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Real-time imaging reveals endothelium-mediated leukocyte retention in LPS-treated lung microvessels

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Abstract

Endotoxemia, a major feature of sepsis, is a common cause of acute lung injury and initiates rapid accumulation of leukocytes in the lung vasculature. Endothelial mechanisms that underlie this accumulation remain unclear, as current experimental models of endotoxemia are less suitable for targeted activation of the endothelium. Toward elucidating this, we used the isolated blood-perfused rat lung preparation. With a microcatheter inserted through a left atrial cannula, we cleared blood cells from a small lung region and then infused lipopolysaccharide (LPS) into microvessels. After a Ringer's wash to remove residual LPS, we infused fluorescently-labeled autologous leukocytes and imaged their transit through the treated microvessels. Image analysis revealed that leukocytes infused 90 min after LPS treatment, were retained more in treated venules and capillaries than untreated vessels. Further, pretreatment with either the intercellular adhesion molecule (ICAM-1) mAb or polymyxin-B blunted LPS-induced leukocyte retention in both microvessel segments. In addition, retention of leukocytes treated *ex vivo* with LPS in LPS-treated microvessels was higher compared to retention of untreated leukocytes. *In situ* immunofluorescence experiments revealed that LPS significantly increased microvessel ICAM-1 expression at 90 min post treatment. Polymyxin pretreatment inhibited this increase. Taken together, the data suggest that LPS increased leukocyte retention in both venules and capillaries and this response was mediated by the increased expression of endothelial ICAM1. Thus, endothelial mechanisms may themselves play a major role in LPS-induced leukocyte retention in lung microvessels. Blunting the endothelial responses may mitigate endotoxin-induced morbidity.

Keywords

Capillaries; Confocal Microscopy; Immunofluorescence; Rhodamine 6G; TLR4; Venules

Introduction

Sepsis, a major cause of acute lung injury, continues to exact a high mortality rate (Erikson 2009, Melamed and Sorvillo 2009). LPS from gram-negative bacteria, a primary cause for the pathogenesis of sepsis, induces rapid leukocyte accumulation in the pulmonary

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vasculature. Established experimental models of endotoxemia, including LPS administration through either the systemic or intra-tracheal route, initiate accumulation of leukocytes by activating them concomitantly with the pulmonary vascular endothelium. While the relevant mechanisms that underlie the increased accumulation have been well defined for leukocytes, those for pulmonary microvascular endothelium remain unclear.

Previous reports suggest that the pulmonary endothelium may contribute to LPS-induced responses. Intratracheal administration of either gram-negative bacteria or aerosolized LPS increases ICAM-1 expression and leukocyte sequestration in an endothelium-dependent manner (Kumasaka et al. 1996, Doyle et al. 1997, Mizgerd et al. 2004, Basit et al. 2006). Moreover, LPS administration into the systemic vasculature augments selectin-dependent endothelial-leukocyte interaction and leukocyte accumulation in lung microvessels (Kuebler et al. 2000). Similarly, intraperitoneal LPS administration induced P-selectin dependent leukocyte accumulation in microvessels and migration into the alveolar space (Kamochi et al. 1999). However, these reports do not separately define the individual contributions of the endothelium and leukocytes to this response. As leukocyte binding to endothelium by itself triggers endothelial signaling and cytoskeletal changes (Wang and Doerschuk, 2001, Wang et al. 2001), the above in vivo models may not selectively define the endothelial role in LPS-induced leukocyte accumulation.

Since activation reduces the deformability of leukocytes, their sequestration in the tortuous pulmonary microvascular network is augmented (Worthen et al. 1989, Aoki et al. 1997, Yoshida et al. 2006). Further, it is well established that LPS signals via the Toll-like Receptor 4 (TLR4) (Poltorak et al. 1998, Chow et al. 1999). In a recent report, it was suggested that endothelial, but not leukocyte, TLR4 plays a predominant role in LPS-induced responses in lung (Andonegui et al. 2003). These findings support the possibility that endothelial mechanisms are important in the induction of LPS-induced proinflammatory responses and leukocyte accumulation, likely during the prolonged response that follows LPS administration. Thus, experimental models that activate the pulmonary microvasculature selectively are necessary to elucidate endothelial signaling that underlies LPS-induced leukocyte accumulation.

Dynamic observations of endothelium-dependent leukocyte sequestration in lung remain limited. Intravital microscopy of untreated lung microvessels reveals that nearly half of all leukocytes entering the pulmonary microvessels transiently stop in capillaries (Gebb et al. 1995, Kuebler et al. 2000). Using similar methods, other studies show that leukocytes accumulate in pulmonary arterioles, capillaries and venules, and that the extent of this accumulation is dependent on blood flow velocity (Kuhnle et al. 1995, Ichimura et al. 2005) and leukocyte activation (Aoki et al. 1997). Though other methods have been used to quantify LPS-induced leukocyte sequestration (Doyle et al. 1997, Kamochi et al. 1999, Basit et al. 2006, Yoshida et al. 2006), they do not adequately establish the dynamic characteristics of the accumulation. Thus, intravital microscopy may better define the dynamics of leukocyte transit and retention in LPS-treated microvessels and help elucidate the endothelial mechanisms that contribute to leukocyte accumulation.

Toward this we utilized isolated blood-perfused rat lungs. In a small region of the lung, we cleared microvessels of all blood cells using a microcatheter and then infused LPS. Subsequently, we infused fluorescently-labeled autologous leukocytes. Real-time fluorescence images captured during the infusion of these untreated leukocytes revealed that their retention was significantly augmented in both LPS-treated capillaries and venules. These data suggested that endothelial mechanisms played a significant role in LPS-induced leukocyte retention.

Material and Methods

Lung preparation

Animals were treated in accord with protocols approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. Animals were given *ad libitum* access to food and water and placed on a 12 hr light-dark cycle. We used adult male Sprague-Dawley rats weighing 250–350g and C57/B6 mice weighing 25–35g, and prepared isolated blood perfused rat and mouse lungs, respectively, as detailed previously (Parthasarathi et al. 2006). Briefly, anesthetized rats and mice were exsanguinated by cardiac puncture. The lungs were then excised and continuously pump-perfused at 14 ml/min (rat) and 1 ml/min (mouse) with autologous blood warmed to 37°C. The isolated lungs were constantly inflated at an airway pressure of 5 cmH₂O. The pulmonary artery and left atrial pressures were maintained at 10 and 3 cmH₂O, respectively. The lungs were positioned under a microscope on a vibration-free air table. The lung surface was kept moist with saline.

Fluorescent dyes and reagents

We used the fluorescent marker rhodamine 6G (R6G; Sigma Aldrich, St. Louis, MO) to label leukocytes. LPS from *E. coli* (serotype 0111:B4) was from Sigma. mAbs against intercellular cell adhesion molecule-1 (ICAM-1) and L-selectin were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated goat anti-mouse secondary Ab was from Invitrogen (Carlsbad, CA). The nuclear stain Hoechst 33342 was from sigma. The TLR4 inhibitor, polymyxin-B and the fluorescent marker, FITC-Dextran 70kD (FDx70) were from Sigma. For leukocyte isolation, we used the Ficoll gradient, Histopaque-10771 (Sigma). Agents were infused into microvessels in calcium-rich HEPES-buffered Ringer's solution (150 mM Na⁺, 5 mM K⁺, 1.0 mM Ca²⁺, 1 mM Mg²⁺, 10 mM glucose and 20 mM HEPES) with 4% dextran and 1% fetal bovine serum. Final Ringer's pH was adjusted to 7.4.

Leukocyte isolation

After exsanguination, we set aside a portion of the autologous blood for leukocyte isolation. This portion was centrifuged at 400g for 30 min with Histopaque-10771 (Norman et al. 1995) in a swinging-bucket centrifuge (Thermo Scientific, Waltham, MA). The buffy coat was separated, re-suspended in phosphate buffered saline, and centrifuged at 250g for 10 min. This procedure was repeated twice. The isolated leukocytes were then incubated in R6G (2 μM) for 10 min. Subsequently, the suspension was centrifuged and the supernatant discarded, to remove unbound R6G. Fluorescence labeling of leukocytes was confirmed using a standard fluorescence microscope. The number of leukocytes in the suspension was quantified using a hemocytometer, and final concentration adjusted to 100,000 cells/ml in Ringer's solution. The leukocyte suspension was maintained at 4°C and warmed to 37°C prior to infusion.

Leukocyte pretreatment

In some experiments, we used leukocytes pretreated *ex vivo* with LPS or polymyxin-B. Toward this, we first isolated leukocytes as above, and incubated them with either LPS (100 ng/ml) (Ploppa et al. 2010) or polymyxin-B (10 μg/ml), for 15 or 30 min, respectively. The suspension was centrifuged and the supernatant discarded. Subsequently, the treated leukocytes were labeled with R6G, as described above.

Leukocyte transit in rat lung microvessels by real-time fluorescence microscopy

A PE10 (BD Biosciences, Sparks, MD) microcatheter was introduced through the left atrial cannula until it met resistance. To establish blood cell-free conditions, we flushed rat lung

microvessels with Ringer's solution as described previously (Parthasarathi et al. 2002). Then, we infused LPS (100µg/ml) into microvessels. Ringer's solution was infused as buffer control. At defined time intervals after LPS infusion, we infused the R6G-labeled leukocytes and imaged their transit through the lung surface microvessels by capturing their fluorescence (546 nm excitation, >560 nm emission) using a BX61WI fluorescence microscope (Olympus, Center Valley, PA) coupled to a CCD camera (ImageEM; Hamamatsu, Bridgewater, NJ). We continuously recorded leukocyte transit through a microvessel network for 3 min and repeated the process for additional microvessels till the end of leukocyte infusion. Subsequently, we flushed the microvessels with Ringer's for 10 min (Ringer's wash) and repeated the imaging. The captured images were recorded using the imaging software, Metamorph (Molecular Devices, Sunnyvale, CA). For ICAM-1 antibody blocking experiments, we first infused LPS. After a 30 min Ringer's wash, we infused 10 µg/ml of the ICAM-1 mAb for 30 min, and then infused the leukocytes. For TLR4 inhibition, polymyxin-B (10 µg/ml) was infused for 30 min prior to LPS infusion. The protocols used in this study including the infusion sequences and the duration of the infusions are summarized in Chart 1. For all protocols, we quantified the number of retained leukocytes in microvessels by analyzing the images at the end of the experiments using Metamorph.

ICAM-1 expression

We determined ICAM-1 expression in microvessels by *in situ* immunofluorescence method, as established previously (Parthasarathi et al. 2006). Briefly, we fixed LPS-treated microvessels with paraformaldehyde, as this allowed determination of the expression levels at a specific time point. We then infused the ICAM-1 primary Ab (10µg/ml) for 30 min followed by Ringer's wash. Subsequently, we infused the Alexa Fluor 488-tagged secondary Ab (10 µg/ml) for 30 min, and then washed off the excess secondary Ab with Ringer's for 30 min. Confocal images of ICAM-1 expression in surface microvessels were obtained using a LSM-710 (Zeiss, Thornwood, NY) confocal imaging system attached to an Axio Examiner.Z1 microscope (Zeiss). We excited the secondary Ab using an Argon laser (488 nm line) and collected the emission (>515 nm) using a 63x/1.0NA objective lens (Zeiss). The images were recorded and analyzed using the Zen 2010 software (Zeiss). The infusion sequences that preceded determination of ICAM-1 expression were as summarized above for leukocyte infusion with the exception of the *ICAM* protocol sequence, which was excluded.

L-selectin expression

Whole blood from rats were divided into two portions, with the first portion used for leukocyte isolation, as described above. The isolated leukocytes were treated with paraformaldehyde (3.75%). The second portion of blood was treated with paraformaldehyde, and then used for leukocyte isolation. Leukocytes isolated from the two portions were separately treated as follows. First they were incubated for 60 min with bovine serum albumin (BSA; 5%), and then overnight with L-selectin mAb (1 µg/ml) at 4°C. Subsequently, the leukocytes were washed with PBS to remove the primary Ab and incubated for 60 min with Alexa Fluor 488-tagged secondary Ab (2µg/ml) and the nuclear stain Hoechst 33342 (2µg/ml). After wash-off to remove excess secondary Ab and nuclear stain, the leukocytes were placed on glass slides for imaging L-selectin immunofluorescence using our Zeiss confocal microscope (as described above for ICAM1). A minimum of three slides were prepared for each leukocyte sample and 5 or more random 600 µm × 600 µm regions were imaged for each slide. For each image, the total leukocytes count using the nuclear stain and the L-selectin-expressing leukocyte count were determined. In addition, for all immunofluorescence images, we determined the fluorescence intensity profile over all the pixels within each image.

Estimating LPS wash-off from infused vessels

We prepared a mixture of LPS and FDx70 (25µg/ml) in Ringer's, while maintaining the final dextran concentration in the mixture at 4%. We infused this mixture into microvessels for 30 min via a microcatheter and followed with a Ringer's wash for 60 min, as similar to our *LPS90* protocol in Chart1. During the infusion and wash, we continuously recorded FITC-dextran fluorescence using our BX61WI fluorescence microscope (495 nm excitation and ; >515 nm emission).

Detecting residual leukocytes in microvessels cleared of blood cells

In local microvessels that were flushed with Ringer's and made blood-cell free, we infused R6G (2µM) for 10 min. Following a Ringer's wash for 5 min, we imaged residual R6G fluorescence in microvessels. Then, we analyzed the images to identify and quantify R6G-labeled residual leukocytes in these microvessels.

Leukocyte retention in mouse lungs

Isolated blood-perfused mouse lungs were allowed to stabilize for 30 min. Then we switched the perfusate to Ringer's+5% BSA, and flushed the blood from the lungs till the lungs became colorless. Subsequently, we perfused the lungs with LPS (100 µg/ml) in Ringer's+BSA or only Ringer's+BSA as control. After 30 min, we drained the LPS-containing or -free Ringer's perfusate, flushed and perfused the lungs with Ringer's+BSA. Sixty minutes after the end of LPS treatment, we added R6G-labelled leukocytes to the perfusate. We imaged leukocyte transit through microvessels and analyzed the images as described above for rat lungs.

Statistics

All data are reported as mean±SEM. All multiple groups were compared with Kruskal-Wallis One Way ANOVA on Ranks followed by pair-wise multiple comparisons by Dunn's method. Two groups were compared with Man-Whitney Rank Sum test (used for Figures 1I and 2G).

Results

LPS increases leukocyte margination in lung microvessels

Infused R6G-labeled leukocytes were detectable by fluorescence microscopy during their transit through the surface microvessels of the lung. To define leukocyte dynamics, we recorded their transit, sequentially in several microvascular networks for three minutes each. The transiting leukocytes exhibited stop-and-go motion as described previously (Gebb et al. 1995, Kuebler et al. 2000) and some of transiting leukocytes became stationary in the microvessels, and remained so for the rest of the recording period. The latter leukocytes were considered retained within the microvascular network. At the end of the Ringer's wash that followed leukocyte infusion, we recorded single images of several networks. Analysis of all the images revealed that under control (Ringer's-treated) condition, only a small number of leukocytes were retained during their transit through the microvessels (Figure 1 A–C). In addition, only a small number were still stationary after the Ringer's wash. In contrast in LPS-treated microvessels, the number of leukocytes that became stationary during infusion and those remaining stationary after the Ringer's wash were higher (Figure 1 D–F). Thus, LPS-treated microvessels retained a greater number of leukocytes.

To determine segment-specific responses, we quantified leukocyte retention separately in venules and capillaries. Since we infused leukocytes continuously, but imaged multiple microvessel networks sequentially, we found that some leukocytes were already retained in

microvessels at the start of an imaging sequence (“*pre*”; Figure 1 A, D). We quantified these separately for all the imaged networks. These “*pre*” values provided a global estimate of leukocyte accumulation over all the imaged networks during the entire infusion period. Overall, the number of “*pre*” leukocytes varied among the networks. We did not detect any correlation between the number of “*pre*” leukocytes in a network and the position of the network in the imaging sequence. These previously retained leukocytes were excluded when quantifying the number of transiting leukocytes retained during the imaging period (“*during*”). Thus the “*during*” values indicate the magnitude of leukocyte retention in a network during the 3-min imaging window.

In control venules and capillaries, the number of stationary leukocytes at the beginning of the imaging sequence was small (Figure 1 G, H). Further, only a small number of additional leukocytes became stationary during the imaging period. However, the number of leukocytes that remained retained in the microvessels after the Ringer’s wash was low (Figure 1 G, H). Since, the number of stationary leukocytes after the Ringer’s wash was similar to that at the start of the imaging period, it is possible that this small increase in retention during infusion could be due to the transient leukocyte retentions described previously (Gebb et al. 1995, Kuebler et al. 2000).

In contrast, in both LPS-treated venules and capillaries, the number of stationary leukocytes at the start of the imaging was more than 2-fold greater than in control vessels (Figure 1G, 1H). Moreover, additional leukocytes were continuously retained within a microvessel network during the imaging period. Though the number of stationary leukocytes decreased during the Ringer’s wash in both LPS-treated venules and capillaries, they remained several-fold above control. Among LPS-treated microvessels, the post-wash stationary leukocyte count was higher in capillaries than venules, suggesting that more leukocytes were retained in capillaries. The augmented counts in venules and capillaries suggest that LPS induced a significant increase in the leukocyte retention in both microvessel segments.

To exclude the possibility that differences in the number of leukocytes entering a microvascular network contributed to contrasting leukocyte retention in control and LPS-treated microvessels, we quantified the ratio of total leukocytes entering to that retained for each network during the 3-min imaging period. The ratio was higher in LPS-treated vessels compared to control, indicating the LPS-induced leukocyte retention was independent of any differences in the number of leukocytes traversing a microvascular network (Figure 1I).

To establish endothelial signaling that underlies the LPS-induced leukocyte retention, we determined the responses at increasing durations after LPS treatment. Images captured at the end of Ringer’s wash revealed that the number of stationary leukocytes in microvessels remained at control levels for *LPS30* and *LPS60* protocols (Figure 2 A, B). Moreover, the images also suggested that in microvessels treated with polymyxin or ICAM-1 mAb, the leukocyte retention was low and similar to control (Figure 2 C, D).

We quantified the responses separately in venules and capillaries for the different treatment conditions. Both in venules and capillaries, *LPS30* and *LPS60* protocols did not increase leukocyte retention above control (Figure 2 E, F). ICAM-1 mAb pretreatment blunted the LPS-induced increase in leukocyte retention. Though the blunting was greater in capillaries than venules, this was not statistically significant. In contrast, in *poly-lung* protocol, the LPS-induced increased retention was completely blocked in both vessel segments.

To determine whether activation or changes in deformability of leukocytes contributed to these observations, we quantified retention of LPS-treated leukocytes in Ringer’s- (*LPS-leuko*) and LPS-treated (*LPS-both*) microvessels. In *LPS-leuko* protocol, retention of LPS-treated leukocytes, was similar in venules, but higher in capillaries, as compared to control

(*Ringer's*) (Figure 2 E, F). However, in *LPS-both* protocol, retention of LPS-treated leukocytes was markedly higher in both venules and capillaries compared to that in *LPS-leuko* protocol (Figure 2 E, F). In addition, retention in *LPS-both* was similar to that in *LPS90* protocol. Further, in both LPS-treated venules and capillaries, retention of polymyxin-B-treated leukocytes (*poly-leuko*) was similar to that in *LPS90* (Figure 2 E, F).

Moreover, L-selectin expression in isolated leukocytes was similar to that in whole-blood, thus excluding our isolation procedure as a source of leukocyte activation (Figure 2 G, H). Further, images taken during infusions of R6G into experimental microvessels cleared of blood cells indicated a complete absence of adherent residual leukocytes in the target vessels (Figure 2 I). This excluded the possibility that the infused LPS may have activated the residual leukocytes in microvessels, and they in turn activated the subsequently infused leukocytes.

To establish that LPS-treated vessels were free of residual LPS after the *Ringer's* wash, we infused into microvessels a mixture of LPS and FDx70 for 30 min, and imaged FITC dextran fluorescence during infusion and subsequent *Ringer's* wash. The fluorescence was high in all vessels during infusion of the mixture, but returned to baseline levels at the end of the 60-min *Ringer's* wash indicating complete LPS wash-off from treated microvessels (Figure 2 J–L).

Taken together these data suggest that leukocyte retention in LPS-treated vessels was not due to activation or changes in deformability of leukocytes. Thus, it is evident that LPS-induced endothelial signaling was the primary factor underlying the increased leukocyte sequestration in the *LPS90* treatment condition. Further, the data indicate that the responses were mediated by endothelial TLR4 and ICAM-1.

As ICAM-1 mAb pretreatment reduced LPS-induced leukocyte retention, we determined endothelial ICAM-1 expression in LPS-treated microvessels as described before (Parthasarathi et al. 2006). *In situ* immunofluorescence images show ICAM-1 expression in microvessels was predominant under *LPS90* protocol (Figure 3 A–E). To determine the ICAM-1 expression in microvascular networks overall, we quantified the average fluorescence intensity of each image. The intensity was highest for images acquired under *LPS90* protocol, indicating that ICAM-1 expression in microvessels was highest under this condition (Figure 3F).

To determine the level of ICAM-1 expression separately in venules and capillaries, we quantified the average intensity along the margins of the respective vessels. ICAM-1 expression in capillaries was lower overall than in venules, but the expression levels varied similarly in both microvascular segments across various treatment conditions (Figure 3 G, H). In both segments, no increase in ICAM-1 was evident under *LPS30* protocol. For *LPS60* treatment condition the expression was higher compared to *Ringer* and *LPS30* protocol. In contrast, the expression was highest under *LPS90* protocol. Polymyxin completely blocked the increases in both vessel segments. This suggests that increases in ICAM-1 expression under the different treatment conditions closely followed the corresponding leukocyte responses in both vessel segments (Figure 2 E, F). These data together with the inhibition of leukocyte retention by ICAM-1 mAb suggest that LPS increased endothelial ICAM-1 expression, which in-turn augmented leukocyte retention in lung microvessels.

To determine whether LPS-treatment induced a similar increase in leukocyte retention in whole lungs, we treated isolated-mouse lung preparations with *Ringer's* or LPS, and added R6G-labeled leukocytes to the perfusate. Fluorescence images of microvessels show higher retention of leukocytes in LPS-treated than *Ringer's*-treated lungs (Figure 4A, B). Analysis of the retention in microvessels over a 3-min recording period indicated that the retention

was higher in both venules and capillaries (Figure 4C). Further, the ratio of total leukocytes entering to that retained for each network during the 3-min imaging period was higher in microvessels of LPS-treated lungs compared to control (Figure 4D). As similar to data from rat lungs, these results indicate that the LPS-induced leukocyte retention was independent of any differences in the number of leukocytes traversing a microvascular network. Thus, these data further support our finding that leukocyte retention is higher in LPS-treated microvessels.

Discussion

In this study, we show using a new experimental model that endothelial activation by LPS increases the number of leukocytes retained during their transit through the microvasculature. The increased retention was dependent on TLR4-mediated LPS signaling and augmented expression of the leukocyte adhesion receptor, ICAM-1 on endothelial cells. These data show for the first time that endothelial mechanisms by themselves play an important role in leukocyte retention in LPS-treated capillaries and venules, in particular.

Experimental models to determine LPS-induced responses in lung microvessels primarily utilize intratracheal or intravenous administration of LPS in intact animals (Kumasaka et al. 1996, Doyle et al. 1997, Kuebler et al. 2000, Mizgerd et al. 2004). Other studies have suggested a role for endothelial mechanisms in LPS-injury using similar models (Kamanochi et al. 1999, Andonegui et al. 2003, Basit et al. 2006). Though these models elicit a clinically-relevant response, due to global activation of multiple cell types, these models may be less suitable for specifically elucidating endothelial contributions to leukocyte retention in pulmonary microvessels. In the present method, lung microvessels were LPS-treated under leukocyte-free conditions. The intravascular LPS was washed off prior to leukocyte infusion into the treated microvessels, as confirmed by the LPS+FDx70 experiments, thus limiting exposure of leukocytes to the infused LPS. Retention of polymyxin-treated leukocytes were similar to that using untreated leukocytes, further suggesting an absence of any residual LPS in the treated microvessels. Moreover we did not detect any residual leukocytes in the target microvessels, thus reducing the possibility that these cells may have activated the subsequently infused leukocytes. In addition, leukocyte L-selectin expression was not modified due to our isolation procedure. Thus, the increased leukocyte retention could be primarily attributed to LPS-induced endothelial signaling.

Activation of leukocytes in tandem with the endothelium limits delineating endothelial contribution to LPS treatment. Activation leads to formation of cortical actin on leukocytes (Yoshida et al. 2006). This reduces their deformability and thus, increases their mechanical sequestration in lung microvessels (Worthen et al. 1989, Yoshida et al. 2006). Reports suggest that retention of LPS-activated leukocytes predominates in lung capillaries due to their reduced deformability (Kuebler et al. 2000, Doerschuk 2001). Our finding that retention of leukocytes treated *ex vivo* with LPS was higher in control capillaries, but not in venules, is in agreement with these reports. These changes in deformability are rapid (Doherty et al. 1994), and hence result in rapid reduction in total leukocyte count in animals administered LPS intravenously (Kuebler et al. 2000). However, it is reported that the leukocyte count partially recovers in a short while, possibly due to increased release from the bone marrow (Sato et al. 1998, Kuebler et al. 2000, Saito et al. 2002). Thus, continued retention of the newly released leukocytes could depend more on endothelial-specific mechanisms. Our data indicating higher retention of untreated leukocytes in both venules and capillaries under the *LPS90* protocol supports this possibility. Further, retention of leukocytes was also high in both venules and capillaries in *LPS-both* protocol, though the magnitude was similar to that in *LPS90*. Hence endothelial mechanisms and not reduction in leukocyte deformability, likely contributed significantly toward LPS-induced leukocyte

retention in pulmonary venules and capillaries. Thus, our data suggest for the first time a predominant role for endothelial mechanisms in LPS-induced pulmonary leukocyte retention.

The present study utilized intravital microscopy of *in situ* lung microvessels to continuously image leukocyte transit and determine their retention in real time. While intravital microscopy of leukocyte transit in lung is previously reported (Gebb et al. 1995, Kuebler et al. 2000,), these studies did not address the specific role of the pulmonary microvascular endothelium in leukocyte retention and accumulation. Further, our current understanding of LPS-induced lung leukocyte accumulation comes from static analysis using lung sections (Kumasaka et al. 1996, Kamochi et al. 1999, Andonegui et al. 2003). In addition, while simultaneous determinations of leukocytes entering and exiting lung have been used to quantify retention (Saito et al. 2002), the data may not reflect transient retentions, an event clearly evident from this study. Thus, while the existing models may provide valuable mechanistic data, intravital microscopy is better suited for dynamic determinations of leukocyte retention during their transit through lung microvessels.

In this study, a major finding was that leukocyte retention increased in LPS-treated venules and capillaries. Thus, under conditions where only the endothelium is LPS activated, venules also contribute to leukocyte retention. The retention remained high in both vessel segments even after the Ringer's wash. Since, the duration between the start of the leukocyte infusion and the end of the Ringer's wash was more than 30 min, the leukocytes retained at the end of the wash were likely to be firmly adhered to the endothelium. While L-selectin has been implicated as mediating retention and adhesion of leukocytes in pulmonary microvessels (Doyle et al. 1997, Kuebler et al. 2000), lack of changes in L-selectin expression in isolated leukocytes compared those in whole blood, limited the possibility that L-selectin mediated the increased leukocyte retention in LPS-treated venules.

Though LPS treatment increased retention in both microvessel segments, capillaries retained a higher percentage of leukocytes transiting through a network. Thus, the magnitude of leukocytes retained was greater in capillaries than venules. However, in this study, we quantified the retention separately for each observation field. In each observation field, there were a larger number of capillaries than venules. Thus, the higher leukocyte retention in capillaries was primarily a function of the higher density of capillaries and their larger overall surface, compared to venules. Hence, the magnitude of leukocytes retained per unit endothelial surface area was likely higher for venules than capillaries.

LPS-treatment increased ICAM-1 expression in both capillaries and venules. Since, endothelial pretreatment with polymyxin blocked the ICAM-1 increase in both microvessel segments, we interpret that the increased expression resulted from LPS-induced endothelial signaling. Moreover as ICAM-1 expression was highest under *LPS90*, and lowest under *LPS30* protocols, it is possible that LPS-induced ICAM-1 expression is the result of increased transcription. This is supported by the higher ICAM-1 expression under *LPS60* than under *LPS30* protocol. Reports indicating that tumor necrosis factor- α transcriptionally regulates ICAM-1 expression in endothelial cells (Ledebur and Parks 1995) and that NF- κ B mediates LPS-induced lung inflammation (Mizgerd et al. 2004) support the possibility that LPS transcriptionally augmented microvessel ICAM-1 expression.

Under *LPS90* protocol, both ICAM-1 expression and leukocyte retention were significantly increased in microvessels. Since, the increase in leukocyte retention was blunted in ICAM-1 mAb-treated venules and capillaries, it is possible that the retention was mediated by ICAM-1. This possibility is further supported by our findings that under *LPS30* protocol, there is no significant increase in either ICAM-1 expression or leukocyte retention. In

addition, under *LPS60* protocol both leukocyte retention and ICAM-1 expression were higher compared to control. Further, as polymyxin inhibited the leukocyte and ICAM-1 increases in both microvessel segments, it is more likely that LPS-induced endothelial ICAM-1 expression is the primary mechanism underlying the augmented leukocyte retention.

At baseline, ICAM-1 expression in venules was higher than in capillaries and similar to that previously reported (Burns et al. 1994). In addition, the LPS-induced increase in ICAM-1 expression was higher in venules than in capillaries, though the magnitude of the increase was almost 6-fold above the respective control level in both vessel segments. The higher ICAM-1 expression in venules supports our interpretation that leukocyte retention as a function of endothelial surface area is likely higher in venules. Thus, LPS-treatment may render venules to be more adhesive for leukocytes.

To define the role of endothelial mechanisms in LPS-induced leukocyte retention, we utilized the *ex vivo* blood perfused lung preparation and delivered agents and cells into microvessels in a local lung region via a microcatheter. The *ex vivo* lung preparation was previously used by several groups toward elucidating leukocyte mechanisms (Grimminger et al. 2000, Van Putte et al. 2005, Yiming et al. 2005, Hu et al. 2010, Hattar et al. 2010, Sharma et al. 2011, Rowlands et al. 2011). Using an *ex vivo* model it is feasible to selectively treat microvessels with LPS, and then infuse naïve leukocytes into these microvessels. Further, visualization of leukocyte transit and retention in real-time, and estimates of differences in capillary versus venular retention are simpler using an *ex vivo* lung preparation. Moreover, incorporating microcatheter-based local infusions facilitated repeated microvessels washes, toward removal of residual cells, then LPS, and finally transiently adhered leukocytes, while ensuring that the vessels remained patent over the entire experimental duration. In addition, we have extensive experience with the microcatheter method (Parthasarathi et al. 2002, Ichimura et al. 2005, Parthasarathi et al. 2006). These advantages also led us to choose the microcatheter delivery over infusing fluorescently-labeled leukocytes into the pulmonary artery.

The *ex vivo* model does have its drawbacks that include a need for leukocyte isolation and infusion of the leukocytes through a microcatheter, procedures that induce leukocyte activation. Further, the flow in the target vessels due to the microcatheter-based infusions may not be representative. However, our results that show lack of differences in L-selectin expression in isolated leukocytes compared to that in whole blood and unchanged retention of polymyxin-treated leukocytes compared to untreated cells, limit concerns on leukocyte activation during isolation and infusion. Further, the retention pattern of leukocytes added to the perfusate of isolated mouse-lung preparations was similar to those infused by microcatheter into isolated rat-lung preparations. Hence, the rat lung model and microcatheter-based infusions used in this study is a model well suited to determine endothelial contribution to LPS-induced leukocyte sequestration.

In conclusion, we show that LPS-increased ICAM-1 expression in both venules and capillaries of the lung. In addition, the LPS-augmented leukocyte retention is mediated by ICAM-1 in both microvascular segments. Thus, endothelial signaling mechanisms play a significant role in mediating LPS-induced leukocyte retention in the pulmonary microvasculature. These mechanisms may work in tandem with mechanical sequestration of activated leukocytes to increase accumulation of leukocytes in the pulmonary microvasculature in response to an endotoxemic insult. Targeting endothelium-dependent mechanisms may mitigate the pathophysiological effects of endotoxemia-induced excessive leukocyte sequestration in pulmonary microvessels.

Highlights

- * Focal LPS delivery into microvessels of isolated lungs by microcatheter
- * Infused fluorescently-labeled autologous leukocytes into LPS-treated vessels
- * Intravital microscopy of leukocyte transit through lung microvessels
- * LPS increased leukocyte retention in pulmonary venules and capillaries
- * Endothelial TLR4 and ICAM-1 inhibition blocked leukocyte retention

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Abbreviations

Ab	Antibody
BSA	Bovine Serum Albumin
LPS	Lipopolysaccharide
ICAM-1	Intercellular Adhesion Molecule
mAb	Monoclonal Antibody
R6G	Rhodamine 6G
TLR4	Toll-like Receptor 4

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Figure 1. Leukocyte retention in rat pulmonary microvessels

(A–F) Fluorescence images show leukocytes retained in lung microvessels under Ringer's-treated (A–C) and LPS-treated (D–F) conditions. The images were extracted from sequences recorded consecutively in 4 to 6 separate microvascular networks per lung during R6G-labeled leukocyte infusion into microvessels. Shown are the first image from a sequence (A, D), the last image from the same sequence (B, E), and images obtained after a 10-min Ringer's wash (C, F). Leukocytes retained prior to start of the imaging sequence could be seen in the first image of a sequence (A, D). Only single images of networks were captured at the end of the Ringer's wash (>10 networks per lung). Scale bars represent 20 microns.

(G, H) Number of retained leukocytes within the field of view in venules (G) and capillaries (H) treated with Ringer's or LPS. The data groups are; leukocytes retained prior to the start of imaging sequence as quantified from the first image (*pre*), total leukocytes retained at the end of the sequence (*during*), and stationary leukocytes at the end of the Ringer's wash (*wash*). mean $\pm$ SE, §- $P < 0.05$ compared to *pre*, *during* and *wash* data groups of Ringer's-treated venules; #- $P < 0.05$ compared to *pre*, *during* and *wash* data groups of Ringer's-treated capillaries; ‡- $P < 0.05$ compared to *wash* data groups of Ringer's-treated capillaries. (I) Ratio of total leukocytes flowing through a network during the 3-min imaging duration to number retained during the same period in Ringer's and LPS-treated venules and capillaries. Mean $\pm$ SE, *- $P < 0.05$ compared to Ringer's-treated capillaries.

Figure 2. Temporal and inhibitor responses

(A–D) Fluorescence images show leukocytes retained in lung microvessels after the Ringer's wash. Images were obtained for *LPS30* (A), *LPS60* (B), *ICAM1* (C) and *polymyxin* (D) treatment conditions as described in Chart 1 in *Methods*. Scale bars represent 20 microns. (E, F) Leukocytes retained in venules (E) and capillaries (F) at the end of the Ringer's wash were quantified for the different treatment conditions as indicated. In both graphs, nomenclature used for each data group corresponds to protocols described in Chart 1. *Ringer* and *LPS90* data groups are repeated from Figure 1 for comparison. Mean $\pm$ SE. For (E) ‡-P<0.05 compared to *Ringer*, *LPS30* and *poly-lung* data groups; § and ¶-P<0.05 compared to *Ringer*, *LPS-leuko*, *LPS30*, *LPS60*, *ICAM*, and *poly-lung* data groups. For (F) #P<0.05 compared to *Ringer*, *LPS30*, *LPS60*, *ICAM* and *poly-lung* data groups; ‡, § and ¶-P<0.05 compared to *Ringer*, *LPS-leuko*, *LPS30*, *LPS60*, *ICAM*, and *poly-lung* data groups. (G, H) L-selectin expression was determined by confocal microscopy imaging in leukocytes treated with paraformaldehyde before isolation (*whole blood*) and after isolation (*isolated*) (blood samples from 3 rats, n=15 images each). (G) Number of leukocytes expressing L-selectin immunofluorescence was quantified in each image and expressed as a percentage of total cells in that image. (H) The immunofluorescence intensity profile for each image (1024 pixels $\times$ 1024 pixels) was quantified as a function of number of pixels. (I) Fluorescence image shows lack of R6G-stained residual leukocytes or platelets in microvessels cleared of blood cells (n=3 lungs, >5 images each). Scale bar represents 20 microns. (J–L) Fluorescence images show fluorescence of FITC-Dextran in microvessels before (J), during (K), and 60 min after (L) infusion of a mixture of LPS+FDx70. Vessel outlines in J, L shown for reference (n=4 lungs). Scale bars represent 20 microns.

Figure 3. ICAM-1 expression in microvessels

(A–E) Confocal images show *in situ* immunofluorescence staining of ICAM-1 in lung microvessels. Images were obtained from *Ringer* (A), *LPS30* (B), *LPS60* (C), *LPS90* (D) and *poly-lung* (E) protocols as described in Chart 1 in *Methods*. (F) Bar graph shows average intensity quantified over the entire ICAM-1 immunofluorescence image for various treatment conditions. As all confocal images were obtained using the same imaging parameters, changes in average image intensity represented changes in ICAM-1 immunofluorescence for all microvessels within an image. In the graph, nomenclature used for each data group corresponds to treatment conditions described in *Methods*. Mean $\pm$ SE. *- $P < 0.05$ compared to *Ringer* and *LPS30* data groups, and §- $P < 0.05$ compared to *Ringer* data group. (G, H) Bar graphs show ICAM-1 immunofluorescence intensity separately in venules and capillaries. The intensity was quantified along lines drawn on the respective vessel margins. In both graphs, nomenclature used for each data group corresponds to treatment conditions described in *Methods*. Mean $\pm$ SE. *- $P < 0.05$ compared to *Ringer*, *LPS30* and *poly-lung* data groups, §- $P < 0.05$ compared to *Ringer* and *LPS30* data groups, and ‡- $P < 0.05$ compared to *Ringer* data group.

Figure 4. Leukocyte retention in mouse pulmonary microvessels

(A, B) Fluorescence images show retention of perfusing leukocytes in microvessels of control (A) and LPS-treated lungs (B). (n=2 lungs for each treatment). Scale bars represent 20 microns. Dashed lines in B represent margins of venules. (C) Number of leukocytes retained within the field of view in venules and capillaries of control (*Ringer's*) or LPS-treated (*LPS*) mouse lungs. Mean $\pm$ SE. $\ddagger$ -P<0.05 compared to *Ringer's venule*, *LPS venule* and *Ringer's capillary* data groups. (D) Ratio of total leukocytes flowing through a network during the 3-min imaging duration to number retained during the same period in control (*Ringer's*) or LPS-treated (*LPS*) mouse lungs. Mean $\pm$ SE. $\S$ -P<0.05 compared to *Ringer's venule*, and *Ringer's capillary* data groups.

Notes:

n=3 lungs for all protocols

dashed lines represent imaging period

leuko = leukocytes; *poly* = polymyxin-B

- *LPS leuko* and *poly leuko* indicate leukocytes treated *ex vivo* with LPS and polymyxin, respectively

Chart 1.