Histol Histopathol (2006) 21: 687-696

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Toll like receptor-4 expression in lipopolysaccharide induced lung inflammation

K.S. Janardhan[1], M. McIsaac[1], J. Fowlie[1], A. Shrivastav[2], S. Caldwell[1], R.K. Sharma[2] and B. Singh[1]

[1]Immunology Research Group and Department of Veterinary Biomedical Sciences and [2]Department of Pathology, Cancer Research Unit, Saskatchewan Cancer Agency, College of Medicine, University of Saskatchewan, Saskatoon, Canada

Summary. Bacterial lipopolysaccharides (LPS) initiate immune response through Toll-like receptor 4 (TLR4). Because many a times host is confronted with secondary bacterial challenges, it is critical to understand TLR4 expression following initial provocation. We studied TLR4 expression in rats at various times after intratracheal instillation of LPS. Although TLR4 mRNA was undetectable in normal lungs, it increased at 6h and 12h and declined at 36h 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. We conclude that TLR4 expression in lung is sustained up to 36 hours and that TLR4 and LPS are localized in the cytoplasm and nuclei of lung cells.

Key words: Innate immunity, TLR4 mRNA, Immuno-electron microscopy, Immunohistochemistry, Nucleus

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 (Dayer et al., 1993; Aderem, 2001; Andonegui et al., 2003). The inflammation thus engendered 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 (Muzio et al., 2000; Zarember and Godowski 2002; Andonegui et al., 2003; Armstrong et al., 2004; Muir et al., 2004). 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, we 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 (Cario et al., 2000; Abreu et al., 2001; Hausmann et al., 2002). These studies highlight the unsettled 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, we determined the expression of TLR4 mRNA and protein at various times following a single intratracheal

Offprint request to: Baljit Singh, BVSc&AH, PhD. Department of Veterinary Biomedical Sciences. University of Saskatchewan, Saskatoon, Canada. e-mail: baljit.singh@usask.ca

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. We 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.

Materials and methods

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).

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.

Tissue collection and processing

Tissues for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blots were snap frozen and stored at -80°C. Lung pieces for histology and immunohistology were fixed in 4% paraformaldehyde for 16 hours followed by dehydration and embedding in paraffin. Lung samples for immunoelectron microscopy were fixed in 2% paraformaldehyde containing 0.1% glutaraldehyde for 3 hours at 4°C followed by dehydration and embedding and polymerization in white resin (London resin company, USA).

Preparation of whole lung homogenates and nuclear fractionation

Frozen lung samples were homogenized in RIPA 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 25000 g for 20 minutes. 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 1.5M sucrose and spun for 15 min at 35000 rpm in Beckman L8-55 ultracentrifuge using Type-45 T1 rotor. Even though, method described by Spector et al. (1998) suggests using 2 M sucrose for rat liver nuclei, we were not able to precipitate nuclei either at 2 M or at 1.75 M. The pallet containing nuclei was washed twice with the lysis buffer and resuspended in 300 µl of RIPA lysis buffer.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen Inc., ON, Canada) following manufacturer's protocol. Thirty mg of frozen lung tissues were homogenized in 600 µL of buffer, using a glass-homogenizer 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'-CATGAAGGC CTCCCTGGTGTT and the reverse primer was 5'-TGCCAGAGCGGCTACTCAGAA. For GADPH (298bp), forward primer was 5'- TGAAGGTCGGTGT GAACGGATTTGG 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 70°C for 10 minutes followed by addition of 9 µl of reaction mixture (5 µl of 5X-revert aid buffer, 0.5 µl 10mM dNTP, 1.5 µl water, 1 µl RNase out and 1 µl Revert Aid enzyme). The samples were held at 42°C for 30 minutes followed by incubation at 85°C 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 25mM dNTP mixture, 2 µl each of 25 pmol forward and reverse primers and 0.25 µl Taq DNA polymerase). After the initial denaturation at 94°C for 3 minutes, 40 cycles were carried out as follows (TLR4: 94°C for 30 sec, 57°C for 30 sec and 72°C for 45 sec; GAPDH: 94°C for 1minute, 65°C for 2 minutes and 72°C for 3 minutes). This was followed by a final extension step at 72°C 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 ImagerTM (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.

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, transferred to a nitrocellulose membrane and blocked with 7.5% skim milk powder in PBS with 0.1% Tween20 (PBST). For whole lung extracts, the membrane was incubated with anti-rat TLR4 (1:100) and actin (1:250) antibodies. For nuclear extracts, the membrane was probed with TLR4 and Lamin (1:200) antibodies. After washing with PBST for 30 minutes, membranes were incubated with anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (1:7500) followed by washing with PBST for 30 minutes. Antigen-antibody complex was visualized using enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech, UK). All the antibodies for Western blots were purchased from Santa Cruz Biotechnology Inc. USA. Use of isotype matched goat immunoglobulins instead of primary antibodies served as a negative control. For TLR4 in whole lung homogenates, spot densitometry was performed using Alpha Imager™ (Alpha innotech corp., USA) and the results were interpreted using the average pixel value. The values were normalized to the expression of actin and are presented as ratio of TLR4: Actin.

Histopathology

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

Immunohistochemistry

Immunohistology protocols have been described previously (Janardhan et al., 2004). Briefly, the sections were deparaffinized, treated with 5% hydrogen peroxide and with pepsin (2 mg/ml in 0.01N hydrochloric acid; Sigma, St. Louis MO, USA) followed by blocking with 1% bovine serum albumin (Sigma, St. Louis MO, USA). Sections were incubated with primary antibodies against rat TLR4 (1:50; M-16, Santa Cruz Biotechnology Inc. CA, USA) or *E. coli* LPS (1:300, Cedarlane Laboratories Limited, ON, Canada) antibody followed by anti-goat HRP-conjugated secondary antibodies (1:100; DAKO Diagnostics Canada Inc., ON, Canada). The antigen-antibody complex was visualized using a color development kit (VIP, Vector laboratories, USA). Controls consisted of staining without primary antibody or with isotype matched immunoglobulin instead of primary antibody. Proper quenching of endogenous peroxidase was confirmed by omitting both primary and secondary antibodies.

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 min) antibodies and anti-goat 15 nm gold-conjugated secondary antibodies (1:100; for 30 min). This procedure was repeated using anti-LPS antibody (1:175) and anti-rabbit 10nm gold-conjugated antibodies (1:100; for 30 min) 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.

Results

Lung inflammation

Lung sections were stained with hematoxylin and eosin to evaluate LPS-induced inflammatory response. Control rats instilled intratracheally with endotoxin-free saline showed normal lung histology (Fig. 1A). Lung sections from rats treated with *E. coli* LPS showed perivascular, peribronchiolar and alveolar infiltration of neutrophils and mononuclear phagocytes (Fig. 1B). 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 (Fig. 1C).

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 (Fig. 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 returned to minimal levels at 36 hours after the treatment.

TLR4 protein expression

We examined TLR4 protein expression with Western blots on lung homogenates (Fig. 3). Lungs from the control and all of the treated rats showed TLR4 protein (Fig. 3). However, we observed two bands for TLR4; one at ~87kD and another at ~69kD. Spot densitometry for TLR4 at ~87kD showed a decrease in TLR4 at 6

hours followed by an increase at later time points. Spot densitometry for TLR4 at ~69kD showed unaltered TLR4 expression at 6 hours followed by an increase at later time points compared to the controls.

TLR4 immunohistochemistry

We used immunohistochemistry to precisely identify the cells expressing TLR4. Lung sections stained only with secondary antibody (Fig. 4A) or isotype-matched antibody (Fig. 4B) lacked any staining and ruled out non-specific binding of antibodies. Previously, using a blocking peptide, we have also shown the specificity of the TLR4 antibody which is used in this experiment (Wassef et al., 2004). The immunohistochemical data are summarized in Table1. 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 (Fig. 4C). Compared to the controls, the 6 hour post-LPS lungs showed intense TLR4 staining in the septum and infiltrating neutrophils (Fig. 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 (Fig. 4E).

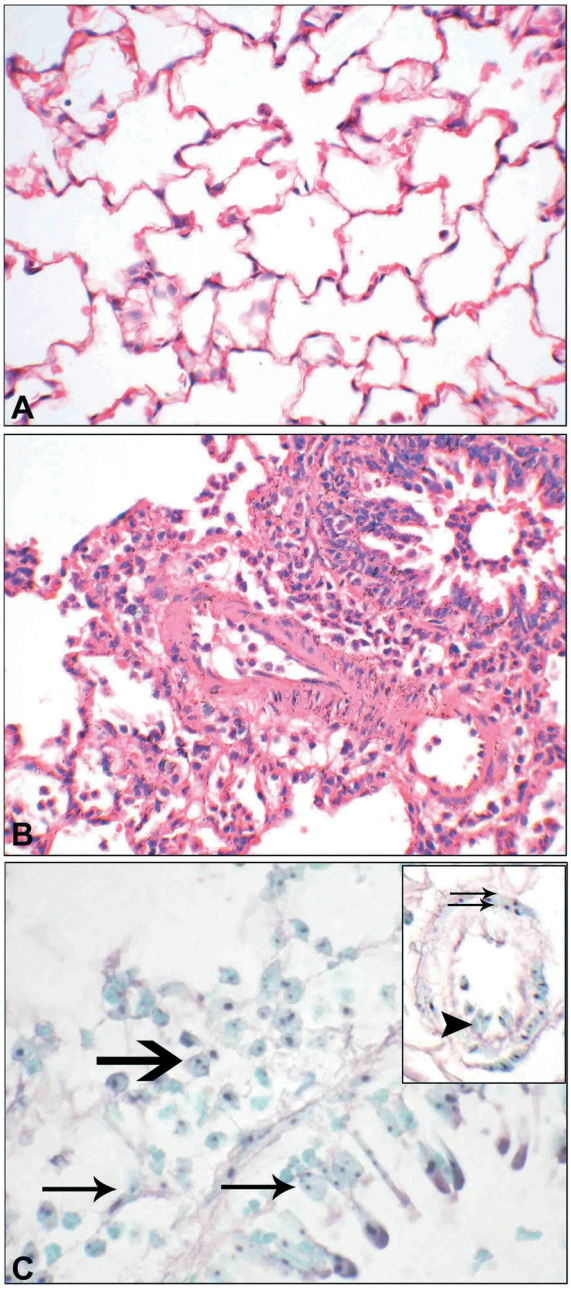


Fig. 1. *E. coli*-LPS induced lung inflammation: Saline treated lungs showed normal histology **(A)**. Instillation of LPS resulted in inflammation characterized by infiltration of inflammatory cells into the perivascular, peribronchiolar and alveolar spaces **(B)**. Instillation of LPS was confirmed using an anti-LPS antibody **(C)**. LPS was localized in the epithelium (arrows), macrophages (large arrow), endothelium (arrowhead) and smooth muscle cells (double arrow). Original magnification A-B, x 400; C, x 1000

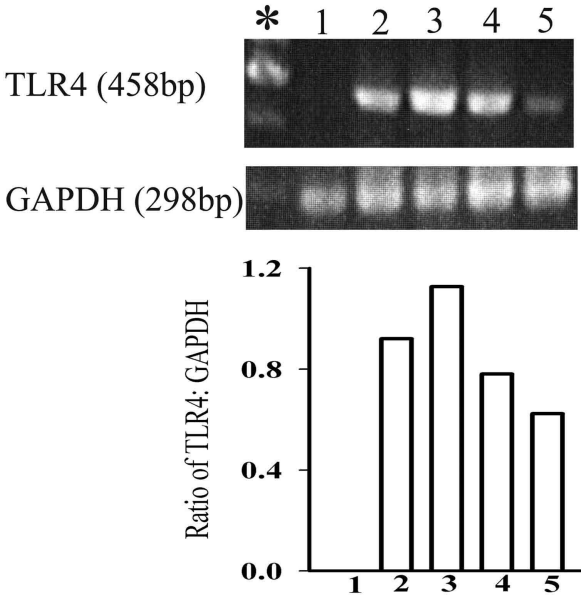


Fig. 2. Expression of TLR4 mRNA in the lung: RT-PCR on RNA extracted from the saline- (1) and LPS-treated [6 hours (2), 12 hours (3), 18 hours (4) and 36 hours (5)] rat lungs showed expression of TLR4 only in LPS-treated lungs. Densitometric evaluation (average pixel value expressed as ratio of TLR4: 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.

TLR4 immunoelectron microscopy

We 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 (Fig. 5A). Lung sections from the control and the treated rats contained TLR4 staining in the macrophages (Fig. 5B), Type I and II alveolar epithelial cells, microvascular and macrovascular endothelium, monocytes, neutrophils (Fig. 5C-F) 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 (Fig. 5B-E). Although LPS and TLR4 were co-localized 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, it was detected in only a few cells (Figs. 5B-E). Saline-treated control lungs showed a negligible labeling for LPS.

TLR4 in lung nuclear extracts

We confirmed immuno-electron 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 (Fig. 6). The blots probed with TLR-4 antibody revealed two bands, approximately 87kD and 69kD, similar to the proteins observed in the crude lung extracts.

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, we 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 immuno-electron 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. We also report co-localization of TLR4 and LPS in the cytoplasm and nucleus of various lung cells.

We 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

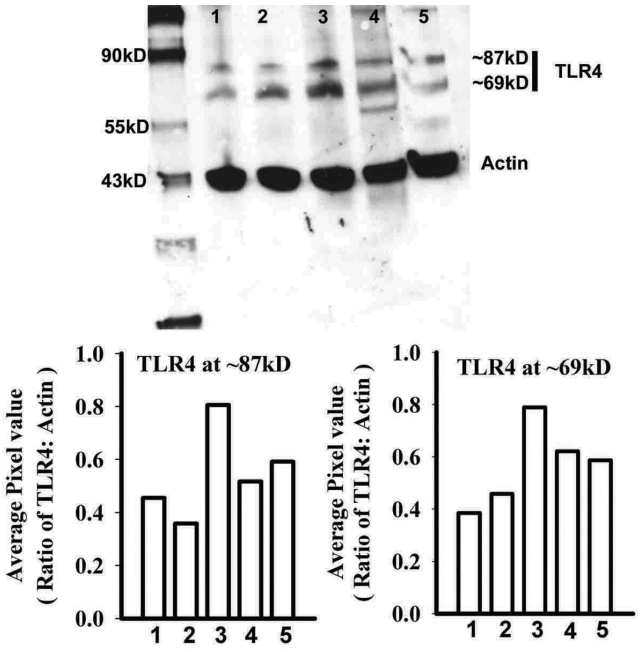


Fig. 3. Expression of TLR4 protein in the lung: Western blots on lung homogenates from the saline (1) - and LPS-treated [6 hours (2), 12 hours (3), 18 hours (4) and 36 hours (5)] rat lungs showed expression of TLR4 at all the time points. There were two bands for TLR4; one at ~87kD and another at ~69kD.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

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

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

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

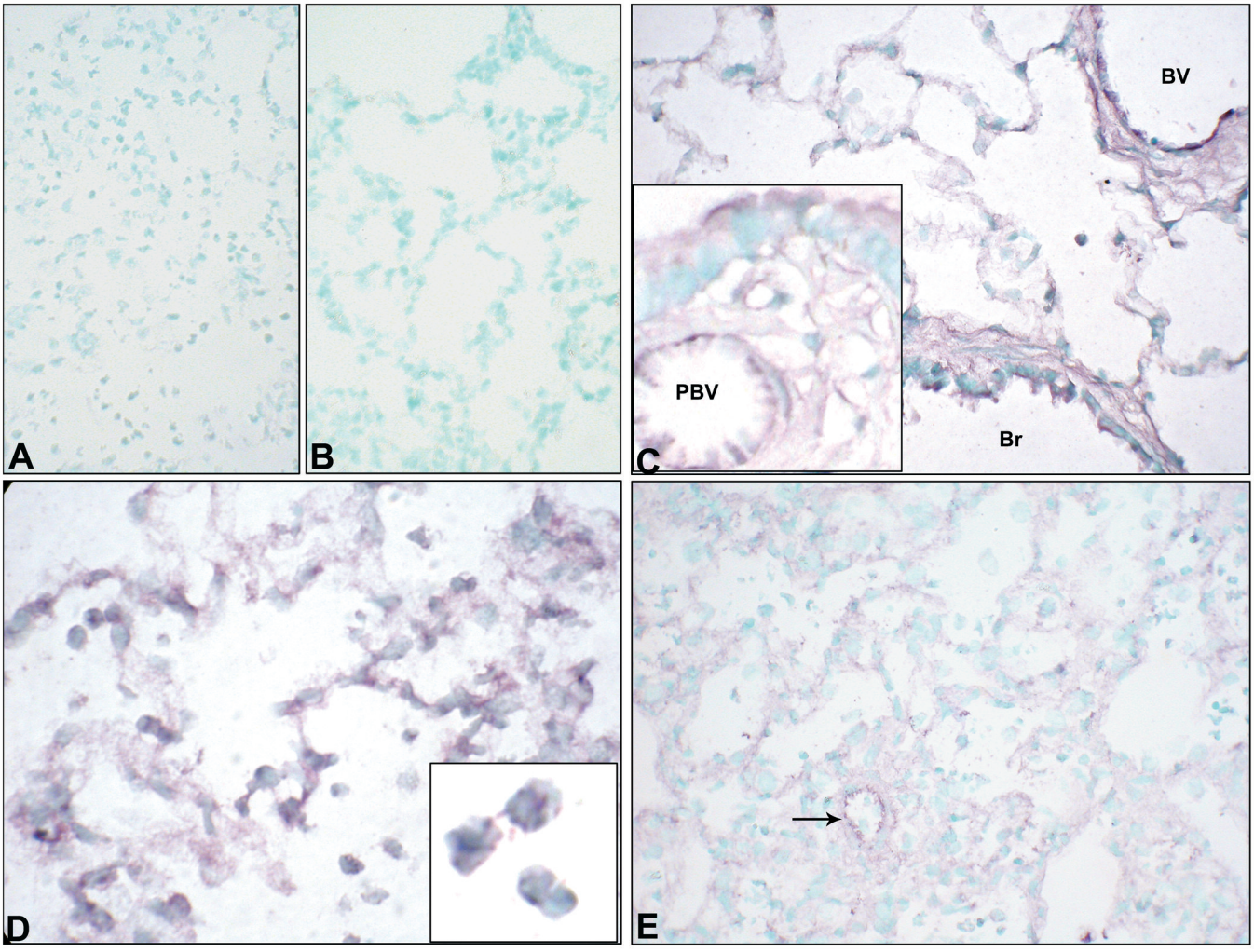
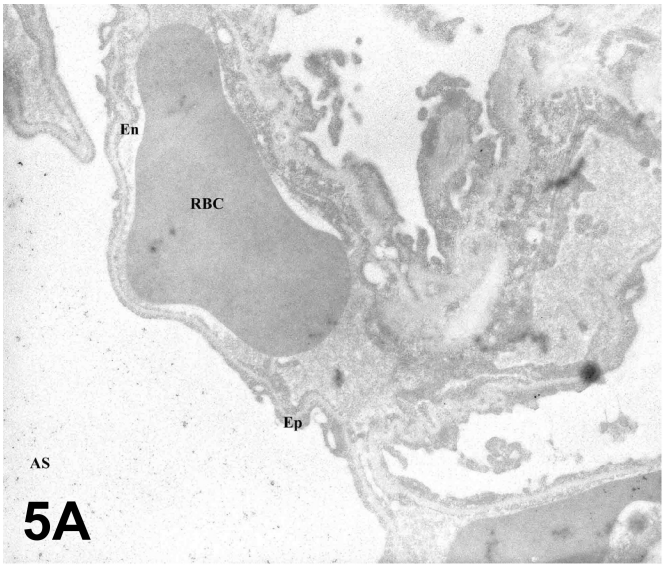
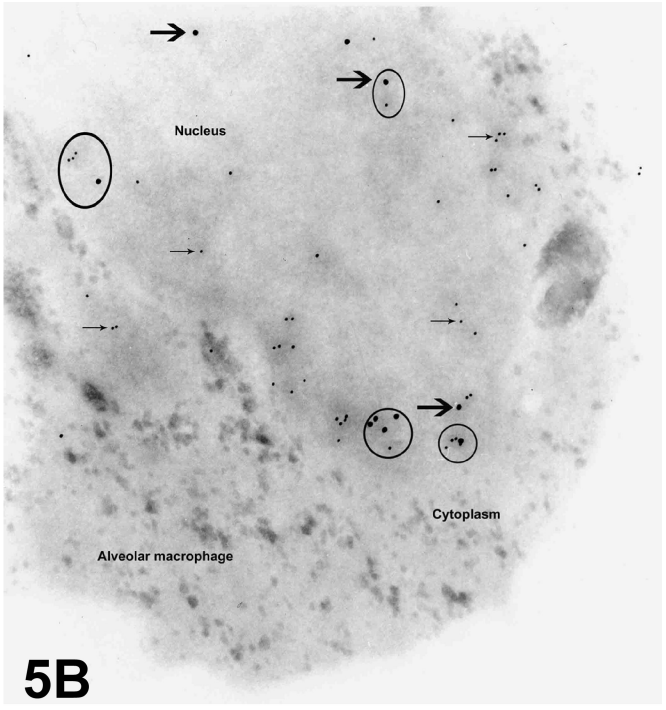
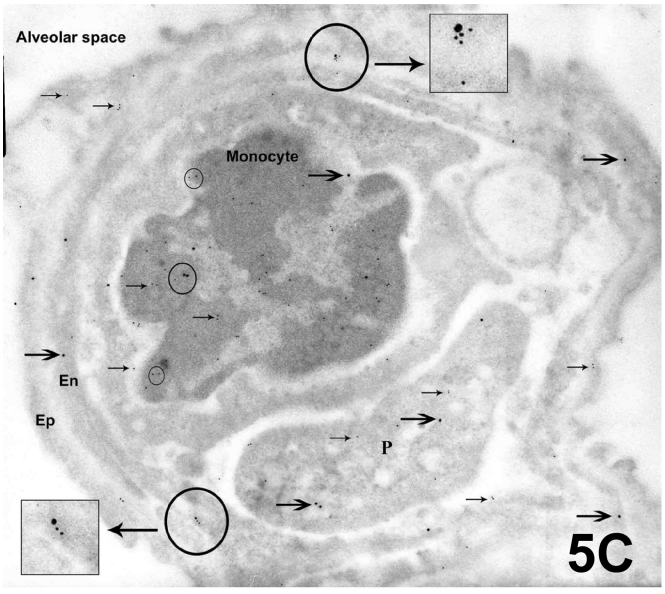


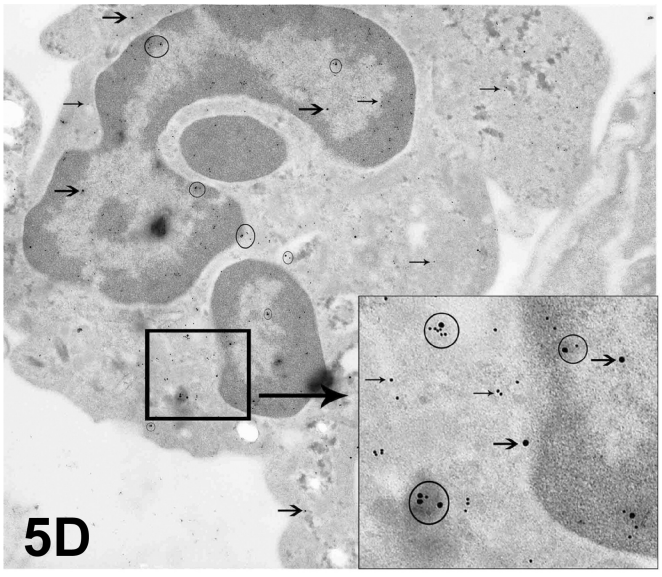
Fig. 4. TLR4 immunohistochemistry. No reaction in the TLR4 antibody omitted **(A)** and isotype matched immunoglobulin treated **(B)** lung sections ruled out non-specific reactions. In saline treated lung sections **(C)**, minimal expression was observed in few of the septal cells and in the bronchiolar 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. Original magnification: A-C, E, x 400; D, x 1,000; Insets, x 2,000

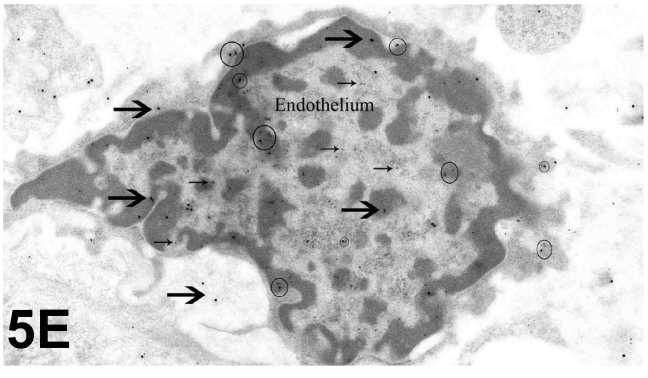
Fig. 5. A. Negative control: Dual immunogold labeling on a lung section with isotype matched rabbit and goat immunoglobulins showed negligible labeling. AS: alveolar space; RBC: Red blood cell; En: endothelium; Ep: epithelium. x 17,500. **B.** Dual labeling for TLR4 and LPS: Electron micrograph of an 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). x 56,000. **C.** Dual labeling for TLR4 and LPS: 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). x 36,000. Insets, x 72,000. **D.** Dual labeling for TLR4 and LPS: 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). x 30,000. Inset, x 52,000. **E.** Dual labeling for TLR4 and LPS: 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). x 30,400. **F.** Dual labeling for TLR4 and LPS: 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). x 30,400

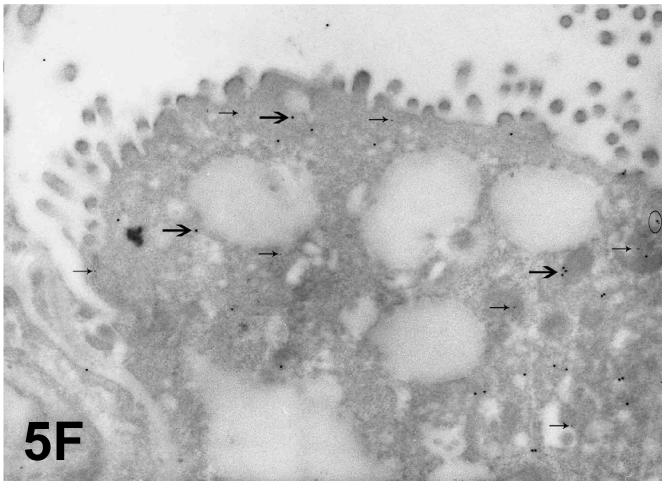

En
RBC
Ep
AS
5A


Nucleus
Cytoplasm
Alveolar macrophage
5B


Alveolar space
Monocyte
En
Ep
P
5C


5D


Endothelium
5E


5F

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 ~87kD and at ~69kD and the first band is closer to the reported molecular weight of regular TLR4. We have not addressed the origin or the function of 69kD band. Although this band could result from proteolysis of TLR4 molecule, we did include sufficient amounts of protease inhibitors in the lysis buffer and we 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 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 80kD (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 preibronchiolar 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.

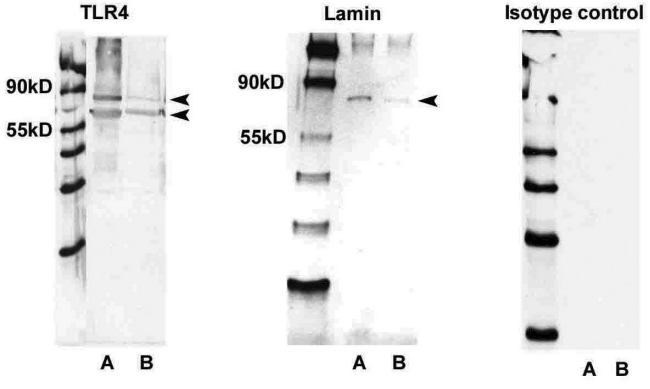


Fig. 6. TLR4 in lung nuclear extracts. Western blots on lung nuclear extracts showed presence of TLR4 in the nucleus. Similar to the crude lung extracts, we observed two bands; one at ~87kD and another at 69kD. Presence of a nuclear protein, lamin, confirmed that it is a nuclear fraction and probing 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.

Our immuno-electron 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* (Hornef et al., 2003; Guillot et al., 2004). 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 (Hornef et al., 2003; Guillot et al., 2004). However, intracellular versus surface localization of TLR4 may have no bearing on LPS responsiveness, because even intracellular interactions of LPS with TLR4 can initiate 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. We further strengthened this observation by demonstrating with Western blots that TLR4 is present 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 our knowledge this is the first report to demonstrate nuclear localization of TLR4. Furthermore, our data show co-localization of TLR4 with LPS in the nuclei of various lung cells. Although we and others have reported rapid trafficking of LPS into the nuclei of macrophages (Kang et al., 1990; Risco et al., 1991; Singh and Atwal 1997), our new data provide first evidence of nuclear localization of an LPS signaling receptor. Our 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 co-localization with LPS in future studies.

To summarize, our study shows that TLR4 expression is sustained, with few cell specific variations, 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 our study creates a need to explore the mechanisms involved in trafficking of TLR4 from intracellular compartments to the cell surface and the functional consequences of TLR4 and LPS localization in the nucleus.

Acknowledgements. The work was supported through a Discovery Grant from Natural Sciences and Engineering Research Council of Canada and a Biomedical Establishment Grant from Saskatchewan Health Research Foundation to Dr. Baljit Singh. Dr. Janardhan is supported through a University of Saskatchewan Graduate Merit Scholarship and Ms. Jennifer Fowlie was a recipient of an Interprovincial Summer Student Research Award from Western College of Veterinary Medicine.

References

Abreu M.T., Vora P., Faure E., Thomas L.S., Arnold E.T. and Arditi M. (2001). Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J. Immunol. 167, 1609-1616.

Aderem A. (2001). Role of Toll-like receptors in inflammatory response in macrophages. Crit. Care. Med. 29, S16-S18.

Akashi S., Shimazu R., Ogata H., Nagai Y., Takeda K., Kimoto M. and Miyake K. (2000). Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J. Immunol. 164, 3471-3475.

Andonegui G., Bonder C.S., Green F., Mullaly S.C., Zbytnuik L., Raharjo E. and Kubes P. (2003). Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. J. Clin. Invest. 111, 1011-1020.

Armstrong L., Medford A.R., Uppington K.M., Robertson J., Witherden I.R., Tetley T.D. and Millar A.B. (2004). Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. Am. J. Respir. Cell. Mol. Biol. 31, 241-245.

Cario E., Rosenberg I.M., Brandwein S.L., Beck P.L., Reinecker H.-C. and Podolsky D.K. (2000). Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing toll-like receptors. J. Immunol. 164, 966-972.

Curtis J.L., Warnock M.L., Arraj S.M. and Kaltreider H.B. (1990). Histologic analysis of an immune response in the lung parenchyma of mice: angiopathy accompanies inflammatory cell influx. Am. J. Pathol. 137, 689-699.

Davidson T.A., Caldwell E.S., Curtis J.R., Hudson L.D. and Steinberg K.P. (1999). Reduced quality of life in survivors of acute respiratory distress syndrome compared with critically ill control patients. JAMA 281, 354-360.

Dayer J.-M., Isler P. and Nicod L.P. (1993). Adhesion molecules and cytokine production. Am. Rev. Resp. Dis. 148, S70-S74.

Espevik T., Latz E., Lien E., Monks B. and Golenbock D.T. (2003). Cell distributions and functions of Toll-like receptor 4 studied by fluorescent gene constructs. Scand. J. Infect. Dis. 35, 660-664.

Fan J., Kapus A., Marsden P.A., Li Y.H., Oreopoulos G., Marshall J.C., Frantz S., Kelly R.A., Medzhitov R. and Rotstein O.D. (2002). Regulation of toll-like receptor 4 expression in the lung following hemorrhagic shock and lipopolysaccharide. J. Immunol. 168, 5252-5259.

Flo T.H., Halaas O., Torp S., Ryan L., Lien E., Dybdahl B., Sundan A. and Espevik T. (2001). Differential expression of Toll-like receptor 2 in human cells. J. Leukoc. Biol. 69, 474-481.

Guillot L., Medjane S., Le Barillec K., Balloy V., Danel C., Chignard M. and Si-Tahar M. (2004). Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like Receptor 4 (TLR4)-dependent signaling pathways: Evidence for an intracellular compartmentalization of TLR4. J. Biol. Chem. 279, 2712-2718.

Hausmann M., Kiessling S., Mesterman S., Webb G., Spottl T., Andus T., Scholmerich J., Ray K., Falk W. and Rogler G. (2002). Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroentrology 122, 1987-2000.

Hollingsworth J.W., Chen B.J., Brass D.M., Berman K., Gunn M.D., Cook D.N. and Schwartz D.A. (2005). The critical role of hematopoietic cells in lipopolysaccharide induced airway inflammation. Am. J. Respir. Crit. Care. Med. 171, 806-813.

Hornef M.W., Normark B.H., Vandewalle A. and Normark S. (2003). Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. J. Exp. Med. 198, 1225-1235.

Hyakushima N., Mitsuzawa H., Nishitani C., Sano H., Kuronuma K., Konishi M., Himi T., Miyake K. and Kuroki Y. (2004). Interaction of soluble form of recombinant extracellular tlr4 domain with md-2 enables lipopolysaccharide binding and attenuates TLR4-mediated signaling. J. Immunol. 173, 6949-6954.

Ichikawa S., Shiozawa Y. and Uchino S. (1996). Immune cell migration through the arterial wall in the murine lung during a pulmonary inflammatory response. Arch. Histol. Cytol. 59, 87-96.

Iwami K.I., Matsuguchi T., Masuda A., Kikuchi T., Musikacharoen T. and Yoshikai Y. (2000). Cutting Edge: Naturally occurring soluble form of mouse toll-like receptor 4 inhibits lipopolysaccharide signaling. J. Immunol. 165, 6682-6686.

Janardhan K.S., Appleyard G.D. and Singh B. (2004). Expression of integrin subunits alphav and beta3 in acute lung inflammation. Histochem. Cell. Biol. 121, 383-390.

Kang Y.-H., Dwivedi R.S. and Lee C.-H. (1990). Ultrastructural and immunocytochemical study of the uptake and distribution of bacterial lipopolysaccharide in human monocytes. J. Leukoc. Biol. 48, 316-332.

Matthay M.A., Zimmerman G.A., Esmon C., Bhattacharya J., Coller B., Doerschuk C.M., Floros J., Gimbrone M.A. Jr., Hoffman E., Hubmayr R.D., Leppert M., Matalon S., Munford R., Parsons P., Slutsky A.S., Tracey K.J., Ward P., Gail D.B. and Harabin A.L. (2003). Future research directions in acute lung injury: Summary of a National Heart, Lung, and Blood Institute Working Group. Am. J. Respir. Crit. Care Med. 167, 1027-1035.

Muir A., Soong G., Sokol S., Reddy B., Gomez M.I., van Heeckeren A. and Prince A. (2004). Toll-Like Receptors in normal and cystic fibrosis airway epithelial cells. Am. J. Respir. Cell. Mol. Biol. 30, 777-783.

Muzio M., Bosisio D., Polentarutti N., D'amico G., Stoppacciaro A., Mancinelli R., van't Veer C., Penton-Rol G., Ruco L.P., Allavena P. and Mantovani A. (2000). Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164, 5998-6004.

Nomura F., Akashi S., Sakao Y., Sato S., Kawai T., Matsumoto M., Nakanishi K., Kimoto M., Miyake K., Takeda K. and Akira S. (2000). Cutting edge: Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. J. Immunol. 164, 3476-3479.

Otte J.M., Cario E. and Podolsky D.K. (2004). Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology 126, 1054-1070.

Pabst R. and Tschernig T. (2002). Perivascular capillaries in the lung: An important but neglected vascular bed in immune reactions? J. Allergy. Clin. Immunol. 110, 209-214.

Punturieri A., Alviani R.S., Polak T., Copper P., Sonstein J. and Curtis J.L. (2004). Specific engagement of TLR4 or TLR3 does not lead to IFN-{beta}-mediated innate signal amplification and stat1 phosphorylation in resident murine alveolar macrophages. J. Immunol. 173, 1033-1042.

Risco C., Carrascosa J.L. and Bosch M.A. (1991). Uptake and subcellular distribution of Escherichia coli lipopolysaccharide by isolated rat type II pneumocytes. J. Histochem. Cytochem. 39, 607-615.

Sha Q., Truong-Tran A.Q., Plitt J.R., Beck L.A. and Schleimer R.P. (2004). Activation of airway epithelial cells by toll-like receptor agonists. Am. J. Respir. Cell. Mol. Biol. 31, 358-364.

Singh B. and Atwal O.S. (1997). Ultrastructural and immunocytochemical study of the pulmonary intravascular macrophages of *Escherichia coli* lipopolysaccharide-treated sheep. Anat. Rec. 247, 214-224.

Spector D.L., Goldman R.D. and Leinwand L.A. (1998). Preparation of nuclei from tissue and suspension cultures. In: Cells. A laboratory manual. Cold Spring Harbor Laboratory Press. New York. pp 43.1-43.6.

Takeda K., Kaisho T. and Akira S. (2003). Toll-like receptors. Annu. Rev. Immunol. 21, 335-376.

Ulich T.R., Watson L.R., Yin S., Guo K., Wang P., Thang H. and del Castillo J. (1991). The intratracheal administration of endotoxin and cytokines: Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. Am. J. Pathol. 138, 1485-1496.

Wassef A., Janardhan K., Pearce J.W. and Singh B. (2004). Toll-like receptor 4 in normal and inflamed lungs and other organs of pig, dog and cattle. Histol. Histopathol. 19, 1201-1208.

Zarember K.A. and Godowski P.J. (2002). Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol. 168, 554-561.

Accepted November 24, 2005