

# LPS differentially regulates adhesion and transendothelial migration of human monocytes under static and flow conditions

Paul F. Bradfield<sup>1</sup>, Caroline A. Johnson-Léger<sup>1,2</sup>, Claudia Zimmerli<sup>1</sup> and Beat A. Imhof<sup>1</sup>

<sup>1</sup>Department of Pathology and Immunology, University Medical Centre, 1 Rue Michel-Servet, CH-1211 Geneva 4, Switzerland

<sup>2</sup>Present address: Merck Serono International S.A., 9 chemin des Mines, 1202 Geneva, Switzerland

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## Abstract

**One of the key components of the innate immune response is the recognition of microbial products such as LPS by Toll-like receptors on monocytes and neutrophils. We show here that short-term stimulation of primary human monocytes with LPS led to an increase in adhesion of monocytes to endothelial cells and a dramatic decrease in transendothelial migration under static conditions. In contrast, under normal physiological flow, monocyte adhesion and migration across a human umbilical vein endothelial cell monolayer appeared to be unaffected by LPS treatment. LPS stimulation of monocytes activated  $\beta_1$  and  $\beta_2$  integrins, but did not increase their surface expression levels. During septic shock, reduction in blood flow as a result of vasodilation and vascular permeability leads to adhesion and accumulation of LPS-stimulated circulating monocytes onto the blood vessel walls. The different findings of monocyte migration under static and flow conditions in our study may offer one explanation for this phenomenon. The rapid engagement of LPS-activated monocytes preventing transendothelial migration could represent a novel mechanism of bacterial exclusion from the vasculature. This occurs during the early stages of sepsis, and in turn may modulate the severity of the pathophysiology.**

## Introduction

At sites of inflammation, signals derived from the underlying tissue induce localized changes in the adhesive properties of the endothelium. Leukocytes are then able to adhere to the endothelium and extravasate in large numbers to the inflammatory lesion. This process has been described as a multi-step adhesion cascade (1–3). The change in the adhesive properties of the endothelium is brought about by the up-regulation of adhesion molecules on the endothelium and this represents a critical early step in the recruitment of leukocytes to sites of inflammation (4). Acute inflammatory processes are characterized by the early infiltration of neutrophils and monocytes, while the chronic inflammatory phase is associated with the recruitment of monocytes and effector memory T cells (5). While a great deal of progress has been made in understanding the molecular events occurring during each step of the adhesion cascade, it remains to be clarified how a tightly adhered leukocyte can 'de-adhere' and go on to cross the endothelium. Clearly, a fine balance must exist between adhesion and release,

which serves to drive migration of the leukocyte through the endothelium. During a localized infection, however, the invading micro-organism may breach the vascular barrier and occupy the blood compartment, directly activating circulating leukocytes via pathogen-associated molecular patterns (PAMPs). LPS is a PAMP that constitute a major component of the outer membrane of gram-negative bacteria. It is a complex glycolipid composed of a hydrophilic polysaccharide and a hydrophobic domain, known as lipid A (6). LPS forms a complex with LPS-binding protein and can signal through membrane-bound CD14 on monocytes and myeloid cells (7). The LPS signal is transmitted across the cell membrane via a co-receptor, Toll-like receptor (TLR)-4 (8).

Severe sepsis is a major cause of death among patients in intensive care units (9). The presence of LPS in blood can set off a cascade of host responses that in extreme cases can lead to sepsis (endotoxemia or blood poisoning). Under normal homeostatic conditions, these responses serve to activate the innate immune response and, ultimately, to clear

*Correspondence to:* B. A. Imhof; E-mail: [beat.imhof@medecine.unige.ch](mailto:beat.imhof@medecine.unige.ch)

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the infection. However, if the host response is not tightly controlled, an overwhelming systemic inflammatory response ensues and can lead to a massive drop in blood pressure, multiple organ failure and fatal septic shock. To understand why endotoxemia leads to such a catastrophic syndrome, it is important to consider the biological function of endotoxin. LPS activates almost every component of the immune system (10). Particularly, circulating monocytes can undergo a rapid activation by LPS within the vasculature. Thus, at the earliest stage of sepsis, monocytes can serve as sentinel cells after a breach of the blood barrier by bacterial endotoxins. Such a massive stimulation of monocytes in the blood also results in the production of pro-inflammatory mediators at later stages, notably tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), IL-1, IL-6 and IL-8. The combined action of these cytokines is known to activate endothelium and leads to increased leukocyte emigration and vascular permeability. This results in leakage of plasma into the tissues and a drop in blood pressure and flow (11, 12).

We have found that the presence of LPS during static transmigration assays had a profound effect on the ability of monocytes to transmigrate across monolayers of human umbilical vein endothelial cells (HUVECs). These observations prompted us to compare the effects of LPS on monocyte migration under static and flow conditions and to investigate a role for LPS on monocytes during the early stages of septicaemia.

## Methods

### *Reagents and antibodies*

For flow cytometry analysis of human PBMCs and monocyte purity, the following antibodies were used: anti-CD3, anti-CD14 and anti-CD19 and CD62L directly conjugated to FITC (PharMingen, San Diego, CA, USA). Immunostaining for CXCR4 was performed using mAb 12G5 directly conjugated to PE (R and D Systems, Abingdon, UK). For integrin staining, antibodies were purified from hybridoma supernatants of cells grown in DMEM supplemented with the serum substitute Ultrosor® (PALL Life Sciences, New York, NY, USA). Antibodies used were 9EG7 (rat anti-mouse  $\beta_1$  integrin, IgG2a) which cross-reacts with human  $\beta_1$  (13), TS1/18 [mouse anti-human leukocyte function-associated antigen-1 (LFA-1),  $\beta_2$  chain, IgG1], TS1/22 (mouse anti-human LFA-1,  $\alpha_L$  chain, IgG1) and MARK-1 (mouse anti-rat kappa L chain, IgG1). For blocking studies, antibodies to  $\beta_1$  (PS2) and  $\beta_2$  integrins (TS1/18) were used. mAbs mAb24 and 15/7 were kind gifts from Nancy Hogg and Elan Pharmaceutical Inc. (San Francisco, CA, USA), respectively. Human stromal derived factor-1 (SDF-1) was also kind gift from RMF Dictagene S.A., (Epalinges, Switzerland). LPS was derived from *Escherichia coli* 055:B5 (3120-25-0; Difco, Detroit, MI, USA and Sigma).

### *Endothelial cells*

HUVECs were isolated by collagenase treatment of umbilical veins (14). HUVECs were maintained in M199 supplemented with 20% FCS (PAA Laboratories, Linz, Austria), 25 mM HEPES, non-essential amino acids, sodium pyruvate, endothelial cell growth supplement (15  $\mu\text{g ml}^{-1}$ ; Upstate Biotechnology, New York, NY, USA) and heparin (4  $\mu\text{g ml}^{-1}$ ; Sigma). Cells were used between passages 2 and 5.

### *Primary lymphocytes and monocytes*

Human PBMCs were obtained from fresh blood of healthy donors. Briefly, citrated blood was separated by Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. For the purification of monocytes, blood was collected from healthy donors into tubes containing EDTA (7.5 mM) and incubated for 1 h at room temperature before the addition of a one-tenth volume of 8% (w/v) Dextran T500 (Pharmacia) in 0.9% NaCl. After 1 h, the plasma-rich top layer was layered onto Nycoprep 1.068 (Axis-Shield, Oslo, Norway) and centrifuged at  $600 \times g$  for 30 min. The monocyte-enriched fraction was collected and washed in either flow assay or static adhesion assay buffer. Monocyte preparations were typically 70–80% pure (CD14+ cells, determined by flow cytometry) with contaminating cells comprised mostly of T-lymphocytes (CD3+). Monocytes used for immunostaining with mAb 12G5 were purified from citrated blood collected from healthy donors using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

### *Flow cytometry*

Cells in suspension were collected, washed in M199/2% FCS and then re-suspended in PBS containing 0.2% BSA and 0.1% sodium azide (flow cytometry buffer) with saturating amounts of antibodies, either uncoupled or directly conjugated to FITC. After a 30-min incubation on ice, cells were washed and analysed directly or incubated with streptavidin-PE for biotinylated antibodies before analysis by flow cytometry (FACScan®; Becton Dickinson Co., Mountain View, CA, USA). Control cell suspensions were incubated with secondary antibody alone.

### *LPS pre-treatment of leukocytes*

Leukocyte suspensions were incubated with LPS (1  $\mu\text{g ml}^{-1}$ ) for 15 min at 37°C. The cells were then washed and re-suspended in either flow or static adhesion assay buffer.

### *Pre-treatment of purified monocytes with functional blocking antibodies to integrins*

Blocking mAb to  $\beta_1$  (PS2) and/or  $\beta_2$  (TS1/18) were added to purified monocytes added at a concentration of 20  $\mu\text{g ml}^{-1}$  for 20 min immediately prior to HUVEC co-culture under flow. Isotype control mAb was added in the absence of the relevant blocking antibody to ensure total antibody concentration was maintained during the course of the flow assay.

### *Static transendothelial migration assay*

HUVECs ( $6 \times 10^4$ ) were cultured on 10 mm diameter polycarbonate membranes (8  $\mu\text{m}$  pore size, Life Technologies) pre-coated with collagen (collagen G, Seromed, 100  $\mu\text{g ml}^{-1}$  in HBSS) in M199 culture medium for 3–4 days. For activation, HUVECs were pre-incubated with TNF $\alpha$  (500 U  $\text{ml}^{-1}$ ) for 4 h. The cultures were washed in M199 with 2% FCS prior to the addition of the PBMCs. The chemokine SDF-1 was added to the lower chamber at a final concentration of 100–200 ng  $\text{ml}^{-1}$ . Washed PBMCs ( $5 \times 10^5$ – $7 \times 10^5$  per insert) or purified monocytes ( $2$ – $3 \times 10^5$  per insert) were

added to each filter and incubated for 2 h at 37°C. Cells were collected from the lower (transmigrated) and upper chamber (unadherent) and counted by light microscopy. In studies using PBMCs, the cells were pooled from quadruplicate wells and stained for CD3 and CD14 to determine the percentage of T-cell and monocyte populations present in each well.

#### *Adhesion assays on HUVECs: FACS analysis of non-adherent populations*

HUVECs were grown to confluence in 3.5-cm tissue culture dishes pre-coated with collagen (100 µg ml<sup>-1</sup>) in HBSS as described. The monolayers were rinsed and the PBMCs added. The cultures were left for 1 h at 37°C and non-adherent cells were recovered.

#### *Static adhesion assays on immobilized ICAM-1*

Purified Intercellular adhesion molecule-1 (ICAM-1) (15) was directly coated onto 96-well plates at 10 µg ml<sup>-1</sup> in 50 µl PBS for 1 h at room temperature. The wells were blocked with 1% BSA for 1 h at room temperature and washed with wash buffer (HBSS, Ca<sup>2+</sup> 1.25 mM, Mg<sup>2+</sup> 0.5 mM and 0.2% BSA). Purified monocytes were pre-incubated with 1% human serum and either an anti-β<sub>2</sub> antibody (TS1/18) or an isotype-matched control at 20 µg ml<sup>-1</sup> for 30 min at 37°C. Cells were plated in triplicate at 2.5 × 10<sup>5</sup> cells per well for 30 min at 37°C. Non-adherent monocytes were removed by washing and adherent cells fixed stained with 0.1% crystal violet in 20% methanol for 10 min. The wells were washed and allowed to air-dry before dissolving the stained fixed cells in methanol and measuring the optical density at 570 nm. Data presented are expressed as a mean of four wells ± SEM.

#### *Static adhesion assays on HUVECs: microscopic analysis of adherent, migrated and non-migrated populations*

HUVECs were cultured in pre-coated 24-well plates for 2–3 days to establish confluent monolayers. Purified monocytes (3 × 10<sup>5</sup> cells per well) were incubated for 60 min at 37°C. Non-adherent cells were removed by gentle washing of the monolayers. Three separate fields were recorded for 2 min each using time-lapse recording facilities. This time period represents a minimum time to confirm transmigration by observing the cell kinetics of phase dark (transmigrated) cells (16). Phase light cells were counted as non-transmigrated cells that remained adherent on HUVEC surfaces. Transmigration was calculated as the percentage of the sum of phase light and phase dark cells and expressed as a mean of three fields.

#### *Flow assays*

For flow assays, glass cover slips (22 × 50 mm) were prepared by coating with 3-aminopropyltriethoxysilane (Sigma) (17). Prior to culturing cells, the slides were sterilized with ethanol and coated with 0.2% gelatine/4% FCS/HBSS. HUVECs (7 × 10<sup>5</sup> per slide) were cultured until confluent (2–3 days) before being attached to a cell adhesion flow chamber (CAF10; Immunetics, Cambridge, MA, USA). Wash buffer (M199, 0.15% BSA and 20 mM HEPES) or a suspension

of monocytes (2 × 10<sup>6</sup> cells ml<sup>-1</sup>) was perfused over the HUVEC monolayer at 0.05 Pa; representative of the shear rates within small venules or capillaries. The flow chamber was mounted on a heated microscope stage (37°C) and observations made using phase-contrast microscopy recorded using time-lapse recording facilities. For the flow assays, the HUVEC monolayer was treated for 4 h with TNFα as described above, which was washed out prior to addition of monocytes. The suspension of monocytes was perfused over the activated HUVECs for 5 min followed by wash buffer for 30 min. Monocytes adhered to the surface of the HUVECs have a phase light appearance, whereas cells that have transmigrated across the monolayer have a phase dark appearance (18, 19). All experiments were carried out using single-field analysis and at least three experiments were performed. Results are presented as mean percentage of total cells per field ± SEM.

#### *Flow assay at decreasing flow rates*

A monocyte (2 × 10<sup>6</sup>–3 × 10<sup>6</sup> cells ml<sup>-1</sup>) suspension was perfused over unactivated HUVECs cultured on slides as described above for 5 min at a flow rate of 0.05 Pa. Adherent cells were counted in three separate fields and represented as a mean total ± SEM. The flow was then reduced to a fixed lower rate for 2 min before adherent cells were counted again in three separate fields. This procedure was repeated for consecutive reductions in flow rate. Flow was then resumed at 0.05 Pa for 1 h before transmigration was assessed by time-lapse recording of a single field for 2 min. Transmigration was represented as a mean percentage (±SEM) of three separate fields at specific time points.

#### *Controlled detachment assay*

The controlled detachment assay was performed as previously described (20). Briefly, a monocyte (2 × 10<sup>6</sup>–3 × 10<sup>6</sup> cells ml<sup>-1</sup>) suspension was perfused over unactivated HUVECs cultured on slides as described above for 5 min at a flow rate of 0.05 Pa. The flow was stopped for 10 min to introduce a period of stasis. The flow was resumed at the same flow rate for 1 min and adherent cells in three separate fields counted. This was repeated using incremental higher wall shear stresses. Results were described as the total number of cells adhered per static field to the cultured HUVEC monolayer for each shear stress. Experimental data were represented as a mean with standard deviation of the counts done in the three fields. Flow was then resumed at 0.05 Pa for 1 h before transmigration was assessed by time-lapse recording of a single field for 2 min. Transmigration was represented as a mean percentage (±SEM) of three separate fields at specified time points.

## **Results**

During a series of transmigration assays, we observed that LPS from *E. coli* consistently reduced transmigration of monocytes across endothelial monolayers. This effect was almost immediate as it occurred within 15 min. It has been reported that LPS can activate the endothelium over a period

of several hours and thereby increase monocyte transmigration. In order to explain our contradictory findings, we investigated whether there is a direct primary short-term inhibitory effect of LPS on primary monocytes.

*LPS pre-treatment of monocytes selectively inhibits transendothelial migration*

We performed transmigration studies using PBMCs pre-treated with or without LPS for 15 min across unactivated HUVECs using the CXCR4 ligand SDF-1, a chemokine that can mediate leukocyte adhesion and transmigration (21). We have established that surface expression levels of the chemokine receptor CXCR4 on monocytes after stimulation with LPS for 15 min remained unchanged (Fig. 1a). This is consistent with a previous study showing down-regulation of CXCR4 only after overnight incubation with LPS (22). Transmigrated cells to SDF-1 were collected from the lower chambers and identified as monocytes or lymphocytes by flow cytometry (data not shown). Pre-treatment of PBMCs with LPS reduced the number of transmigrated monocytes, but not T-lymphocytes, which do not have receptors for LPS (Fig. 1b). The inclusion of polymyxin B, a molecule that sequesters and neutralizes LPS, abrogated this effect (data not shown) (23, 24). Since excess LPS was removed by washing the monocytes prior to use, LPS must have an irreversible effect on monocyte function. Delaying the addition of LPS-pre-treated monocytes to the endothelial monolayers for 2 h did not abrogate this effect (data not shown). Therefore reduced transmigration was not due to a carry-over of LPS by the monocytes to the endothelium. A dose response was carried out and showed that pre-treatment with LPS at a concentration of  $1 \mu\text{g ml}^{-1}$  gave optimal inhibition of monocyte migration across unactivated HUVECs (data not shown). Based on this, we used LPS at  $1 \mu\text{g ml}^{-1}$  throughout this study. In human serum, an equivalent amount of LPS (ranging from 0.05 to  $2.08 \mu\text{g ml}^{-1}$ ) would create a life-threatening septic shock (25, 26).

*The adhesion of monocytes to HUVECs is altered following pre-incubation with LPS*

The inhibition of monocyte transmigration suggested that the effect might be due to changes in the adhesive properties of the monocytes. Indeed, LPS is known to induce monocyte adhesion (27) and cell spreading of macrophages (28). Transmigration depends on an adhesive step but the cell must then be able to de-adhere in order to cross the endothelium. It was therefore possible that, upon treatment with LPS, the monocytes had become too adhesive and could no longer detach from the HUVECs. To test this hypothesis, confluent monolayers of unstimulated HUVECs were incubated with PBMCs, which had been pre-treated with LPS. Non-adherent cells were subsequently harvested and analysed by flow cytometry for leukocyte sub-populations. This demonstrated a reduction in the number of monocytes but no effect was seen with T cells (Fig. 1c and d). This showed that LPS pre-treatment induced a change in the adhesive properties of monocytes, and this may be linked to their inability to migrate across endothelium.

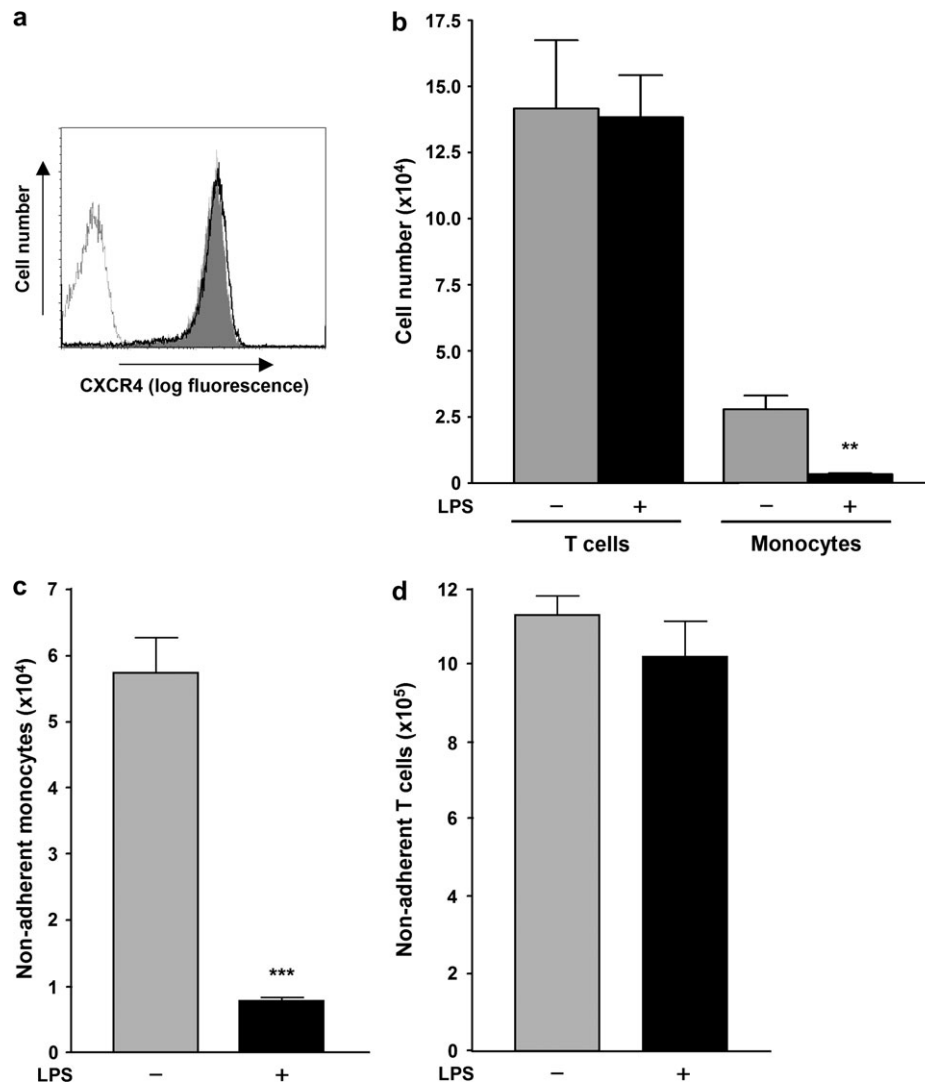
*LPS pre-treatment of monocytes increases adhesion to HUVECs but inhibits monocyte transmigration*

To exclude an indirect effect of LPS, monocytes were purified from PBMCs populations. They also showed increased adhesion and reduced transmigration to SDF-1 after LPS pre-treatment (Fig. 2a). So far, we have investigated the role of LPS-activated monocytes on non-inflammatory endothelium. *In vivo*, however, the endothelium would be exposed to LPS resulting in inflammatory activation. As part of this study, we employed adhesion assays to address the latter phase of sepsis, specifically where endothelial cells have been exposed to a battery of inflammatory agents for prolonged periods. Previous reports have shown that activating endothelium with LPS induced expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) and increased adhesion of mononuclear cells (29–33). However, stimulating HUVECs with TNF $\alpha$  was considered to be more representative of an *in vivo* scenario where the endothelium would not just be exposed to LPS in the vascular compartment, but a milieu of stronger inflammatory stimulants that promote transmigration released by adherent or recruited monocytes. This led us to investigate interactions of LPS-pre-treated monocytes with fully activated endothelium. Surprisingly, similar levels of increased adhesion were seen with activated and unactivated endothelium (Fig. 2a). Similarly, LPS pre-treated monocytes transmigrated less efficiently through activated and unactivated endothelium than non-treated monocytes. Thus, reduced transmigration is due to a process regulated by the monocytes and not by the vascular endothelium. However, the intriguing observation was that the majority of the monocytes remained firmly attached to the endothelial monolayer (Fig. 2b).

To study how LPS may affect monocyte positioning in the endothelium, we carried out static adhesion assays using time-lapse video microscopy to observe the dynamics of monocyte interactions with unactivated HUVECs under static conditions. These experiments were performed in the absence of SDF-1 to establish if the endothelium plays a more proactive role in regulating monocyte transmigration. Interactions of adherent monocytes with HUVECs were analysed and classified as adherent (phase light) or transmigrated cells (phase dark) (18, 19). Even in the absence of SDF-1, monocytes were observed to transmigrate under static conditions confirming that unactivated HUVECs have an intrinsic capability to support monocyte transmigration. Furthermore, LPS-pre-treated monocytes were observed to remain adherent to HUVEC monolayer surfaces and transmigrated at much lower levels than non-treated monocytes (Fig. 3). Therefore, LPS pre-treatment has rendered the monocytes more adherent and unable to transmigrate through the endothelium.

*LPS pre-treatment of monocytes activates  $\beta_2$  integrins but does not alter the level of expression*

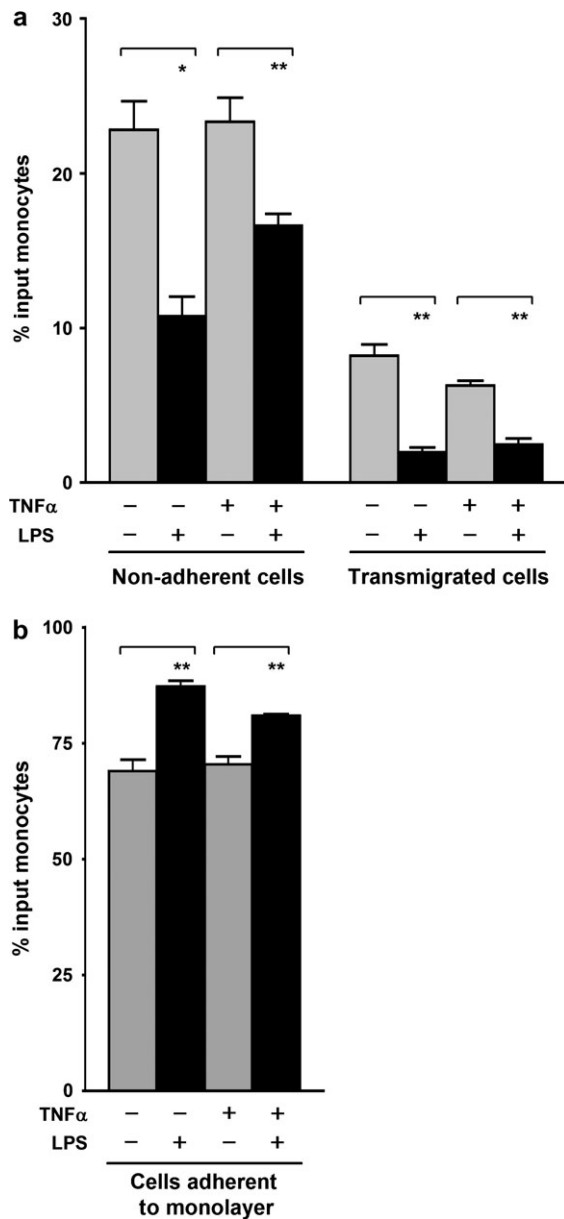
Integrins play an important role in the adhesion of leukocytes to the endothelium (34). Interestingly, it has been shown that adhesion induced by LPS is mediated at least in part by LFA-1 (35, 36). To confirm a role for  $\beta_2$  integrins in our experimental system, we carried out adhesion experiments using



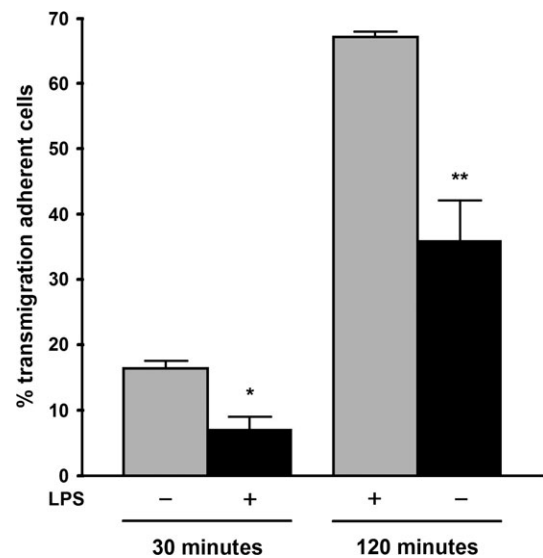
**Fig. 1.** LPS inhibits the transendothelial migration, increases the adhesive properties of monocytes but does not affect expression of chemokine receptor CXCR4. (a) Pre-treatment of monocytes with LPS does not alter surface expression of chemokine receptor CXCR4 on monocytes. Flow cytometric analysis of purified monocytes immunostained with mAb 12G5-PE confirmed CXCR4-surface expression levels were identical for untreated (grey fill) and LPS-pre-treated monocytes (dotted line). Control monocytes were unlabelled (dotted line). (b) Transmigration of monocytes and T cells. PBMCs were added to confluent monolayers of HUVECs grown in transwell culture inserts and incubated for 120 min in the presence of human SDF-1 added to the lower chamber. PBMCs were pre-incubated with medium alone (grey bars) or LPS for 15 min ( $1 \mu\text{g ml}^{-1}$ ) (black bars). Transmigrated cells collected from the lower chamber were counted by light microscopy and total T cells or monocyte counts were generated from flow cytometry using mAbs to CD3 and CD14, respectively. Asterisks indicate significance of  $P < 0.01$  (\*\*). The data are representative of two independent experiments. (c and d) Adhesion of monocytes and T cells. PBMCs were pre-incubated in medium alone (grey) or LPS (black) (at  $1 \mu\text{g ml}^{-1}$ ) for 15 min at  $37^\circ\text{C}$ , and then added to confluent monolayers of HUVECs in 35 mm tissue culture dishes or six-well plates. Following a 60-min incubation, the dishes were washed and the non-adherent cells were counted and stained for CD3 and CD14 to determine the percentage of each cell type present. Data are presented as the mean number of (c) non-adherent monocytes ( $P < 0.001$ ) or (d) T cells calculated from triplicate dishes or wells. Error bars are SEM. Statistical analyses were performed using Student's *t*-tests. The data are representative of two independent experiments.

purified monocytes on recombinant ICAM-1 as a ligand for LFA-1. Indeed, LPS pre-treatment induced increased adhesion of monocytes to ICAM-1, and this was blocked by an antibody against  $\beta_2$  integrins (Fig. 4a). However, this was not a product of increased expression levels of  $\alpha_L$  or  $\beta_2$  integrins during LPS pre-treatment (Fig. 4b and c). Similarly, expression levels of  $\beta_1$  integrins was unaffected by LPS pre-treatment (Fig. 4d) in contrast to a recent study, showing LPS-mediated up-regulation of  $\beta_1$  integrin (37). We then

postulated that increased monocyte adhesion was associated with a change in integrin activation on monocytes pre-treated with LPS (38). To do this, we used mimetic mAbs 15/7 and mAb24 which recognize epitopes associated with activation of  $\beta_1$  and  $\beta_2$  integrins, respectively (39, 40). Flow cytometric analysis using these antibodies confirmed increased activation of  $\beta_1$  integrin (Fig. 4e) and  $\beta_2$  integrin (Fig. 4f) after LPS pre-treatment, which is consistent with increased monocyte adhesion to HUVECs.



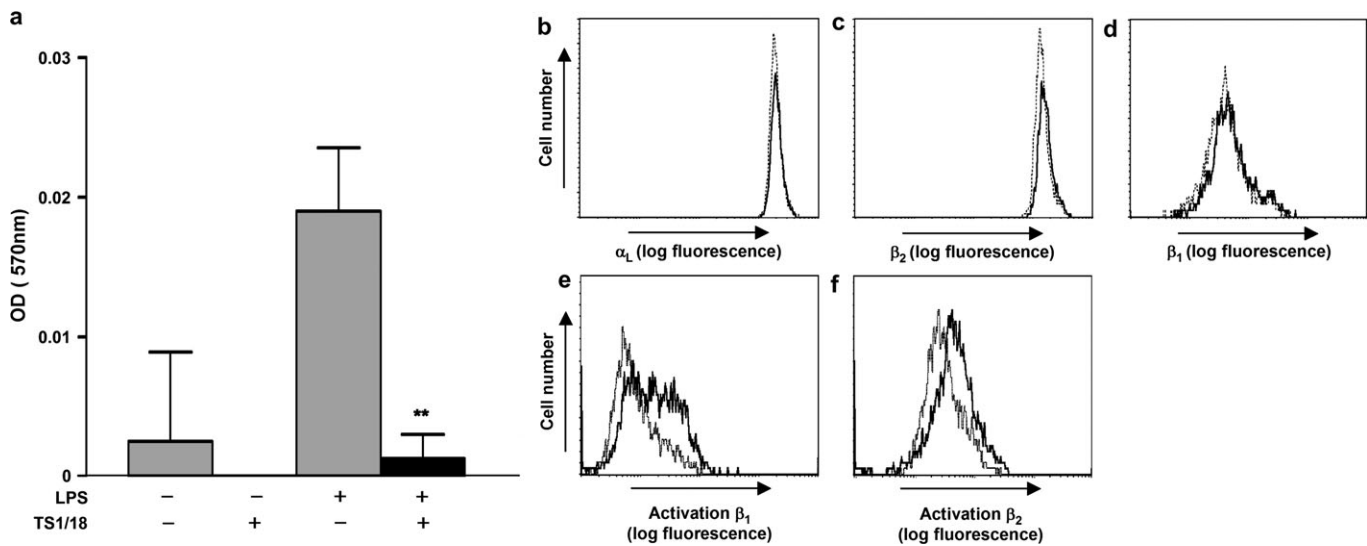
**Fig. 2.** The effect of LPS pre-treatment on monocyte adhesion and transmigration on resting and activated endothelium. (a) Adhesion and transmigration of monocytes. Purified monocytes, LPS treated (black) or non-treated (grey), were incubated on HUVECs cultured on transwell culture inserts for 120 min in the presence of human SDF-1 added to the lower chamber. Non-adherent cells were collected from the top chamber by gentle washing and counted by light microscopy. The underside of the transwell was washed gently with PBS and 2 mM EDTA and pooled with transmigrated cells collected from the lower chamber and counted by light microscopy. (b) LPS pre-treatment of monocytes increases accumulation in the HUVEC monolayer. For each well, the sum of cells collected from the top chamber (non-adherent) and the bottom chamber (transmigrated) was subtracted from the total cells added. Results are presented as the mean percentage of total cells added from three separated transwells in a single experiment  $\pm$  SEM. Statistical analyses were performed using Student's *t*-tests comparing groups marked by brackets. Asterisks indicate significance of  $P < 0.01$  (\*) and  $P < 0.001$  (\*\*). The data are representative of two independent experiments.



**Fig. 3.** Video analysis of LPS pre-treatment on monocyte transmigration under static conditions. Purified monocytes, LPS treated (black) or non-treated (grey), were incubated and recorded by video time-lapse microscopy on HUVEC monolayers for 30 or 120 min. Non-adherent cells were removed by gentle washing and discarded. Transmigration was assessed by counting monocytes that had transmigrated (phase dark) or had not transmigrated (phase grey). Transmigration was expressed as a percentage of the sum of total monocytes per field (non-transmigrated cells plus transmigrated cells). Results are presented as the mean percentage migration from three separated fields filmed for 2 min in a single experiment at each time point  $\pm$  SEM. Statistical analyses were performed using Student's *t*-tests comparing  $\pm$ LPS at both time points.

#### *Transendothelial migration of monocytes under physiological flow is LPS independent*

We have demonstrated that LPS pre-treatment blocks monocyte transmigration across HUVECs under static conditions. However, leukocyte interactions with the vascular wall *in vivo* occur under flow, which induces a minimum shear stress of 0.05 Pa at the monocyte:endothelial interface. We therefore decided to investigate monocyte interactions with endothelium under flow conditions as observations made so far may be linked to the use of static migration assays. Previous studies have shown that physiological flow plays a critical role in modulating transendothelial migration of lymphocytes (41). We investigated if flow was sufficient to overcome the increased adhesiveness and reduced transmigration of LPS-pre-treated monocytes on activated endothelium. Flow assays were carried out at a physiological flow rate (0.05 Pa) on TNF $\alpha$ -activated HUVECs. Pre-treatment of LPS did not affect the number of monocytes captured (data not shown). We have shown that the capture of monocytes pre-treated with and without LPS was mediated by  $\beta_1$  and  $\beta_2$  integrins, as blocking mAbs PS2 and TS1/18 reduced monocyte adhesion (Fig. 5a) (42–45). In contrast to static transmigration assays, LPS pre-treatment of monocytes had no effect on transmigration with TNF $\alpha$ -activated HUVECs (Fig. 5b). No adhesion or transmigration of monocytes was observed with unactivated HUVECs, irrespective of LPS pre-treatment (data not shown). Differences between activated and unactivated HUVECs demonstrate that despite the



**Fig. 4.** LPS pre-treatment of monocytes activates  $\beta$  integrins and increases adhesion to ICAM-1, but does not affect integrin expression levels. (a) Blocking monocyte adhesion by using an anti- $\beta_2$  integrin antibody. LPS-pre-treated or untreated monocytes were incubated on immobilized ICAM-1 in the presence of blocking antibodies to the integrin subunit  $\beta_2$  (TS1/18) or an isotype control. Non-adherent cells were removed by washing and the remaining adherent cells stained with crystal violet. Results are presented as the mean of the OD at 570 nm of separate four wells  $\pm$  SEM. Statistical analysis was performed using the Student's *t*-test comparing groups  $\pm$ TS 1/18 for LPS-treated monocytes. The data are representative of two independent experiments. (b–d) Expression levels of integrins. Flow cytometric analysis investigating (b)  $\alpha_L$ , (c)  $\beta_2$  and (d)  $\beta_1$  integrin subunit expression demonstrated that expression levels were identical for untreated (line) and LPS-pre-treated monocytes (dotted line). (e and f) Activation state of integrins. Analysis by flow cytometry demonstrated an increased activation state of  $\beta_1$  and  $\beta_2$  integrins by increased display of epitopes recognized by the mimetic mAbs (e) 15/7 and (f) mAb24 on monocytes pre-treated with LPS (line) compared with untreated monocytes (dotted line). Isotype control profiles were contained in the first log of fluorescence (data not shown).

higher activation state of integrins on LPS-pre-treated monocytes, trans migratory cues are required from activated endothelium, and the application of shear stress alone on unactivated endothelium is not sufficient. This is consistent with a lack of selectin expression on unactivated HUVECs, which is necessary for the initial tethering and rolling of leukocytes onto the luminal surface from free flow (46).

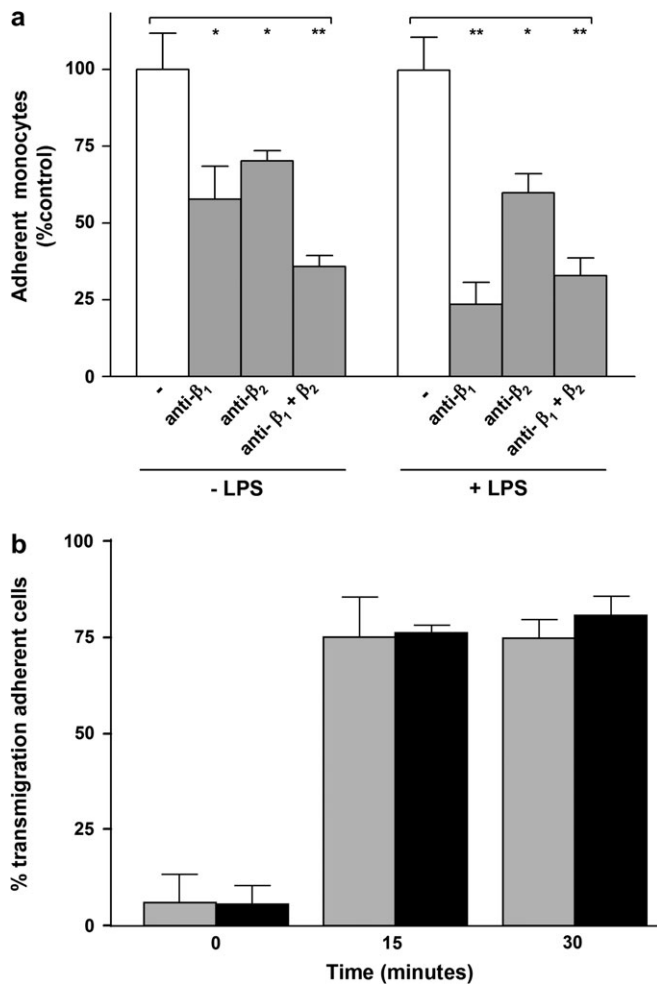
Investigating monocyte interactions with HUVECs under decreasing flow conditions to near-stasis has helped to understand apparent inconsistencies and limitations in using static assays. At flow rates of 0.025 Pa and below, higher numbers of LPS-pre-treated monocytes adhered to the unactivated HUVEC monolayer, which increased dramatically as the flow rate was incrementally reduced (Fig. 6a). In contrast, untreated monocytes remained largely non-adherent with only a modest increase in the number of adherent cells observed at the lowest flow rates tested. These monocytes showed a gradual increase in transmigration >1 h with LPS-pre-treated monocytes showing lower levels of transmigration over this time period (Fig. 6b). This monocyte adhesion was mediated by  $\beta_1$  and  $\beta_2$  integrins, as it was dramatically reduced using blocking antibodies, with and without LPS pre-treatment (Fig. 6c). To consolidate our observations, monocytes that were allowed to adhere under static conditions to HUVECs were exposed to augmented flow rates. When flow was incrementally increased, monocyte adhesion was gradually reduced with ~30% remaining adherent even under very high flow rates (Fig. 7a). However, LPS-pre-treated monocytes were more resistant to higher shear stresses than non-treated cells. These monocytes were still able to transmigrate with LPS-pre-treated cells

showing an ~40% reduction in transmigration compared with non-treated cells (Fig. 7b).

## Discussion

Sepsis is a contributing factor in up to 200 000 deaths per year in the USA, and the incidence of sepsis continues to increase worldwide due to increased frequency of invasive procedures and widespread bacterial antibiotic resistance (10). The mortality rate in patients with septic shock ranges from 20 to 80%. Mortality is related to the severity of both the sepsis and the underlying disorder that is invariably present. The primary trigger in septic shock elicited by gram-negative bacteria is endotoxin or LPS. Whereas LPS itself is chemically inert, the presence of LPS in blood can induce an overwhelming inflammatory response (10), leading to a severe and rapid drop in blood pressure culminating in multiple organ failure.

LPS interacts with CD14, which then complexes with TLR4 on the surface of monocytes and neutrophils (8). This interaction activates signal transduction pathways that in turn activate integrins (47, 48). Integrin activation plays a critical role in the multi-step adhesion cascade that describes the passage of inflammatory cells from the blood into the tissue at sites of inflammation (1). TLR4 is thought to engage LPS through a common motif suggesting that any differential effect between bacterial strains, particularly under saturating conditions, is unlikely (49, 50). Later studies have shown that TLR2 can also recognize LPS from the bacteria *Porphyromonas gingivalis* (33). Leukocyte recruitment during inflammation involves multiple adhesive interactions under shear



**Fig. 5.** Adhesion and transmigration of LPS pre-treated monocytes on activated HUVECs under flow. (a) Monocyte capture from flow by activated HUVECs is mediated by  $\beta_1$  and  $\beta_2$  integrins. Monocytes pre-treated with blocking mAbs to  $\beta_1$  integrins (PS2) and  $\beta_2$  integrin (TS1/18) showed a significant decrease in capture from physiological flow. Monocytes pre-treated with LPS showed a similar profile. Monocytes were counted in three separate fields after bolus delivery (0 min) and presented as a mean  $\pm$  SEM. (b) LPS does not affect monocyte transmigration under flow. Monocytes, LPS pre-treated (black) or untreated (grey bars), were flowed over HUVEC monolayers. Transmigration was assessed at 0, 15 and 30 min. Transmigration is calculated as a percentage of the sum of cells adhered to the surface of the endothelium (phase light) and transmigrated cells (phase dark). Results are presented as the mean of three independent experiments  $\pm$  SEM. No significant difference was seen between monocytes pre-treated with LPS or untreated.

stress. However, sepsis is characterized by drop in shear stress at the vascular interface. This may prove a critical factor in leukocyte extravasation, as it has been shown that transmigration of lymphocytes is increased by the application of shear stress at the lymphocyte:endothelial interface (41). This requires contact between lymphocytes and activated endothelium promoting a higher turnover of focal adhesion sites consisting of integrins, thus facilitating the lymphocyte movement through the endothelial barrier (41). Observations made with neutrophils suggest that shear stress plays a role in transmigration under restricted

endothelial inflammatory conditions (51). In particular, one experimental model using neutrophils has shown that low-level occupancy of  $\beta_2$  integrins can mediate increased transmigration only under certain shear stress conditions. In contrast, transmigration through chronically activated endothelium expressing high levels of  $\beta_2$  integrin ligands and E-selectin was shown to be shear independent. This model is consistent with our observations using monocytes and would imply that the activation of integrins on circulating leukocytes by the presence of LPS in the vascular compartment is a critical event in determining the pattern of leukocyte transmigration during the different phases of sepsis.

In the scenario presented in this study, LPS stimulation of circulating monocytes would lead to integrin activation and entrapment on luminal surfaces. We have shown that monocytes transmigrate under flow irrespective of LPS pre-treatment. However, under static or sub-physiological flow conditions, LPS activation leads to arrest of the monocyte on the luminal surface whereas non-activated monocytes readily transmigrate across endothelium. This reflects the critical nature shear wall stress plays by favouring more transient interactions, allowing adherent leukocytes to scan the luminal wall for further signals that promote transmigration. The equivalent of near-static conditions may be reached during septic shock, where there is a drastic reduction in blood flow rate due to vasodilation and increased endothelial permeability. As far as we know, this is the first study to document a decrease in monocyte transmigration under static conditions following treatment of human monocytes with LPS. Several other studies that look at the effects of LPS on monocyte migration, which appear contradictory have been published over the past decade. It has been shown that LPS stimulation of endothelial cells induced increased leukocyte transendothelial migration (52). Shen *et al.* (53) also observed an increase in the migration of monocytes across LPS-stimulated HUVECs, when LPS was present for the duration of the experiment. The authors concluded that the effect of LPS was primarily on the endothelial cell since pre-treatment of monocytes with LPS had no effect on their subsequent transmigration. However, we observed that monocyte migration was inhibited after pre-treatment with LPS when endothelium has not been exposed to LPS. These discrepancies may not only be due to different experimental conditions, but also the source of monocytes used. We have used primary monocytes purified from freshly isolated blood, whereas Shen *et al.* used differentiated HL-60 cells. Confirmation of increased monocyte adhesion was seen in another study which demonstrated monocytes treated with LPS adhered more firmly to HUVEC monolayers and serum-coated plastic surfaces (27). Of particular interest was the finding that LPS acted on the monocyte within 25 min to stimulate adhesion to unactivated HUVECs. By contrast, to render the endothelial cells more adhesive for untreated monocytes required 1.5–2 h pre-treatment of the HUVECs with LPS (27). In accordance with these studies, our static experiments have shown that LPS pre-treatment of monocytes increased adhesion and this is paralleled by reduced transmigration. However, LPS pre-treatment had no effect on monocyte transmigration using activated endothelium under normal physiological flow. Therefore, LPS stimulation may alter

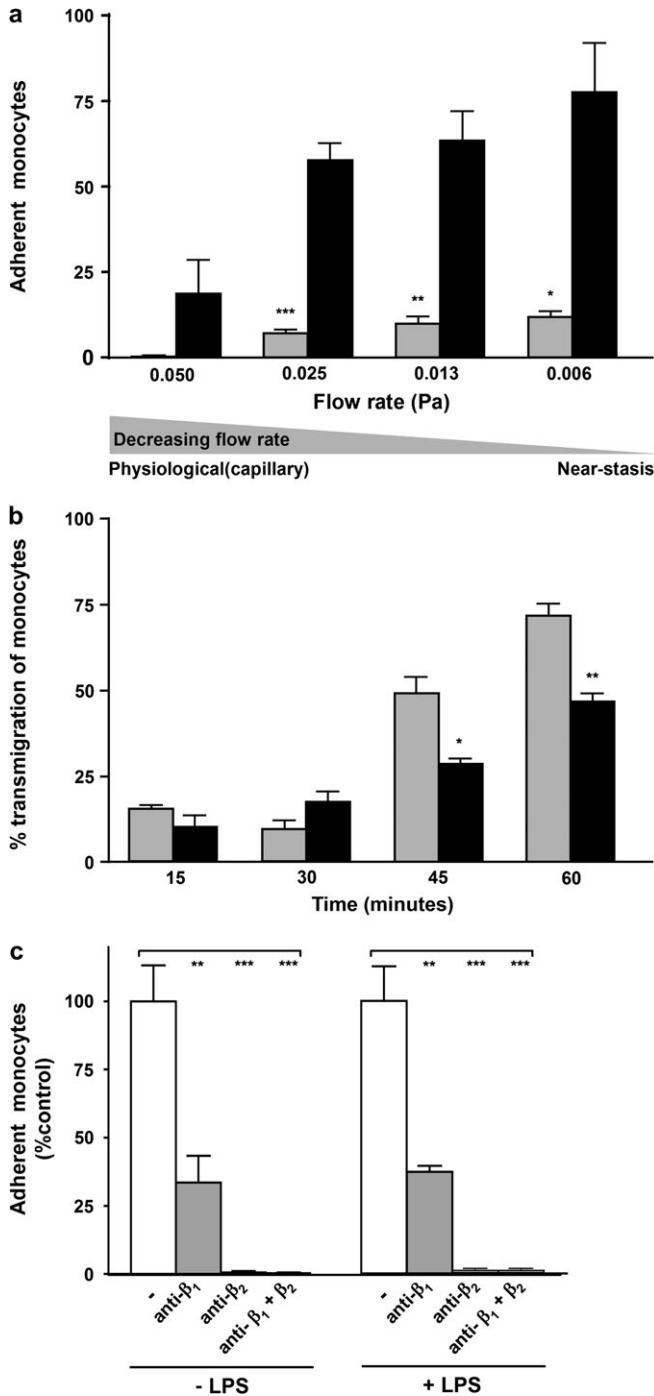


monocyte migration predominantly in situations where blood flow rate is reduced, such as during septic shock.

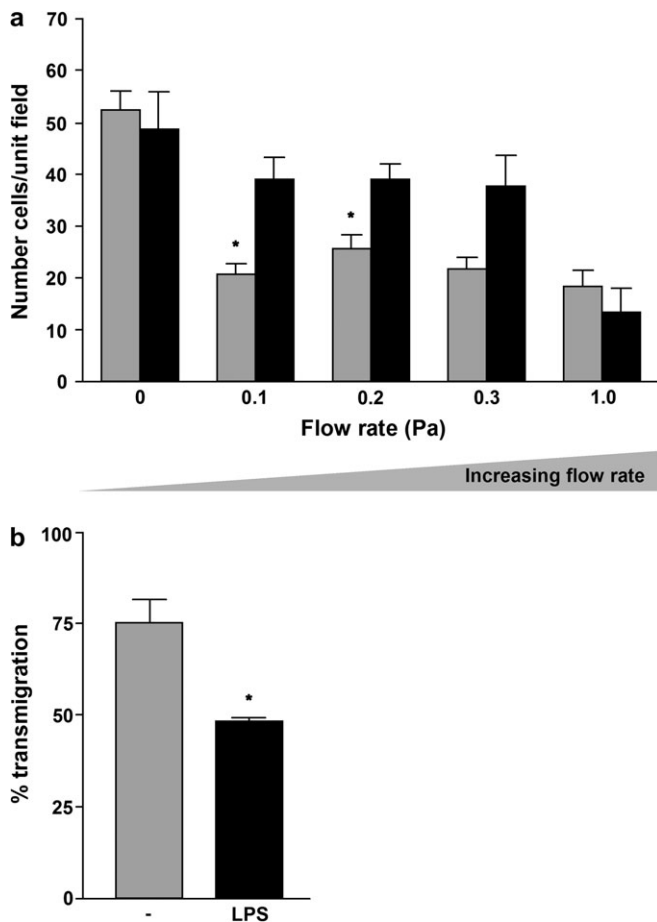
We have shown that activation of integrins on circulating monocytes occurs rapidly upon exposure to LPS, whereas endothelial cells require a number of hours to express gene products, such as ICAM-1 and VCAM-1, that are known to regulate transmigration of monocytes across the endothelial barrier (54). The binding of LPS-pre-treated monocytes under sub-physiological flow rates to the luminal wall may only be relevant in the early stages of sepsis, as prolonged expo-

sure of endothelial cells to LPS within the vascular compartment leads to activation of the endothelium. The binding of activated circulating monocytes to the luminal wall may represent a mechanism of how the vascular compartment can permit the rapid exclusion of bacteria by phagocytosis, exerted by the adhering monocytes on the luminal surface of blood vessels. Such a mechanism may represent an event between bacteraemia, where low levels of bacteria are present but blood pressure is normal and the patient appears asymptomatic, and septicaemia, where the vascular compartment becomes overwhelmed with bacteria. However, if LPS exposure *in vivo* induces circulating monocytes to become more adherent and accumulate on the vascular wall in areas where normal shear forces are no longer operating, then this may contribute to their deleterious effects in endotoxemia since they will release their battery of cytokines directly into the bloodstream. It is important to note that under normal blood flow and non-inflammatory conditions, the higher shear rate at the luminal wall will not permit attachment of monocytes out of flow. This strategy of removing bacteria from the vasculature by entrapment is also employed by neutrophils. Activated neutrophils adherent to vascular surfaces have been shown to release web-like structures of DNA called neutrophil extracellular traps, which actively ensnare bacteria in the circulation (55).

It is clear that the conditions of LPS pre-treatment used in this study may not accurately reflect a concentration or time of exposure of monocytes within the vascular compartment during the early stages of sepsis *in vivo*. While many studies have documented the levels of endotoxin and severity of sepsis, it has proven difficult to extrapolate from quantitative values of endotoxins measured clinically to the concentration of LPS used in this study (56). The kinetics of bacterial clearance during bacteraemia and sepsis is highly dependant on many factors, such as bacteria type, host tolerance and response, and the presence of other inflammatory stimuli (56–58). This apparent heterogeneity in response is reflected in many clinical studies that have documented the levels of



**Fig. 6.** At sub-physiological flow rates, monocytes pre-treated with LPS show increased adhesion but reduced transmigration on unactivated HUVECs. (a) Monocytes pre-treated with (black) or without (grey) LPS were flowed over cultured unactivated HUVEC monolayers at 0.05 Pa. Cells adhered after 5 min were counted in three separate fields. The flow rate was then reduced in incremental steps for 2 min and the cells counted as before. Adhesion is represented as the mean of total cells per field  $\pm$  SEM. (b) Monocyte transmigration on unactivated HUVECs >1 h. Transmigration is determined by video recording a single field for 2 min and counting both adherent and transmigrated cells. Time points were 15 min (three fields counted starting at 12, 14 and 16 min after washout), 30 min (27, 29 and 31 min), 45 min (42, 44 and 46 min) and 60 min (57, 59 and 61 min). Transmigration of monocytes is represented as the mean percentage of total cells per field  $\pm$  SEM. (c) Monocyte capture after decreased incremental flow is mediated by  $\beta_1$  and  $\beta_2$  integrins. Monocytes pre-treated with blocking mAbs to  $\beta_1$  (PS2) and  $\beta_2$  integrin (TS1/18) showed a significant decrease in capture compared with isotype controls on unactivated HUVECs during reduced flow. Monocyte pre-treated with LPS showed a similar profile. Monocytes were counted in three separate fields after flow was reduced in 2-min incremental steps to 0.006 Pa and presented as a mean  $\pm$  SEM. Statistical analyses were performed using non-paired Student's *t*-tests comparing groups  $\pm$ LPS at each time point.



**Fig. 7.** After flow stasis, monocytes pre-treated with LPS show greater adhesion but reduced transmigration on unactivated HUVECs. (a) Monocytes pre-treated with (black) or without (grey) LPS were flowed over cultured unactivated HUVEC monolayers at 0.05 Pa. Flow was stopped for 10 min to allow monocytes to come under contact with the HUVEC monolayer. Total number of cells was counted per unit field under static conditions before flow was resumed and increased incrementally every 1 min with three separate fields counted for each flow rate. Adhesion is represented as the mean of total cells per field  $\pm$  SEM. (b) Monocyte transmigration on unactivated HUVECs after 1 h. Transmigration is determined by video recording a single field for 2 min and counting both adherent and transmigrated cells. Three separate fields were filmed at time points 57, 59 and 61 min. Transmigration of monocytes is represented as the mean percentage of total cells per field  $\pm$  SEM. Statistical analyses were performed using non-paired Student's *t*-tests comparing groups  $\pm$ LPS at each time point.

endotoxin in patients with sepsis where concentration ranges recorded vary substantially (26, 56). The conditions of LPS pre-treatment chosen in this study may therefore represent an event that occurs *in vivo* during the early phase of a progressive bacterial infection in the vascular compartment.

While severe sepsis continues to be a major cause of death, further investigation of this model *in vivo* with humans may prove difficult, as this mechanism appears to play a role in the early phase of sepsis, possibly when the patient is showing little or no clinical symptoms. Therefore, further investigation is required in animal models of sepsis to fully elucidate the kinetics of leukocyte trafficking in the early and latter stages of septicaemia. Targeting monocyte migration may prove to be a useful approach to the therapy of septic

shock, a syndrome that has so far proven extremely difficult to treat.

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### Abbreviations

HUVEC	human umbilical vein endothelial cell
LFA-1	leukocyte function-associated antigen-1
PAMP	pathogen-associated molecular pattern
TLR	Toll-like receptor
TNF $\alpha$	tumour necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule-1

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