Critical role of P-selectin-dependent leukocyte recruitment in endotoxin-induced intestinal barrier dysfunction in mice

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Keywords: sepsis, leukocyte, endotoxin, intestinal barrier, P-selectin

Running head: Leukocyte-recruitment in intestinal leakage.

Abstract

Objective: To define the importance of leukocyte recruitment in endotoxin-induced gut permeability.

Materials and methods: 31 male C57BL/6 mice were challenged with lipopolysaccharide (LPS). Ileal permeability was measured in Ussing chambers and leukocyte-endothelium interactions studied with intravital fluorescence microscopy after 18 h.

Results: LPS caused a clear-cut increase in leukocyte accumulation and intestinal permeability. Immunoneutralisation of P-selectin not only reduced leukocyte recruitment significantly (54% reduction) but also abolished endotoxin-induced intestinal leakage. Intestinal levels of pro-inflammatory chemokines increased markedly in response to LPS but were not influenced by inhibition of P-selectin *in vivo*.

Conclusion: The present study shows not only that endotoxin-induced leukocyte recruitment is mediated by P-selectin but also that sepsis-associated intestinal leakage in the gut is largely regulated by leukocyte accumulation. Thus, our novel data demonstrate a critical link between P-selectin-dependent leukocyte recruitment and gut barrier failure in endotoxemia.

Introduction

Despite substantial efforts to improve surgical treatment, antimicrobial therapies and immunomodulatory regimes, gram-negative sepsis remains a common cause of mortality in intensive care patients [1, 2]. Lipopolysaccharide (LPS) constitutes the major portion of the outer membrane of most clinically relevant gram-negative bacteria found in human infections [3]. The host response to LPS challenge is associated with disturbed gut integrity and increased leukocyte infiltration [4]. Indeed, increased intestinal leakage through the epithelial cell lining constitutes a key feature in the pathophysiology of sepsis by facilitating bacterial translocation and passage of toxic substances from the gastrointestinal tract [5]. Notably, several studies have reported that pro-inflammatory substances can increase the permeability of epithelial cell monolayers *in vitro* [6, 7]. However, these *in vitro* systems lack the presence of leukocytes and the potential role of leukocyte recruitment in mediating endotoxin-induced intestinal leakage *in vivo* remains therefore elusive.

LPS binds to the cell surface receptor CD14 on tissue macrophages and activates Toll-like receptor-4 [8], which, in turn, initiates intracellular signaling cascades that converge on specific transcription factors regulating gene expression of pro-inflammatory mediators, such as cytokines and chemokines [9]. Indeed, tissue recruitment of leukocytes is dependent on the formation and action of CXC chemokines, including macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) [10]. Several studies have shown that leukocyte accumulation is a multistep process, comprising initial rolling along the microvascular endothelium followed by firm leukocyte adhesion and migration [11]. Leukocyte rolling is considered to be a precondition for the subsequent adhesion and extravasation of leukocytes and is largely dependent on the

function of the selectin family of adhesion molecules (L-, E-, and P-selectin) [12, 13]. An accumulating body of data indicates that P-selectin supports most rolling adhesive interactions *in vivo* [14] although the detailed role of P-selectin in endotoxin-induced leukocyte-endothelium interactions in the small intestine has not previously been studied.

Based on the above considerations, the main aim of this study was to define the importance of leukocyte recruitment in endotoxemia-associated gut leakage. For this purpose, we used a monoclonal antibody against P-selectin, which we found to be effective in blocking LPS-provoked leukocyte-endothelium interactions in the intestine.

Methods

Animals

Male C57BL/6 mice (Taconic Europa, Ry, Denmark) weighing between 20-22 g were maintained at 12 h dark and 12 h light cycles and had free access to standard pellet food (R3, Lactamin AB, Kimstad, Sweden) and water *ad libitum*. Anaesthesia was achieved by intraperitoneal (i.p.) injection of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel Switzerland) and 2.5 mg xylazine (Jansen Pharmaceutica, Beerse, Belgium) per 100 g body weight. All experiments were approved by the local Animal Ethic's Committee at Lund University.

Experimental protocol

Mice were allocated to one of the following groups: 1) negative control group received 0.3 ml sterile saline i.p. ; 2) positive control group received 2 mg lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4 (Sigma Chemical Co, St Louis, MO, USA) per 100 g body weight, dissolved in 0.3 ml sterile saline i.p. ; 3) control antibody group received 40 µg of an isotype-matched rat antibody IgG (clone R3-34, BD Biosciences Pharmingen, San Diego, CA, USA) dissolved in sterile saline to a total volume of 0.2 ml intravenously (i.v.) by a lateral tail vein injection, immediately followed by i.p. administration of LPS as described above; 4) anti-P-selectin antibody group received 40 µg of a monoclonal antibody against mouse P-selectin (clone RB40.34, BD Biosciences Pharmingen) dissolved in sterile saline to a total volume of 0.2 ml i.v. immediately followed by LPS as described above. Permeability studies and intravital microscopy were conducted 18 h after LPS treatment.

Intestinal permeability studies

Mice in the separate groups were anaesthetised and a midline laparotomy was performed. The ileocaecal junction was identified and 3-4 cm of distal ileum was harvested while carefully removing the mesentery. The intestine was cut open along the mesenteric side, rinsed in Krebs´ buffer and mounted in a modified Ussing diffusion chamber [15] (Harvard Apparatus, Holliston, MA, USA) using inserts allowing for a 0.25 cm^2 exposed area of intestine. The chambers were filled with 3 ml Krebs´ buffer that was continuously bubbled with carbogen (95% O_2 and 5 % CO_2) at 37 ºC. The experiment started within 45 min after harvesting the intestinal segment by first replacing the buffer in the serosal (recipient) reservoir with 3 ml fresh Krebs´ buffer and then the buffer in the mucosal (donor) reservoir with 3 ml Krebs´ buffer containing sodium fluorescein (molecular weight 376 Da, Sigma Chemical Co, St Louis, MO, USA) at a concentration of 0.1 mg/ml. The chambers were covered to prevent light exposure. After 60 min specimens were taken from the serosal chambers for spectrofluorometry (SpectaMax Gemini, Molecular Devices, Sunnyvale, CA, USA). Samples were serially diluted in duplicates and measured at an excitation wavelength of 485 nm and emission wavelength of 525 nm. Known amounts of sodium fluorescein were dissolved in Krebs´ buffer to make a standard curve, which was used to determine the amount of sodium fluorescein passage in the experiments.

The transepithelial potential differences were measured immediately before start of the experiment (t = 0) and after 60 min, using electrodes imbedded in 3 M KCl agar connected to a standard commercial voltmeter (APPA 63N Multimeter, APPA Technology Corp, Taipai, Taiwan). Potential difference < 3.0 mV at t = 0 excluded a specimen from the experiment.

Intravital microscopy

Mice were anaesthetised and placed on a heating pad (37 ºC) to maintain body temperature. A polyethylene catheter (PE-10) with an inner diameter of 0.28 mm was inserted into the jugular vein and used to administer marker solutions and additional anaesthesia. A midline laparotomy was performed and the most distal part of ileum was carefully exteriorised. The mouse was then put under an inverted Olympus microscope (IX70, Olympus Optical Co, GmgH, Hamburg, Germany), equipped with different lenses (x10/NA 0.25 and x40/NA 0.60). The image was televised using a charge-coupled device video camera (FK 6990A-IQ, Pieper GmbH, Schwerte, Germany) and recorded on videotape (Panasonic HR S8600 S-VHS recorder) for later off-line evaluation. Analysis of rolling and adhesion was made in 3 – 6 distal ileum venules with an inner diameter of 25 – 50 µm and with stable blood flow. Blood perfusion was studied after contrast enhancement of the plasma with i.v. injection of 0.1 ml fluorescein isothiocyanate-dextran (molecular weight 150 000, 5 mg/ml, Sigma Chemical Co, St Louis, MO, USA) followed by illumination with blue light (excitation wavelength 490 nm, emission wavelength 510 nm).

In vivo labelling of leukocytes was done by i.v. injection of 0.1 ml rhodamine 6G (molecular weight 479, 0.5 mg/ml, Sigma Chemical Co, St Louis, MO, USA) followed by illumination with green fluorescent light (excitation wavelength 530 nm, emission wavelength 560 nm). Leukocyte rolling was determined by counting the number of leukocytes passing a reference point in the venule during a 20 sec observation period and expressed as cells/min. Firm adhesion was determined by counting the number of leukocytes adhering and remaining stationary along a 100 µm segment of the venular endothelium for 20 sec. The results are expressed as cells/mm venule length. Blood flow velocity was analysed off-line by means of a computer image analysis programme

(CapImage, Zeintl, Heidelberg, Germany). The velocity is expressed as mm/sec and wall shear rate calculated from the formula wall shear rate = 8([velocity/1.6]/venular diameter) [16].

ELISA

The levels of chemokines MIP-2 and KC were determined in distal ileum tissue. After intravital microscopy a segment of the distal ileum was harvested, rinsed of faecal matter, weighed and put in PBS containing 1% PEST (penicillin and streptomycin) (Gibco/Invitrogen, Carlsbad, CA, USA) and 0.1 mg/ml amphotericin B (Fungizone, Bristol Myers Squibb, NY, NY, USA) for 60 min. The tissue was then incubated in Dulbecco´s modified Eagle's medium solution containing 10% fetal calf serum, 1% PEST and amphotericin B for 24 h in 37 ºC in a 12-well plate. The medium was harvested, centrifuged for 10 min at 3000 rpm and the supernatant was then collected and frozen in -20 ºC. Analysis was made using a quantitative sandwich enzyme immunoassay technique with polyclonal antibodies specific for murine MIP-2 and KC (R&D Systems, Minneapolis, MN, USA). The minimum detectable amount of protein in these kits is 1.5 pg/ml. Each sample was analysed in duplicates and optical density read at 450 nm (Milenia Kinetic Analyzer, DPC, Los Angeles, CA, USA).

Myeloperoxidase (MPO)

Two cm of distal ileum were harvested and weighed for MPO assay from the negative control group (n=4), control antibody group (n=8) and anti-P-selectin antibody group (n=5). After homogenisation in 1.5 ml 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical, St Louis, MO, USA) MPO was extracted and purified. Absorbance was

measured in a spectrophotometer at 450 nm and the MPO activity calculated from a standard curve and corrected for sample weight.

Systemic leukocyte counts

20 µl peripheral blood was mixed with Turks solution (0.2 mg gentian violet/ml glacial acetic acid, 6.25% v/v) in a 1:10 dilution. Using a Burker chamber, leukocytes were counted and differentiated as mononuclear (MNL) or polymorphonuclear (PMNL).

Statistical analysis

Data are presented as mean values ± SEM. Statistical evaluations were performed using one way analysis of variance (ANOVA) followed by multiple comparisons between groups (Holm-Sidak´s method). For non-parametric data Kruskal-Wallis one way analysis of variance on ranks followed by multiple comparison between groups (Dunn´s method) was used. $P < 0.05$ was considered significant and n represents the number of animals.

Results

Endotoxin-induced intestinal permeability

Challenge with LPS markedly increased sodium fluorescein permeability by 104% in the distal ileum. Thus, after 60 min in the Ussing-chamber, the concentration of sodium fluorescein was 0.22 ± 0.01 µg/ml on the serosal side of distal ileum in LPS-treated mice ($n = 11$), as compared to 0.11 ± 0.01 µg/ml in negative control mice ($n = 8$, $P < 0.05$) (Fig. 1). In control antibody-treated mice, LPS significantly increased intestinal permeability by 68% (0.19 ± 0.01 µg/ml, $n = 6$, $P < 0.05$ *vs* negative control). Notably, pre-treatment with the anti-P-selectin antibody completely abolished LPS-induced permeability, normalising passage of sodium fluorescein across distal ileum down to control levels (0.12 ± 0.01 µg/ml, $n = 6$, $P < 0.05$ *vs* LPS). Electrical potential difference across the intestinal membrane remained constant and was not affected by LPS or any of the antibodies ($n = 6 - 11$) (Table 1).

Endotoxin-induced leukocyte recruitment

Intravital microscopy revealed that baseline leukocyte rolling was 12 ± 1 cells/min in negative control mice. Concomitant with increased permeability, it was observed that LPS significantly increased leukocyte rolling up to 30 ± 6 cells/min ($n = 6$, $P < 0.05$ *vs* negative control) (Fig. 2). In contrast, we found that immunoneutralisation of P-selectin decreased leukocyte rolling down to baseline levels, *i.e.* 6 ± 2 cells/min ($n = 6$, $P < 0.05$ *vs* control antibody + LPS) (Fig. 2).

The number of leukocytes firmly adhering to the venular wall was 1.0 ± 0.4 cells/mm in the negative control group and increased significantly after LPS-challenge up to 48 ± 9

cells/mm (*n* = 6, *P* < 0.05 *vs* negative control) (Fig. 3). Administration of the control

antibody had no effect on leukocyte adhesion in endotoxemic mice (48 ± 5 cells/mm).

However, pre-treatment with the anti-P-selectin antibody reduced the number of adherent

leukocytes down to 22 ± 5 cells/min, corresponding to 54% reduction in LPS-induced

leukocyte adhesion (n = 6, *P* < 0.05 *vs* control antibody + LPS).

There were no significant differences in vessel diameter or blood flow velocity between

the different experimental groups (Table 2). Moreover, differential count of the peripheral

leukocytes revealed a 96% reduction of MNL in the LPS-treated mice (Table 3). This

reduction was absent in the endotoxemic animals pre-treated with the anti-P-selectin

antibody. No significant changes in PMNL were observed between the groups.

Moreover, tissue infiltration of leukocytes was evaluated by measuring intestinal levels of

MPO. We found that LPS increased MPO activity in the intestine by more than two-fold (n

= 4-8, *P* < 0.05 *vs* negative control). Notably, it was observed that pre-treatment with the

anti-P-selectin antibody reduced MPO levels from 0.7 ± 0.1 units/mg down to 0.4 ± 0.1

units/mg (n = 5-8, *P* < 0.05 *vs* control antibody + LPS).

Endotoxin-induced chemokine production

LPS challenge significantly increased protein levels of CXC chemokines in the intestine

(Fig. 4 a and b). Thus, baseline values of MIP-2 and KC in the small intestine were 35.5 ±

10.6 pg/mg and 66.5 ± 18.7 pg/mg, respectively. LPS resulted in more than a three-fold

increase in tissue chemokines, *i.e.* MIP-2 increased up to 154.4 ± 35.0 (*n* = 7, *P* < 0.05 *vs*

negative control) (Fig. 4 a) and KC up to 177.0 ± 28.6 (*n* = 7, *P* < 0.05 *vs* negative control)

(Fig. 4 b). Interestingly, immunoneutralisation of P-selectin had no effect on the levels of

MIP-2 or KC in intestine of endotoxemic mice. No differences in tissue chemokines were found between the control antibody group and the anti-P-selectin group.

Discussion

This study demonstrates a fundamental role of leukocytes in mediating sepsis-associated intestinal barrier dysfunction. Indeed, inhibition of P-selectin not only blocked endotoxin-induced leukocyte rolling and adhesion, but also abolished the increased intestinal permeability in endotoxemic animals. These novel findings suggest a causal link between on one hand leukocyte accumulation and on the other hand intestinal leakage in sepsis. Moreover, it was found that inhibition of P-selectin did not exert any effect on local production of pro-inflammatory mediators in the intestine, suggesting that actual infiltration of leukocytes is relatively more important than any direct effect of pro-inflammatory mediators on tissue cells during induction of intestinal permeability in sepsis. Thus, our data indicate that targeting P-selectin and leukocyte-endothelial interactions may be a useful approach to attenuate sepsis-induced intestinal leakage.

An intact mucosal barrier function maintained by the intestinal epithelial cells is crucial in preventing toxic substances in the gut lumen from entering the body. The integrity of the epithelial cell lining in the gut mucosa is also critical in preventing the huge load of potentially pathogenic bacteria contained within the lumen to translocate into normally sterile compartments of the body. These protective functions of the intestinal mucosa are delicately balanced with the task of absorbing water, ions and nutrients from the intestinal lumen and also allowing small quantities of antigens to permeate and interact with the gut immune system [17]. In sepsis this balance is disturbed, resulting in an increased permeability. Indeed, sepsis is also associated with leukocyte activation and recruitment. However, the relation between leukocyte-endothelial interactions and increased permeability across the intestinal wall is not known. The present study is the first one to explore the definitive role of leukocytes in gut barrier dysfunction associated with a

systemic inflammatory response *in vivo*. We used a model based on systemic

administration of LPS, which increased leakage in the gut by more than 100%. Notably,

using this model, we found that immunoneutralisation of P-selectin not only abolished

intestinal recruitment of leukocytes but also markedly decreased endotoxin-induced

permeability in the distal small intestine. In this context it is important to note that P-

selectin expression is limited to endothelial cells and circulating platelets [18] and is

neither expressed in the extravascular matrix nor on tissue cells in the intestinal wall,

excluding the possibility of any effect of the anti-P-selectin antibody on targets beyond the

intestinal microvasculature. MPO activity is a useful indicator of leukocyte recruitment,

and our results shows that LPS treatment induces a significant increase in leukocyte

recruitment in the intestine. This effect was markedly reduced by inhibition of P-selectin,

which is in line with our findings on leukocyte rolling and adhesion. Taken together, these

novel findings suggest that leukocyte recruitment plays a critical role in mediating

intestinal barrier dysfunction induced by endotoxin.

Moreover, it is interesting to note that inhibition of P-selectin reduced leukocyte

recruitment and intestinal leakage, but had no concomitant effect on tissue production of

pro-inflammatory mediators (MIP-2 and KC) in the intestinal wall. The fact that the

intestinal levels of MIP-2 and KC were intact in anti-P-selectin antibody-treated animals

suggests that leukocyte recruitment is relatively more important than the local production

of pro-inflammatory mediators in the pathophysiology of sepsis-associated gut barrier

dysfunction. This notion is somewhat in contrast to previous *in vitro* studies reporting that

pro-inflammatory mediators exert powerful effects on the permeability across epithelial

cell monolayers [6, 7]. However, it is of importance to note that these *in vitro* studies lack

the presence of leukocytes, which we, herein, have shown to play a key role in sepsis-

associated intestinal leakage *in vivo*. Although the predominant impact on the intestinal tissue seems to be regulated by accumulated leukocytes, our data do not exclude that local mediators also may contribute to increased leakage by exerting direct and leukocyte-independent effects in the intestinal tissue.

The underlying mechanism behind the leukocyte-mediated intestinal barrier dysfunction observed herein remains elusive. Indeed, leukocytes contain numerous potent and tissue damaging substances, such as reactive oxygen species, bactericidal permeability-increasing proteins, matrix metalloproteinases, defensins and elastases [19]. Upon release in the intestinal wall these may contribute to disrupted intestinal integrity and increased macromolecular passage across the epithelial cell lining. In this study, we used sodium fluorescein as a marker of intestinal permeability. It is generally held that sodium fluorescein permeates paracellularly across the intestinal epithelium [20]. Paracellular integrity is maintained by tight junctions, which consists mainly of different transmembrane proteins [21]. Interestingly, it has been shown that neutrophil-derived proteases have the capacity to disrupt the function of tight junctions [22] which may help to explain, at least in part, the underlying mechanism of leukocyte-dependent gut barrier dysfunction in sepsis. Besides, it may be worth noting that leukocyte migration *per se* may also cause physical disruption of the mucosal integrity and thereby increase intestinal leakage [23].

When inducing permeability changes in the gastrointestinal tract by means of exogenous administration of LPS, it is of importance to determine if the increased permeability is due to any effect on the viability of the enterocytes. For this purpose we measured the electrical potential difference, which is maintained by ATP-dependent ion pumps in the basolateral

membrane of intact enterocytes. Throughout the 60 min of this experiment, we did not find any significant difference in potential difference between LPS-treated and control mice when the intestinal segments were mounted in the Ussing chambers, indicating that LPS did not have any adverse effect on tissue viability. This notion is in accordance with the findings of Wells et al reporting that LPS has no effect on enterocyte morphology or viability [24].

In conclusion, this study demonstrates for the first time that leukocyte recruitment *per se* is critical in mediating sepsis-associated intestinal barrier dysfunction. Interestingly, the accumulation of leukocytes seems to be more important than pro-inflammatory mediators in regulating LPS-induced intestinal leakage. Our data show that targeting P-selectin function decreases pathological leakage in the gut through inhibition of leukocyte accumulation. Thus, these novel findings suggest that therapies directed against recruitment of leukocytes may be a useful strategy against gut barrier failure in sepsis.

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Figure Legends

Figure 1

Mucosal-to-serosal permeability of sodium fluorescein (µg/ml) in distal ileum. Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. Values are mean ± SEM. #$P < 0.05$ *vs* negative control and *$P < 0.05$ *vs* positive control (LPS) or control-antibody + LPS.

Figure 2

Leukocyte rolling (cells/min) along the endothelium of postcapillary venules in the submucosa of distal ileum was measured by use of intravital microscopy. Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. Values are mean ± SEM. #$P < 0.05$ *vs* negative control and *$P < 0.05$ *vs* positive control (LPS) or control-antibody + LPS.

Figure 3

 Leukocyte adhesion (cells/mm) on the endothelium of postcapillary venules in the submucosa of distal ileum was measured by use of intravital microscopy. Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. Values are mean ± SEM. #$P < 0.05$ *vs* negative control and *$P < 0.05$ *vs* negative control, positive control (LPS) or control-antibody + LPS.

Figure 4

Levels of the a) macrophage inflammatory protein-2 (MIP-2) and b) cytokine-induced neutrophil chemoattractant (KC) in distal ileum were measured by ELISA. Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. Values are mean ± SEM and #$P < 0.05$ *vs* negative control.

Table 1. Potential difference

	n	t = 0	t = 60
Negative control	8	7.2 ± 0.5	5.8 ± 0.7
PBS + LPS	11	7.5 ± 0.4	4.7 ± 0.5
Control ab + LPS	6	5.9 ± 0.7	4.9 ± 0.9
Anti-P-selectin ab + LPS	6	6.2 ± 1.6	4.6 ± 0.7

Potential difference (mV) across intestinal segments mounted in Ussing chambers. Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. t = 0 indicates the time point immediately prior to addition of sodium fluorescein on the mucosal side of the intestinal segments and t = 60 at the end of the experiment. Values are mean ± SEM.

Table 2. Haemodynamic parameters.

	n	Diameter (µm)	Velocity (mm/sec)	Wall shear rate (sec^{-1})
Negative control	6	29.4 ± 2.9	1.0 ± 0.1	142 ± 23
PBS + LPS	6	37.7 ± 3.8	0.8 ± 0.1	105 ± 24
Control ab + LPS	6	35.1 ± 2.0	0.8 ± 0.1	95 ± 13
Anti-P-selectin ab + LPS	6	32.3 ± 2.5	0.7 ± 0.1	102 ± 15

Haemodynamic parameters of distal ileum venules. Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. Values are mean ± SEM. Wall shear rate = 8([velocity/1.6]/venular diameter.

Table 3. Peripheral leukocyte counts.

	n	Mononuclear leukocytes (10^6/ml)	Polymorphonuclear leukocytes (10^6/ml)
Negative control	6	2.3 ± 0.1	0.6 ± 0.3
PBS + LPS	6	$0.1 \pm 0.01*$	0.2 ± 0.05
Control ab + LPS	6	$0.1 \pm 0.01*$	0.1 ± 0.04
Anti-P-selectin ab + LPS	6	0.6 ± 0.2	0.5 ± 0.2

Peripheral leukocyte counts. Values are mean $\pm$ SEM. * $P < 0.05$ *vs* negative control.