in other organs, such as peritonitis and dermatitis there is also a documentation of very early increase in monocyte/macrophage numbers either before or along with increase in neutrophil numbers (Henderson *et al.* 2003; Issekutz and Issekutz. 1993; Issekutz *et al.* 1981). Such observations on the early increase in the monocyte/macrophage numbers have not been made in the context of lung inflammation. This could be either due to the primary focus of previous studies on monocyte/macrophage increase in the late phase of inflammation (Doherty *et al.* 1988; Li *et al.* 1998; Yamamoto *et al.* 1998) or because most of the investigators have used bronchoalveolar lavage (BAL) to assess migration of inflammatory cells into inflamed lungs (Fillion *et al.* 2001; Maus *et al.* 2002b; Ulich *et al.* 1991; Yamamoto *et al.* 1998). Although BAL is an useful tool to assess migration of inflammatory cells into the lungs, it does not account for inflammatory cells that are present in the septum which includes cells in the microvasculature and interstitial space (Li *et al.* 1998). Since monocytes/macrophages in inflamed lungs, irrespective of their location, can significantly contribute to inflammation by producing cytokines and free oxygen radicals (Elias *et al.* 1985a; Elias *et al.* 1985b; Li *et al.* 1998; Maus *et al.* 2002a), it is important to assess the total number of monocytes/macrophages accumulated in the lung instead of examining only those which migrate into the airspace. Therefore, I decided to examine the pattern of total monocyte/macrophage population up to 36 hours post-LPS treatment in the lung.

MCP-1, a CC chemokine, is a major chemoattractant for monocytes and is produced by various cells including neutrophils (Burn *et al.* 1994; Sakanashi *et al.* 1994; van Coillie *et al.* 1999). There is evidence that neutrophils regulate the monocyte increase in the late phase of C5a and LPS induced lung inflammation in rabbits and mice, respectively (Doherty *et al.* 1988; Maus *et al.* 2002b). It is also suggested that MCP-1 release by neutrophils could possibly play a role in late monocyte/macrophage increase in inflamed lungs (Yamamoto *et al.* 1998). But there is no direct evidence to show that neutrophils are the major source of MCP-1 in the lung. Therefore, I wanted to assess the role of neutrophils on MCP-1 concentration and its effect on

monocyte/macrophage numbers in inflamed lungs. To address these questions, I conducted an *in vivo* quantitative study in rats and evaluated increase in total monocytes/macrophage numbers, without making a distinction between alveolar, interstitial and microvascular, in inflamed lungs. Then, I investigated the role of neutrophils in relation to MCP-1 expression in monocytes/macrophage increase in acute lung inflammation. The data show an early, in addition to previously established late, increase in monocytes/macrophage numbers. The data also shows that neutrophil depletion inhibits both early as well as the late increase in monocyte/macrophage numbers without affecting concentrations of MCP-1 in the lung.

6.3. Materials and methods

6.3.1. Rats and treatment groups

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and experiments were conducted according to the Canadian Council on Animal Care Guidelines. Specific pathogen-free, ten-week-old, male Sprague-Dawley rats were procured from Charles River laboratories, Canada. Rats were maintained in the animal care unit and were acclimatized for a period of one week. Rats were randomly divided into eight groups of five each.

6.3.2. Acute lung inflammation

Rats were anaesthetized by intraperitoneal administration of xylazine (20 mg/kg) and ketamine (100 mg/kg). Trachea was exposed surgically and endotoxin-free saline (Sigma, St.Louis MO, USA) or *E. coli* LPS (250 µg; serotype 0128:B12; Sigma, St.Louis MO, USA) was instilled intratracheally. Animals were euthanized at 1, 3, 6, 12, 24, 30 and 36 hours (n = 5 each) post-treatment. Control animals (n = 5) were euthanized at 6 hours post saline treatment.

6.3.3. Tissue collection and processing

Described in Section 3.3.4.

6.3.4. Immunohistology

Procedure is described under Section 3.3.6. Tissue sections were stained using rat monocyte/macrophage (1:75; ED-1, Serotec Inc**.** NC, USA), rat MCP-1 (1:300; Torrey Pines

Biolabs, Inc. TX, USA), *E. coli*-LPS (1:300, Cedarlane Laboratories Limited, ON, Canada) and HRP-conjugated antibodies (1:100; Dako cytomation, ON, Canada).

6.3.5. Quantification of monocytes/macrophages and neutrophils

Neutrophils were counted in hematoxylin and eosin (H&E) stained lung sections while monocytes/macrophages were counted in ED-1 immunostained lung sections. I did not use immunohistochemistry for identifying neutrophils as these cells are easily identifiable by their morphology. Cells were counted in 10 high power fields (100× objective)/section from each of the six lung pieces from every rat. Area of the field was calculated using a stage micrometer (Tissue area: 0.025 mm^2/field, 0.25 mm^2/section, 1.5 mm^2/rat). The fields for counting the cells were randomly selected and those fields containing larger blood vessels, bronchioles and larger airways were excluded (Mizgerd *et al.* 1997).

6.3.6. Immunoelectron microscopy

The procedure is described in Section 3.3.8. Sections were labeled using ED-1(1:50), MCP-1(1:250) and 15 nm gold-conjugated secondary antibodies (1:100).

6.3.7. Induction of neutropenia and lung inflammation

Eight rats were given anti-neutrophil antibody (0.3 ml/100 g; intraperitoneal; Catalogue number AIA51140; Accurate Chemicals, NY, USA) (Riedemann *et al.* 2004; Sir *et al.* 2000). Differential counts were performed on peripheral blood samples before and after the treatment to confirm the induction of neutropenia (Sir *et al.* 2000). Before antibody treatment the differential count of neutrophil in peripheral blood was 12.65±2.3 % (mean±SD). After 24 hours of the antibody treatment the neutrophil counts declined to 0% in six rats and 1% in two rats. After 24 hours of anti-neutrophil antibody treatment, rats were anaesthetized to instill 250 µg of *E. coli*-LPS intratracheally followed by euthanasia at 3 hours (n=4) or 24 hours (n=4) after the treatment. Tissues were collected and processed for light microscopy, immunohistochemistry and ELISA.

6.3.8. Quantification of MCP-1 in lung homogenates

Purified anti-rat MCP-1 (clone C4) and biotinylated anti-rat MCP-1 (clone B4) and recombinant rat MCP-1 were purchased from BD Biosciences, ON, Canada. Lung samples were homogenized in HBSS (0.1 g/ml) containing protease inhibitor cocktail (100 µl/10 ml; Sigma-

Aldrich Co, MO, USA). Microtiter plates (Immulon 4 HBX, VWR CAN LAB, AB, Canada) were coated with 50 µl of purified anti-rat MCP-1 antibody (10 µg/ml) and incubated at 4°C overnight. After 12 hours, plates were washed with PBS containing 0.05%-Tween (PBST) before incubating with 200 µl of blocking buffer (1% BSA in PBS) for one hour at 37°C. Plates were washed 5 times with PBST and incubated with 100 µl standard or samples in duplicates for two hours at 37°C. After adding 100 µl of biotinylated anti-rat MCP-1 antibody (2 µg/ml) diluted in blocking buffer with Tween, plate was incubated at 37°C for an hour. This was followed by incubation with streptavidin-HRP (1:2500 in PBS; DAKO A/S, Denmark) for 30 minutes at 37°C. The reaction was visualized using TMB substrate (Mandel Scientific, ON, Canada) and reaction was stopped using 50 µl of 1M sulfuric acid, followed by reading at 450 nm.

6.3.9. Statistical analyses

All values are presented as mean±SE, unless otherwise mentioned. Differences between two groups were tested using independent-samples *t*-test and more than two groups were compared using one-way analysis of variance with Fisher's LSD for post hoc comparisons. Statistical significance was accepted at $P<0.05$.

6.4. Results

6.4.1. Monocyte/macrophage and neutrophil kinetics in acute lung inflammation

Monocyte/macrophage and neutrophil counts were performed in sections stained with ED-1 antibody, which recognizes both monocytes and macrophages (Figure 6.1A) and H&E, respectively. ED-1 positive cells in control animals consisted of alveolar macrophages and few monocytes in the septum (Figure 6.1B). Inflamed lungs, however, showed increased numbers of monocytes/macrophages in the lungs (Figures 6.1C and D). Electron microscopy confirmed that at 1 hour and 3 hours of LPS treatment most of the septal cells were monocytes and were in the septal microvessels (Figure 6.1E).

Quantitative analyses showed elevated numbers of monocytes/macrophages at 1 hour (20.16±1.01; P=0.053), 3 hours (22.66±2.32; P=0.019), 6 hours (23.77±1.48; P=0.012) and 12 hours (23.9±2.19; P=0.011) post-LPS challenge compared to the controls (9.25±0.89; Figure 2); however, there were no differences between these post-LPS treatment time points. Second increase in monocyte/macrophage numbers occurred at 24 hours (38.23±6.97; $P<0.001$) 30 hours

(38.72±4.21; P<0.001) and 36 hours (46.21±6.79; P<0.001) compared to controls (Figure 6.2). In contrast to monocytes/macrophages, neutrophils in the lungs increased steadily till 24 hours compared to the control (6 hours: 26.30±5.33, P=0.027; 12 hours: 38.00±7.42, P=0.001; and 24 hours: 57.14, P<0.001) followed by a decline at 30 hours (33.36±5.08; P=0.019) and 36 hours (35.33±4.24; P=0.031) compared to 24 hours (Figure 6.2).

6.4.2. Effect of neutropenia on number of monocytes/macrophages in lungs

I determined the role of neutrophils in the early as well as the late increase of monocytes/macrophages by provoking acute lung inflammation in neutropenic rats. The antibody reduced the differential count of neutrophils in peripheral blood from 12.65±2.3% (mean±SD) before the treatment to 0% in six rats and 1% in two rats at 24 hours after the treatment. The antibody used in our experiment has been shown not to affect the monocyte and alveolar macrophage numbers in rats (Snipes *et al.* 1995). Lungs from neutropenic rats demonstrated a reduction in monocyte/macrophage numbers at 3 hours and 24 hours post-LPS administration compared to non-neutropenic time-matched LPS-challenged rats (Figures 6.3 A-C; 22.66±2.32 v/s 11.92 ± 0.46, P= 0.001 and 38.23±6.97 v/s 12.19 ± 2.25, P= 0.015, respectively). Immunohistology for the LPS showed staining in the alveolar epithelium, macrophages, bronchiolar epithelium and smooth muscle cells (Figure 6.3D) and ruled out the possibility that reduction in monocyte/macrophage numbers is due to improper instillation of LPS.

6.4.3. MCP-1 expression and concentrations in lung

To obtain an insight into implications of MCP-1 as a downstream signal in neutrophil-dependent monocyte/macrophage increase, I first confirmed expression of MCP-1 in neutrophils using immunohistochemistry (Figure 6.4A) and immunoelectronmicroscopy (Figure 6.4B). MCP-1 staining was observed in alveolar septa as well as neutrophils in the septum. Lung sections stained as the controls for immunohistology (data not shown) or immunoelectron microscopy showed no staining (Figure 6.4C). Next, I determined MCP-1 concentrations in lung homogenates from non-neutropenic and neutropenic rats at 3 and 24 hours post-LPS treatment (Figure 6.4D and 6.4E). I chose 3 and 24 hours post-LPS intervals because significant increases in monocyte/macrophage numbers occurred at these times points (Figures 6.1 and 6.2). Compared to saline treated controls (126.19 ± 46.97 pg/ml), MCP-1 concentrations increased at 3 hours (1260 ± 46.08 pg/ml; P<0.001) and 24 hours (1201 ± 195.16 pg/ml; P<0.001) post-LPS

treatment in non-neutropenic LPS-treated rat lungs (Figure 6.4D). However, there were no differences in MCP-1 concentrations at 3 hours and 24 hours time points (P = 0.717). MCP-1 concentrations were also similar between LPS-challenged non-neutropenic and neutropenic rats at 3 hours (1260.18 ± 46.08 pg/ml v/s 1269.31± 190.35 pg/ml; P = 0.822) and 24 hours (1201.86 ± 195.16 pg/ml v/s 815 ± 263.11 pg/ml; P= 0.304) post-treatment (Figure 6.4E).

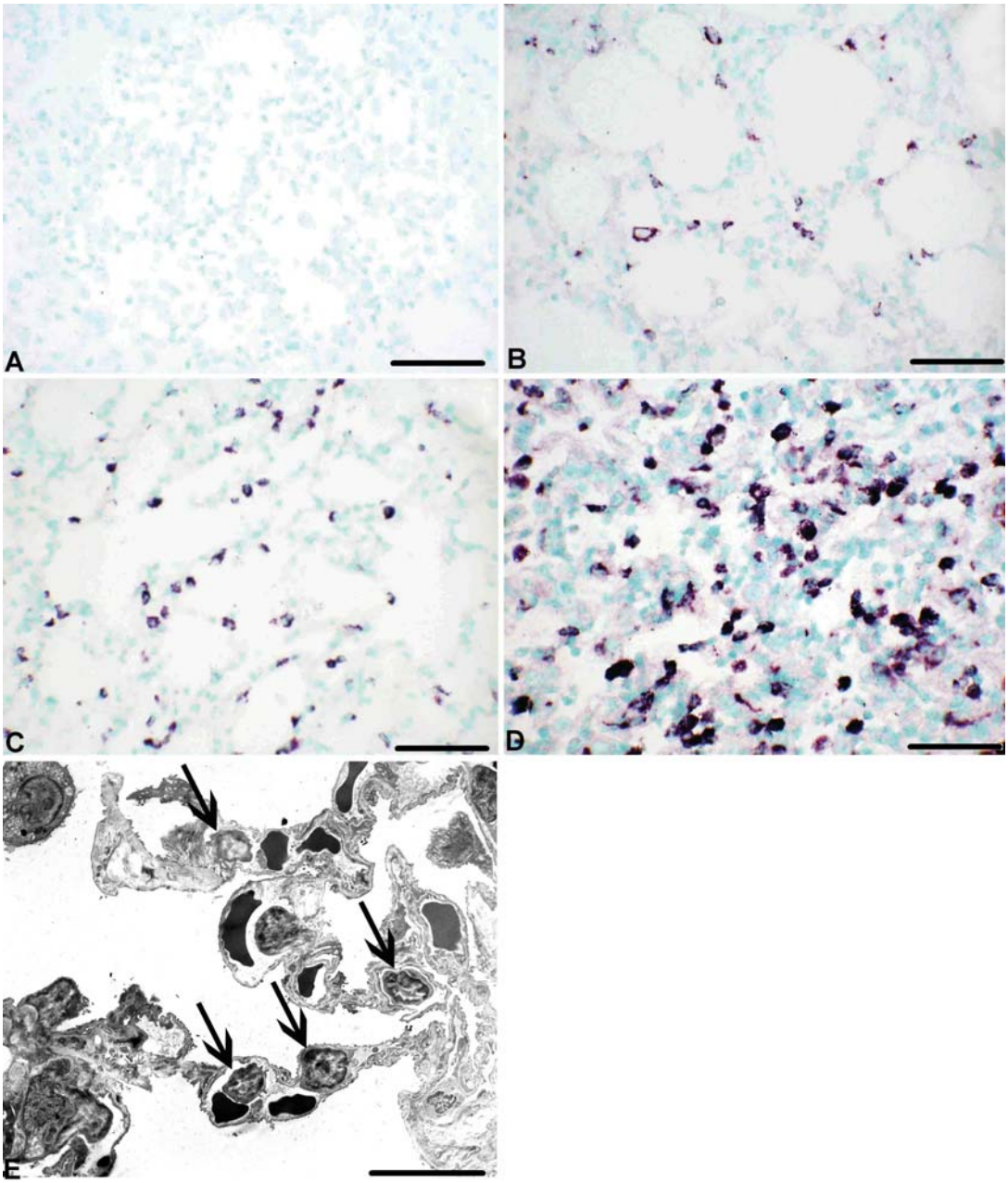

Figure 6. 1. Monocyte/macrophage increase in acute lung inflammation.

Monocytes/macrophages were identified using a monoclonal antibody, ED1. Staining with an isotype matched immunoglobulin did not show any reaction (A). Compared to saline treated controls (B), monocyte numbers appeared to increase at 3 hours post-LPS-treatment (C). The increase in monocyte numbers was much higher at 24 hours post-LPS-treatment (D). E. Electron microscopic observations showed that the early increase in the monocyte/macrophage numbers were mainly due to the presence of monocytes (arrows) mainly in the lung microvasculature. Magnification- A-D:×400; E: ×1600

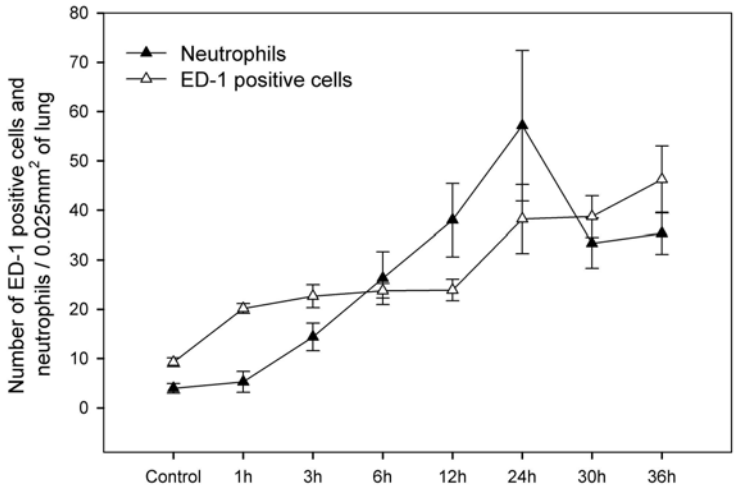

Figure 6. 2. Monocyte/macrophage and neutrophil kinetics in acute lung inflammation.

Number of monocytes/macrophages and neutrophils in lungs at various time points after LPS treatment are shown. Increase in monocyte/macrophage numbers was biphasic; the early increase was observed at 3 hours post-LPS-treatment and the late increase occurred at 24 hours post-LPS-treatment. Neutrophils increased from 6 hours to 24 hours followed by a decline at 30 and 36 hours post-LPS treatment. For monocytes/macrophages, time points from 3 hours to 36 hours are different from control; 1 hour, 3 hours, 6 hours and 12 hours are not different; 24 hours, 30 hours and 36 hours are different from 12 hours. For neutrophils, 6 hours to 36 hours time points are different from controls; 30 hours and 36 hours are different from 24 hours.

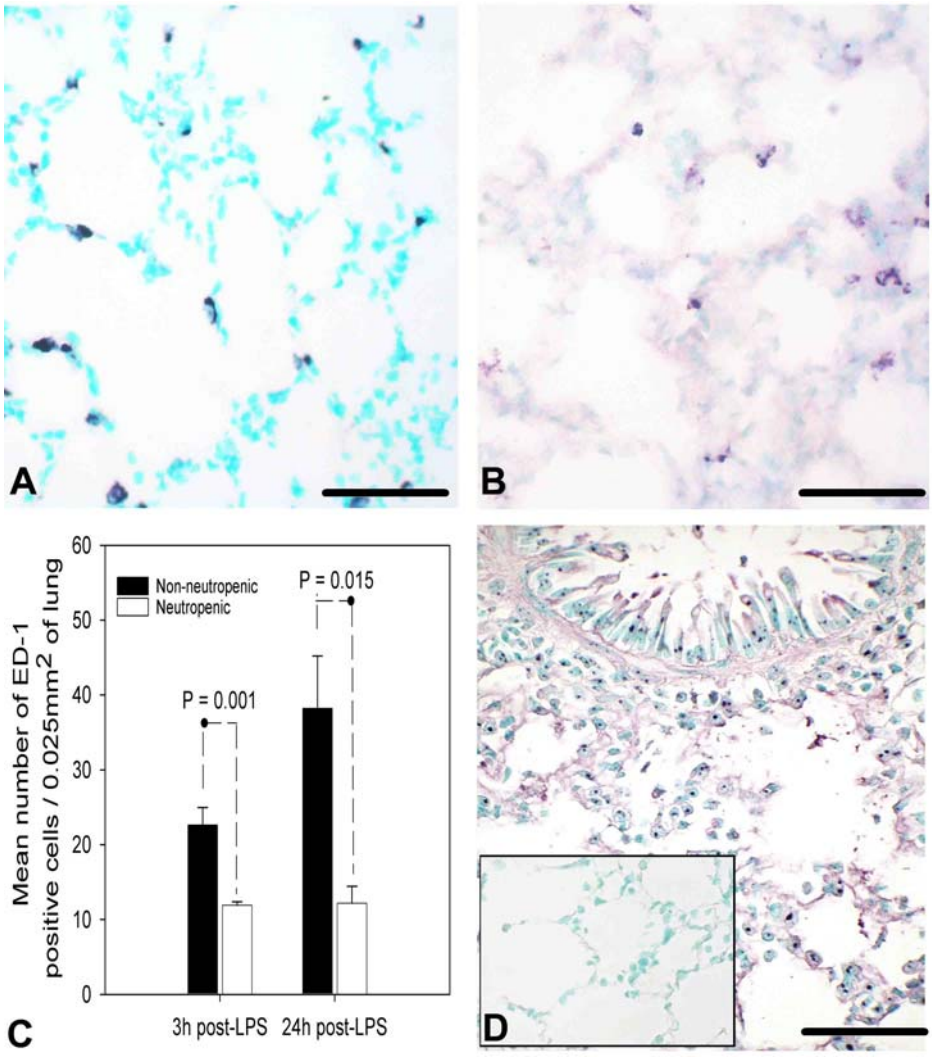

Figure 6. 3. Monocyte/macrophage kinetics in non-neutropenic and neutropenic rat lungs.

Monocyte/macrophage numbers in the neutropenic rat lungs at 3 hours (A) and 24 hours post-LPS-treatment (B) appeared to be less compared to non-neutropenic rat lungs (compare with Figures 1C and D). Quantification of monocyte/macrophage numbers showed a significantly lower number of monocytes in neutropenic rat lungs (C). D: The LPS localization using anti-LPS antibody in the bronchial epithelium, smooth muscle cells and alveolar epithelium confirmed proper instillation of LPS. Inset: staining with an isotype-matched immunoglobulin showed no reaction. Magnification- A, B and D: ×400

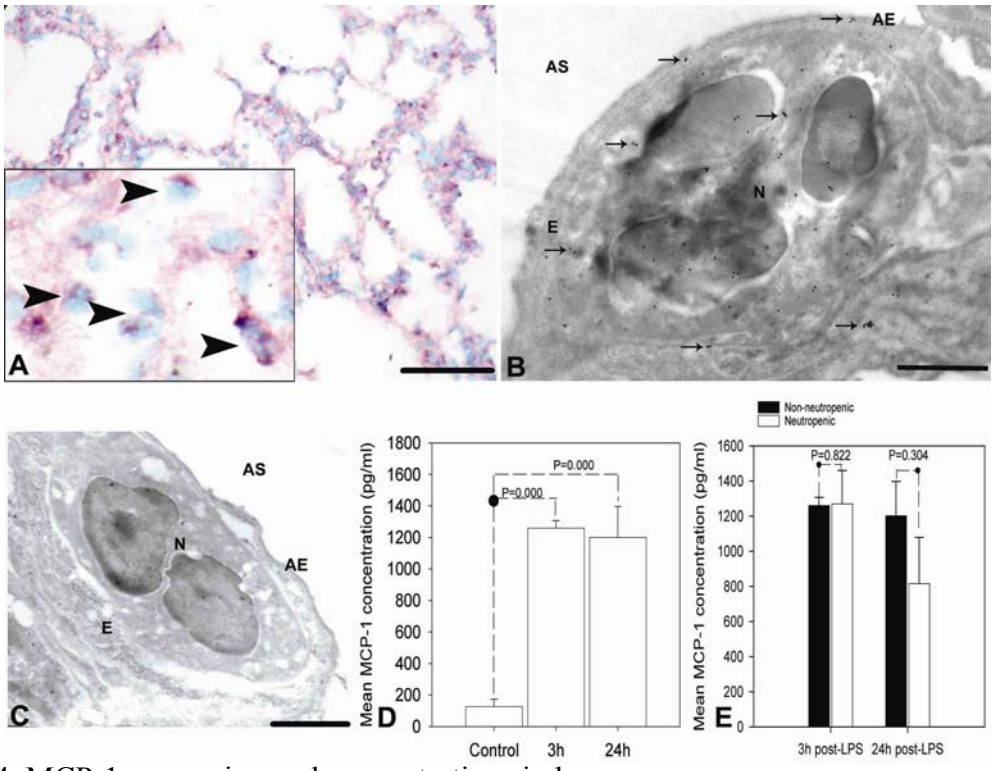

Figure 6. 4. MCP-1 expression and concentrations in lung.

A. At 3 hours post-treatment, MCP-1 expression was present in the neutrophils (arrowheads). B. Immunoelectron microscopy confirmed the presence of MCP-1 in neutrophils. C. Lung section stained with only secondary antibody lacked any labeling and ruled out non-specific binding (N: neutrophil; AE: alveolar epithelium; E: microvascular endothelium; AS: alveolar space; Arrows indicate gold particles labeled for MCP-1). D and E show concentration of MCP-1 in lung homogenates. Compared to controls, the concentrations of MCP-1 in lung homogenates were increased at 3 and 24 hours post-treatment. But there was no difference between 3 and 24 hours time points (D). E). Similar concentrations of MCP-1 in neutropenic rats compared to non-neutropenic rats at both 3- and 24 hours after LPS treatment. Magnification- A:×400 and inset:×1,000; B:×18,000; C:×13,000

6.5. Discussion

In this manuscript, I report an early increase in monocyte/macrophage numbers in acute lung inflammation. The data further demonstrate that neutrophil depletion inhibits both early as well as the late increase of monocytes/macrophages without affecting MCP-1 concentrations in inflamed lungs.

First, I wanted to know if there is an early increase in monocyte/macrophage numbers in inflamed lungs, as observed in peritonitis and dermatitis (Henderson *et al.* 2003; Issekutz and Issekutz. 1993; Issekutz *et al.* 1981). For this I undertook a detailed quantification of monocyte/macrophage numbers in normal and inflamed lungs. This became necessary because of lack of data on *in situ* quantification of monocytes/macrophages in inflamed lungs. Most of the previous studies have used only BAL to quantify cells such as mononuclear phagocytes and neutrophils in normal and inflamed lungs (Fillion *et al.* 2001; Maus *et al.* 2002b; Ulich *et al.* 1991; Yamamoto *et al.* 1998). Despite usefulness of BAL analyses in evaluation of cells that have migrated into the air spaces, it does not provide information on the inflammatory cells present in the septa (Li *et al.* 1998). For example, recent data convincingly showed that although bromo-deoxyuridine labeled monocytes disappeared from the peripheral blood into alveolar septa within 1 hour of their infusion, an increase in their numbers in BAL was observed after 48 hours (Goto *et al.* 2004). Therefore, BAL may not capture early increase in monocyte/macrophage numbers in the septum. Because intravascular monocytes/macrophages can influence the course of lung inflammation, it is important to undertake direct *in situ* quantification of all the monocytes/macrophages in unlavaged lungs.

These experiments resulted in an observation of an early increase in ED-1 positive monocyte/macrophage numbers in inflamed lungs. Previously, the early increase of monocytes/macrophages may have been missed due to reliance on BAL analyses or the studies' primary focus on the late, post-neutrophilic increase of monocytes/macrophages (Doherty *et al.* 1988; Fillion *et al.* 2001; Li *et al.* 1998; Maus *et al.* 2002b; Ulich *et al.* 1991; Yamamoto *et al.* 1998). The early increase in the cell numbers could be either due to increased recruitment of monocytes or due to local proliferation of macrophages (van oud Alblas and van Furth. 1979). Local proliferation of macrophages could be one of the pathways, mainly in chronic lung inflammation (Bitterman *et al.* 1984) and such an event is very unlikely at 3 hours after the LPS stimulation (van oud Alblas and van Furth. 1979), time at which I observed an increase in

monocyte/macrophage numbers. The electron microscopic observations showed that the early increase in ED-1 cells was largely due to the monocytes present in septal microvessels, which may be a prelude to their migration into the interstitium and alveolar spaces. I counted ED-1 cells without making a distinction between the alveolar, microvascular and the interstitial because both macrophages and monocytes, whether present in the alveolar space or interstitium or microvasculature can influence the inflammatory process by producing free oxygen radicals and cytokines (Elias *et al.* 1985a; Elias *et al.* 1985b; Li *et al.* 1998; Maus *et al.* 2002a). Furthermore, monocytes are the source of renewal of pulmonary macrophages (van oud Alblas and van Furth. 1979) and produce more cytokines, such as IL-1β, than alveolar macrophages (Elias *et al.* 1985a). Therefore, an early increase in monocytes/macrophages in inflamed lungs may significantly impact the course of inflammation.

Next, I addressed the role of neutrophils in relation to MCP-1 expression in the early as well as the late phase of monocyte/macrophage recruitment by inducing lung inflammation in neutropenic rats. The data confirms the previously demonstrated role of neutrophils in the late phase of monocyte/macrophage increase in lung (Doherty *et al.* 1988; Maus *et al.* 2002b). Because early recruitment of monocytes/macrophages in lung inflammation has not been reported so far, the importance of neutrophils in this early monocyte/macrophage increase in the lung remains unexplored. Now, my experiments provide the data to show that neutrophil depletion blocks early increase in monocyte/macrophage numbers in inflamed lungs. Although neutropenia inhibited early increase in monocyte/macrophage in inflamed lungs, at 3 hours, the non-neutropenic LPS-challenged rats had very few neutrophils in their lungs. Therefore, the data suggest that even fewer numbers of neutrophils may mediate the early increase of monocyte/macrophage numbers and underscores the complexity of cellular and molecular interactions in the recruitment of monocytes/macrophages in lung inflammation. Interestingly, inhibition of monocyte/macrophage recruitment in neutropenic animals was not accompanied by expected suppression of MCP-1 expression. Consistent with previous observations, my observation with light and electron immunocytochemistry showed MCP-1 in neutrophils (Burn *et al.* 1994; Sakanashi *et al.* 1994; van Coillie *et al.* 1999). Because neutrophil depletion did not alter MCP-1 concentrations in inflamed lungs, these cells may not be major contributors of MCP-1. An intriguing fact remains that elevated concentrations of MCP-1 were not accompanied by an increase in numbers of monocytes/macrophages in inflamed lungs of neutropenic rats. At this

stage, I do not have an explanation for this apparent discord between MCP-1 expression and lack of monocyte/macrophage recruitment in inflamed lungs. One of the possibilities may be that neutrophil depletion alters the production of other mediators of inflammation such as IL-1β, IL-8, fibronectin and elastin, which participate in monocyte/macrophage sequestration (Abraham. 2003; Doherty *et al.* 1990; Fillion *et al.* 2001; Gerszten *et al.* 1999; Parsey *et al.* 1998; Senior *et al.* 1980; Yamamoto *et al.* 1998). Another explanation could be that an interaction between neutrophils and MCP-1 is required to signal monocyte/macrophage increases in inflamed lungs. Nevertheless, the data show significance of neutrophils as well as complexity of cellular and molecular interactions in the recruitment of monocytes/macrophages in inflamed lungs.

Despite novel observations on the early increase of monocyte/macrophage increase in acute lung inflammation, there are certain limitations to this study. First, I did not study the functional implication of early monocyte/macrophage increase. Next, I observed that even with increased MCP-1 concentration, monocyte/macrophage increase does not occur in the absence of neutrophils. Neither my study nor previous literatures offer any explanation about the possible mechanism. Since monocytes/macrophages are capable of influencing the inflammatory process, further studies including MCP-1 blocking in neutropenic animals are warranted to establish the functional significance of early increase in monocyte/macrophage numbers and to identify the complex interaction between neutrophils and MCP-1. Lastly, it will be important to explore, if neutrophil depletion alters expression of any other monocyte/macrophage chemoattractants.

CHAPTER 7: GENERAL DISCUSSION AND FUTURE DIRECTIONS

The overall objective of my experiments was to understand the mechanisms of neutrophil recruitment into the lungs and contribution of neutrophils to the inflammatory process. Specifically, I set out to understand the role of integrin $\alpha_v\beta_3$ in neutrophil migration, understand the modulation of expression of TLR4 and the role of neutrophils in monocyte recruitment in acute lung inflammation (Figure 2.1).

1) First, I evaluated the role integrin $\alpha_v\beta_3$ as a molecule mediating integrin β_2-independent neutrophil migration in the lungs (Chapters 3 & 4). I chose *S. pneumoniae* for my experiment, because this organism is known to induce integrin β_2-independent neutrophil migration in lungs (Doerschuk *et al.* 1990) and is a clinically important pathogen (Finn and Jenkinson. 2006; Kadioglu and Andrew. 2004). In the first experiment (Chapter 3) I compared expression of integrin subunits α_v and β_3 on neutrophils in response to *E. coli* and *S. pneumoniae*, known inducers of integrin β_2-dependent and –independent pathways, respectively (Doerschuk *et al.* 1990). I chose to study individual subunits α_v and β_3 instead of the heterodimer $\alpha_v\beta_3$ because, both the subunits are required for the functioning of the heterodimer and understanding one subunit will indirectly indicate the function of the heterodimer (Horton. 1997) . My experiment led to interesting observations that the expression of the integrin subunits is reduced in response to *E. coli* whereas the expression was unaltered in *S. pneumoniae* infection. This implied that integrin $\alpha_v\beta_3$ might have a role in *S. pneumoniae* induced neutrophil recruitment. But mere expression of integrin expression on neutrophils is not the proof of function. Therefore, I decided to evaluate the function of integrin $\alpha_v\beta_3$ by focusing on integrin subunit β_3 in mice. I focused selectively on β_3 because, unlike promiscuity of α_v, it dimerizes only with α_v. Fortunately, integrin $\beta_3^{-/-}$ mice and the function blocking antibodies were available. The data from antibody blocking and the knock out mice studies show that integrin subunit β_3 and therefore, integrin $\alpha_v\beta_3$ is not critical for *S. pneumoniae* induced neutrophil recruitment in the lungs.

2) While search for the molecule mediating integrin β_2-independent pathway still continues, my experiments make a significant contribution in the area of neutrophil recruitment by ruling out of the role of integrin $\alpha_v\beta_3$. My experiments along with the previous studies (Doerschuk *et al.* 1990; Mizgerd *et al.* 1996; Ridger *et al.* 2001; Tasaka *et al.* 2002) rule out the roles of all possible integrins that are expressed on neutrophils, in their recruitment in response to *S. pneumoniae*. My experiments rule out the requirements of integrin $\alpha_v\beta_3$ in the interactions of neutrophils with both endothelium and extracellular matrix. This creates a challenge to identify molecules involved in the neutrophil recruitment in conditions not regulated by β_2 integrins. In this situation we can think of few other possibilities. Many, instead of one single integrins might be involved in the neutrophil recruitment. Therefore, blocking multiple molecules may be more rewarding than the reductionist approach taken by me. Because of the complexity involved in blocking many molecules, we can also try to dampen some of the signaling molecules driving the inflammatory process. For example, molecules such as TLR4 and lipopolysaccharide binding protein are known to play important roles in innate immunity against *S. pneumonia* (Paterson and Mitchell. 2006). More understanding on these might help us to understand mechanisms that regulate the neutrophil recruitment in *S. pneumoniae* induced pneumonia. Another possibility could be to evaluate the role of several other molecules such as PECAM-1, JAM and CD99 in the context of lung inflammation. Since these molecules are essential for transendothelial migration (Britta Engelhardt. 2004; Muller. 2001), we could probably regulate neutrophil recruitment in response to multiple pathogens by interfering with one single molecule.

3) During evaluation of integrin subunit β_3's role using function blocking antibody and isotype matched antibodies, I observed reduced neutrophil recruitment in response to immunoglobulin (both isotype and β_3 antibody) treatment. While we need to interpret the finding with caution, it is exciting to consider future studies evaluating the mechanisms and effect of immunoglobulin treatment on lung inflammation in response to variety of inflammatory stimuli. It gains more importance in the light of recent finding that immunoglobulins interfere with adhesion of neutrophils onto the endothelium (Gill *et al.* 2005). A good way to start will be to evaluate the adhesion of neutrophils onto pulmonary microvascular endothelial cells *in vitro*, using different stimuli and immunoglobulin treatments.

4) Another novel observation was localization of integrins in the nucleus (Chapter 3) of neutrophils and endothelial cells. There are only two other reports on the nuclear localization of the integrins (Merono *et al.* 2002; Miller *et al.* 1987). Because of the integrin's ability to bind with many proteins, it will be interesting to see if they play a role in transporting some of the proteins into the nucleus. On the other hand, they might directly regulate gene expression and nuclear architecture. However, it is difficult to predict the function of these molecules unless we explore the proteins with which these molecules are interacting. *In vitro* experiments such as immunoprecipitation of integrins from cytoplasmic and nuclear fractions will help us to answer some of these questions.

5) In addition to the integrins, TLR4 has been shown to regulate neutrophil recruitment in the lung (Andonegui *et al.* 2003; Hollingsworth *et al.* 2005). But, there was no information available on the pattern of expression of TLR4 in lung inflammation, specifically at different times after the induction of inflammation. My experiments (Chapter 5) revealed that TLR4 expression is increased at 6 hours, the time of neutrophil increase, and reduced at 12-36 hours, the time of a decline in neutrophil migration, after the LPS treatment (Ulich *et al.* 1991). Thus, the expression pattern observed in my experiments supports the previous observations that TLR4 plays a role in neutrophil recruitment. This opens an exiting opportunity to think about therapeutic strategies to modulate TLR4 in inflammatory situations. One might argue that modulating TLR4 expression may interfere clearance of bacteria from the lung, but recent findings suggested that it may not be the case (Lee *et al.* 2005).

6) While understanding the expression of TLR4 in lung inflammation, I also observed localization of TLR4 and LPS in the nucleus. Although nuclear localization of LPS has been reported previously (Kang *et al.* 1990; Kang *et al.* 1992; Risco *et al.* 1991; Singh and Atwal. 1997), my study is the first to localize TLR4 in nucleus of various lung cells *in vivo*. Although, localization of LPS in the nucleus has been known for more than 25 years (Kang *et al.* 1990) and there is an evidence to its ability to bind with nuclear histone (Hampton *et al.* 1988), the function of LPS in the nucleus is not seriously examined. It was proposed that LPS in the nucleus might regulate the transcriptional activity (Kang *et al.* 1990; Kang *et al.* 1992; Risco *et al.* 1991). However, after the ability of TLR4 to recognize LPS and initiate signaling events was recognized

(Medzhitov *et al.* 1997), not much has been addressed on the localization of LPS. It is important and interesting to understand the molecules involved in transport of these molecules into the nucleus. Equally important will be to test if both TLR4 and LPS in the nucleus initiate anti-inflammatory signals to control the inflammation. Immunoprecipitation and electrophoretic mobility shift assays on nuclear fractions could provide important answers to these questions.

7) The other interesting observation in my experiments was the presence of two fractions of TLR4. One fraction was similar to the regular TLR4 in terms of molecular weight, while the other fraction was of lower molecular weight. Previously, a naturally occurring soluble form of TLR4 has been identified and a recombinant protein without transmembrane and cytoplasmic domain has been generated (Hyakushima *et al.* 2004; Iwami *et al.* 2000). Both of these variants of TLR4 have been shown to suppress TLR4 mediated signaling. In this regard, it is important to further characterize the lower molecular weight TLR4 observed in my experiments. It might have a role similar to other soluble forms reported and could probably be a potential mechanism involved in the regulation of inflammatory process.

8) My last objective was to address the mechanism of neutrophil dependent monocyte/macrophage recruitment in the lung. Specifically, I wanted to test if MCP-1 produced by neutrophils is the mechanism regulating neutrophil dependent monocyte/macrophage recruitment in lungs. Although, neutrophils expressed MCP-1, they were not the major source as depleting neutrophils did not affect the MCP-1 levels. However, my experiments confirmed the existence of neutrophil dependent monocyte recruitment in the lungs showing the critical role of neutrophils in regulating inflammatory process in the lung. Thus, we still do not understand the mechanism of monocyte/macrophage recruitment regulated by neutrophils and it is possible that other chemokines might be involved in regulating this mechanism. Also, I made an observation that monocyte/macrophage number increases in the lungs very early in the inflammatory process. Since monocytes/macrophages can significantly modulate the inflammatory process, functional significance of early recruitment of these cells in the inflammatory process needs to be addressed.

To conclude, my experiments furthered the understanding on the leukocyte recruitment in lung inflammation. In the process, my experiments also raised some important questions that are discussed above (depicted in Figure 7.1).

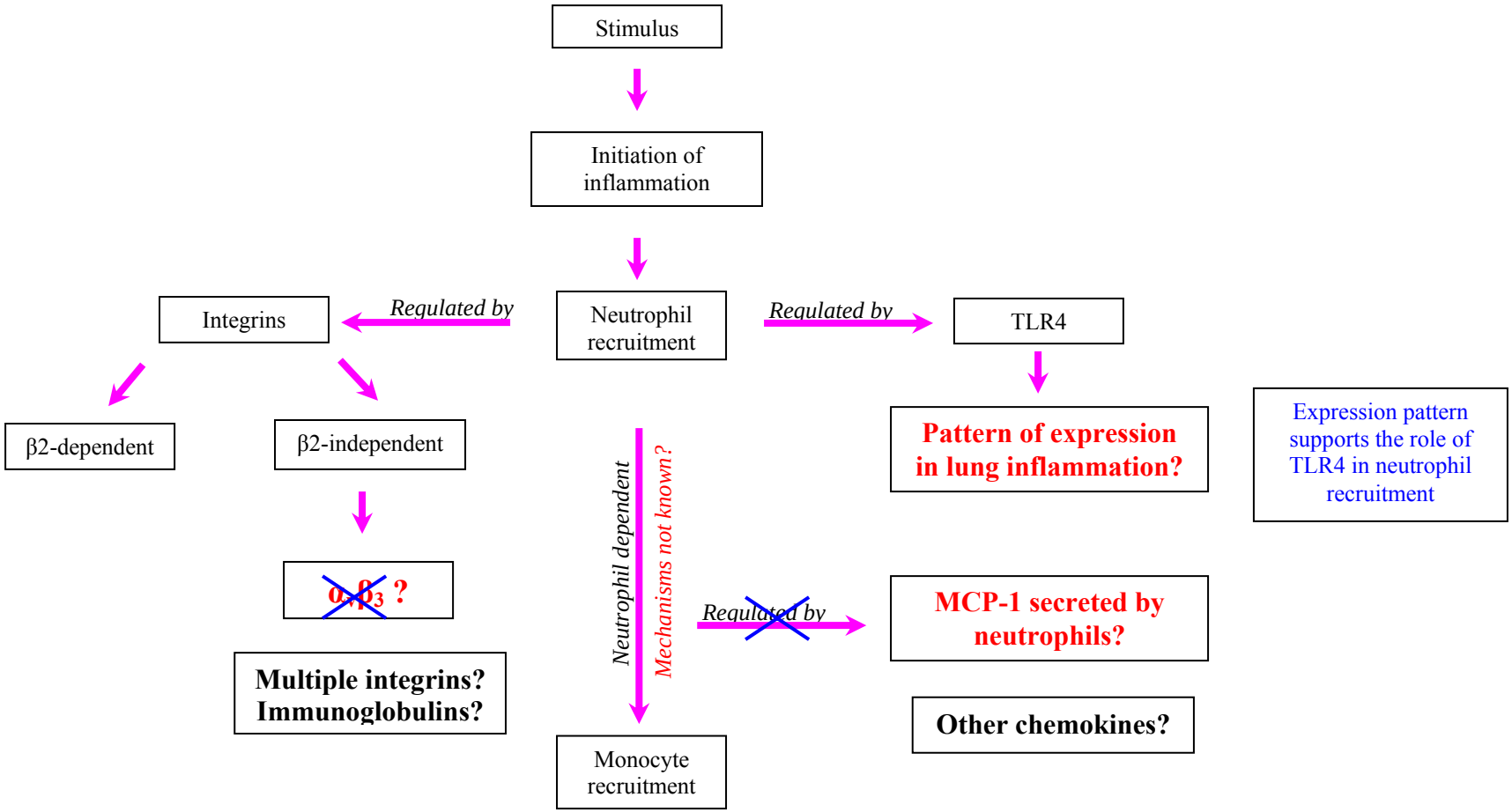

Figure 7. 1. What did my experiments contribute to the understanding of acute lung inflammation?

The questions asked before the experiments are in red. The answers to those questions, obtained from my experiments, are in blue. The questions presented in the black are some of many questions that my experiments have raised.

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