Research article

Innate Immunity

16(5) (2010) 278–287

ISSN 1753-4259 (print)
10.1177/1753425909341885

Monitoring of endothelial cell activation in experimental sepsis with a two-step cell culture model

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The aim of this work was to establish and characterize a cell culture model for lipopolysaccharide (LPS)-induced activation of human endothelial cells. Monocytic THP-1 cells were stimulated for 4 h with 10 ng/ml LPS from *Pseudomonas aeruginosa* in media containing 10% human plasma. Culture supernatants containing LPS and factors secreted by THP-1 in response to stimulation were applied to human umbilical vein endothelial cells (HUVECs). Nuclear factor-κB (NF-κB) activity, expression of adhesion molecules, and cytokine secretion were quantified. In addition, the effect of adsorptive removal of tumour necrosis factor-α (TNF-α) from the THP-1 culture supernatant on HUVEC activation was assessed. After 4 h of stimulation, THP-1 cells secreted various mediators including TNF-α (854 ± 472 pg/ml), interleukin (IL)-8 (2069 ± 710 pg/ml), IL-18 (305 ± 124 pg/ml), IL-10 (14 ± 5 pg/ml), and IL-1β (24 ± 11 pg/ml). Stimulated HUVECs showed significantly increased NF-κB activity and secreted high amounts of IL-6 and IL-8. Additionally, adhesion molecules ICAM-1 and E-selectin were increased both in the culture supernatant and at the cell surface. Removal of TNF-α from the THP-1 culture supernatant prior to HUVEC stimulation resulted in a decrease in NF-κB activity, expression of adhesion molecules, as well as IL-6 secretion. The cell culture model established in this study permits the monitoring of LPS-induced endothelial activation, which plays a central role in sepsis and may serve to assess the effect of mediator modulation by methods such as extracorporeal blood purification.

Keywords: cytokines, blood purification, endothelial dysfunction, inflammation, lipopolysaccharide

Introduction

Sepsis is a clinical syndrome defined by the presence of both infection and a systemic inflammatory response.[1] In the US, sepsis affects approximately 750,000 patients annually. The overall mortality rate is about 30%, rising to 50% or greater in patients with septic shock,[2] despite best available intensive care. Although the mortality rate has decreased slightly in recent years, the incidence of sepsis has been continuously increasing, leading to a tripling of the number of sepsis-related deaths over the past 20 years.[3,4] Care for septic patients results in hospital costs of US$16 billion annually in the US, exclusive of posthospitalization care and indirect costs.[3]

The septic process is initiated by pathogen-associated molecular patterns (PAMPS), highly conserved structural motifs that are either located on the surface of pathogens or released during bacterial lysis.[5,6] The main activators of the host response are lipopolysaccharide (LPS) in the case of Gram-negative bacteria, and peptidoglycan in the case of Gram-positive infection. Recognition of PAMPS by pattern recognition receptors (*e.g.* Toll-like receptors, TLRs) expressed on monocytes/macrophages, neutrophils, and endothelial cells results in wide-spread activation of the innate immune response. This process comprises both humoral and cellular components[7,8] and includes massive activation of complement and coagulation cascades.[9,10] In this context, the

Received 3 March 2009; Revised 2 April 2009, 29 May 2009; Accepted 2 June 2009

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endothelium, a major dynamic tissue of the human body, plays a central role in sepsis pathogenesis.[11,12] Vascular endothelial cells have various functions such as the control of vessel wall permeability, regulation of blood flow and quiescence of circulating leukocytes. During sepsis, endothelial cells are affected by different mechanisms including the direct interaction with pathogens or pathogen components, or the action of host-derived factors such as cytokines, chemokines, serine proteases, complement, fibrin and changes in oxygen supply. Endothelial activation results in a loss of the anticoagulant properties of the endothelial surface, loss of barrier function, microcirculatory dysfunction, and perturbed blood flow to the organs. The initial pro-inflammatory phase is followed by the release of an array of anti-inflammatory molecules; it has become clear, in recent years, that most deaths from sepsis are actually caused by a substantially impaired immune response or hyporesponsiveness of monocytes.[13,14]

Current treatment modalities for sepsis are limited, and therapies directed towards single inflammatory mediators have failed to improve survival.[15,16] This can be explained by the fact that the inflammatory cascade is highly redundant and interdependent, thus precluding single modality therapy. Recombinant human activated protein C, the only new drug that has been approved by the FDA for the treatment of severe sepsis,[17] exerts anticoagulant as well as anti-inflammatory and anti-apoptotic effects, underscoring the potential benefit of therapies that target several levels of the septic process. Given the pleiotropic role of many mediators in sepsis, modulation of mediator concentrations rather than complete blockade might be the method of choice. Under this aspect, strategies such as extracorporeal blood purification are superior to systemic administration of antagonists, and there is consensus that the application of extracorporeal blood treatment methods in sepsis is useful.[18] In addition to diffusive and convective techniques for mediator removal, a number of selective or specific adsorbent-based methods have been developed[19,20] and tested in small clinical studies[21,22] with promising results. Still, it remains a key question which mediators of the inflammatory process should be targeted with extracorporeal techniques and at which time points.

Thus, the aim of this study was to develop and characterize a cell culture model for experimental sepsis. The model is based on activation of THP-1 cells, a monocytic cell line, with LPS, and subsequent stimulation of human umbilical vein endothelial cells (HUVECs) with the supernatant of the stimulated THP-1 culture. Endothelial activation was monitored with various read-out assays including cytokine release, expression of adhesion molecules, and activity of nuclear factor κB (NF-κB). Additionally, the effect of adsorptive tumour necrosis factor-α (TNF-α) removal from the THP-1 cell culture supernatant prior to HUVEC stimulation was determined.

It is demonstrated that the model allows assessment of the influence of modulation of single mediators or defined mediator combinations on endothelial activation, and thus may be a useful tool for the development of supportive therapies in endotoxemia/sepsis.

Materials and Methods

Cell culture media and reagents

The cell culture media RPMI-1640 (RPMI), Medium 199 (M199) and Hank's Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), ethylene diamine tetraacetic acid disodium salt (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin-streptomycin (PS) and LPS from *Pseudomonas aeruginosa* were purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant human LPS-binding protein (LBP) and recombinant human sCD14 were from R&D Systems (Minneapolis, MN, USA). Phycoerythrin (PE)-conjugated mouse anti-human CD54 (anti-ICAM-1) and PE-Cy5-conjugated mouse anti-human CD62E (anti-E-selectin) monoclonal antibodies were purchased from BD Biosciences (San Diego, CA, USA). Protease inhibitor cocktail set I (100 ×) and phosphatase inhibitor cocktail set II (100 ×) were purchased from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) was obtained from HyClone (Thermo Fisher Scientific, Waltham, MA, USA). Unfractionated heparin was from Baxter (Vienna, Austria). Cell culture flasks and well plates were purchased from Greiner Bio-One (Kremsmünster, Austria) except otherwise stated. Human plasma from healthy volunteers was obtained from a local plasma donation centre.

Cells and cell culture

Primary HUVECs were isolated from umbilical cord veins. Cords were provided by the local hospital after informed consent of the donors and kept at 4°C in sterile HBSS. HUVEC isolation was performed within 10 h after delivery. Cannulated umbilical veins were perfused with M199 containing 0.02 M HEPES and 100 μM penicillin-streptomycin (M199/HEPES/PS) at 37°C to wash out remaining blood. The veins were filled with dispase (BD Biosciences Europe, Vienna, Austria) and incubated at 37°C for 15 min. After incubation, the dispase solution containing the endothelial cells was flushed from the cord by perfusion with M199/HEPES/PS. Cells were sedimented at 500 *g* for 5 min,

resuspended in M199/HEPES/PS containing 20% FBS, 15 IU/ml heparin and 10 µg/ml endothelial cell growth supplement (ECGS; Upstate, Billerica, MA, USA) and transferred to a 75-cm^2 cell culture flask. One day after isolation, cells were washed with M199/HEPES/PS and supplied with fresh culture medium. After reaching confluency (*i.e.* after 3–5 days), HUVECs were subcultured at a split ratio of 1:2 using 0.02% EDTA for cell detachment. Human umbilical vein endothelial cells were used between passages 4–7 for the experiments described below.

The human monocytic cell line THP-1, originating from a male infant with monocytic leukaemia, was obtained from the American Type Culture Collection (No. TIB-202). THP-1 cells were cultured in RPMI-1640 supplemented with 10% FBS, 0.02 M HEPES and 100 µM penicillin-streptomycin (RPMI/FBS/HEPES/PS) at densities between 0.1–1 $\times 10^6$ cells/ml. All cell cultures were maintained in humidified atmosphere (5% CO_2, 37°C).

Optimization of THP-1 stimulation with LPS

Secretion of TNF-α by THP-1 was used to define optimal stimulation conditions. The influence of various factors (presence of human plasma, LBP, or sCD14) on TNF-α secretion by THP-1 was assessed by stimulating aliquots of 500 µl THP-1 culture (1×10^6 cells/ml) with 10 ng/ml LPS in 24-well plates in different media (Table 1). Since plasma obtained from the plasma donation center contained citrate as anticoagulant, it was heparinized (60 IU unfractionated heparin/ml) prior to addition to medium M199 to avoid coagulation caused by complexation of citrate with Ca^{2+}contained in the medium, and $CaCl_2.2H_2O$ was added to M199/plasma to restore the correct Ca^{2+} concentration of the cell culture medium.

The effect of various LPS concentrations on TNF-α secretion by THP-1 cells was tested by stimulating 500 µl THP-1 culture (1×10^6 cells/ml) with 1, 10, and 100 ng/ml LPS, respectively, in 24-well plates for 4 h in M199/plasma. After stimulation, THP-1 cells were pelleted at 1000 *g*, the supernatants were frozen and kept at–70°C until determination of TNF-α concentration.

Preparation of conditioned media

Cultures of THP-1 cells (1×10^6 cells/ml) were stimulated with 10 ng/ml LPS in M199/plasma for 4 h. After stimulation, the cells were pelleted at 1000 *g* for 5 min, and the supernatants (conditioned media) were aliquoted and stored at –70°C until further use. Control media were obtained by the same procedure without using LPS.

Stimulation of HUVECs

Human umbilical vein endothelial cells were grown to confluency in 75-cm^2 cell culture flasks, washed with 5 ml M199/HEPES/PS and stimulated with 10 ml of conditioned media or control media. At defined time points, supernatants were aspirated, centrifuged at 1000 *g*, aliquoted, and stored at –70°C until further analysis. The cells were washed with 3 ml of ice-cold PBS and detached with 3 ml 0.02% EDTA. After addition of 3 ml PBS, cells were pelleted at 500 *g* for 5 min and used for flow cytometry analysis or for the preparation of nuclear and cytoplasmic extracts.

Quantification of cytokines and soluble adhesion molecules

The cytokines TNF-α, IL-1β, IL-18, IL-10, IL-6, IL-8, as well as the soluble adhesion molecules sE-selectin and sICAM-1 were quantified by ELISA (all from Bender MedSystems, Vienna, Austria; sICAM-1 from Biosource) according to the instructions of the manufacturer. For the experiments monitoring the effect of TNF-α removal on HUVEC activation, cytokines were quantified using the Bio-Plex™ Cytokine Array from Biorad (Vienna, Austria) according to the instructions of the manufacturer.

Flow cytometry analysis

After HUVEC stimulation, cells were detached using 0.02% EDTA and washed with PBS containing 0.1% sodium azide. Human umbilical vein endothelial cells were stained by incubation with PE-conjugated anti-ICAM-1, PE-Cy5-conjugated anti-E-selectin, or with the

Table 1. Media used for stimulation of THP-1 with LPS

Medium	Ingredients
M199/FBS	M199/HEPES/PS, 10% FBS
M199/FBS/sCD14/LBP	M199/FBS, 1 µg/ml sCD14, 0.5 µg/ml LBP
M199/plasma	M199/HEPES/PS, 10% human plasma
M199/plasma/sCD14/LBP	M199/plasma, 1 µg/ml sCD14, 0.5 µg/ml LBP

respective IgG control antibodies in phosphate-buffered saline containing 0.1% sodium azide and 2% fetal bovine serum on ice for 30 min. After two washing steps (as above), cells were analyzed on a FACScan flow cytometer (Becton Dickinson Austria, Vienna, Austria). Using the CellQuest software (Becton Dickinson), 10,000 gated cells were presented in histograms.

Preparation of nuclear and cytoplasmic cell extracts

In its inactive form, NF-κB is bound to a family of cytoplasmic inhibitory proteins. Degradation of these inhibitors results in translocation of NF-κB into the nucleus and subsequent gene activation. To quantify NF-κB in the nucleus, nuclear and cytoplasmic fractions were prepared with the NucBuster™ Protein Extraction Kit (Novagen, VWR International, Vienna, Austria) with slight modifications of the protocol recommended by the manufacturer. All steps were performed on ice or at 4°C, except when noted. For a confluent HUVEC culture of a 75-cm^2 flask, the pelleted cells were washed with 500 µl ice-cold PBS and centrifuged for 5 min at 500 *g*. The cell pellet was dissolved in 75 µl NucBuster Reagent 1 supplemented with protease inhibitor cocktail set I and phosphatase inhibitor cocktail set II (0.75 µl each). After vortexing, the samples were incubated on ice for 15 min, vortexed again and centrifuged at 16,000 *g* for 5 min. The supernatant (cytoplasmic fraction) was aliquoted and frozen at–80°C. The nuclear pellet was washed with 250 µl ice-cold PBS. The pelleted nuclei were dissolved in 25 µl NucBuster Reagent 2 supplemented with protease inhibitor cocktail set I and phosphatase inhibitor cocktail set II (0.3 µl each) and dithiothreitol (1 mM). The samples were vortexed, incubated on ice for 5 min, vortexed again and centrifuged at 16,000 *g* for 5 min, and the supernatants (nuclear extracts) were aliquoted and stored at –70°C until further analysis. Protein content of nuclear extracts was determined with the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

Experiments to monitor the effect of TNF-α removal on HUVEC activation were performed on a small scale (12-well plates, 2×10^5 HUVECs/well, see below). For these experiments, nuclear extracts were prepared using the Nuclear Extract Kit and protein was quantified with the ProStain Protein Quantification Kit, which is more sensitive than the BCA assay. Both kits were obtained from Active Motif (Rixensart, Belgium).

Nuclear factor-κB p65 assay

Nuclear NF-κB was determined using the NoShift™ Transcription Factor Assay Kit and the NoShift NF-κB (p65) reagents (all from Novagen, VWR International, Vienna, Austria). Briefly, 20 µg of nuclear protein was incubated on ice with biotin-labeled wild-type DNA (10 pmol) for 30 min. Then, the mixture was applied to a streptavidin-coated plate and incubated for 1 h. Wells were washed three times and treated with primary antibody (mouse anti-p65) for 1 h, washed three times and incubated with the anti-mouse secondary antibody for 30 min. After five washing steps, the substrate was added and the reaction was stopped after 30 min by addition of 1 N HCl. The absorbance was read at 450 nm to quantify nuclear NF-κB.

In the experiments to monitor the effect of TNF-α removal on HUVEC activation, NF-κB activity was determined with the more sensitive TransAM NF-κB p65 chemiluminescence assay (Active Motif) according to the manufacturer's protocol. Briefly, 1.25 µg of nuclear cell extract per well was incubated in a 96-well plate coated with an oligonucleotide containing the NF-κB consensus site. Bound NF-κB was detected using the antibody to NF-κB p65 and an HRP-conjugated secondary antibody leading to a chemiluminescence readout.

Preparation of an adsorbent for TNF-α

A chimeric human–mouse monoclonal anti-TNF-α antibody (Infliximab, Centocor, Leiden, The Netherlands) was immobilized onto cyanogen bromide (CNBr)-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden). All steps were performed under sterile conditions. Lyophilized Sepharose was swollen in 1 mM HCl and washed on a glass filter for 15 min. Sepharose (2 g wet weight) was washed three times with 0.1 M $NaHCO_3$ containing 0.5 M NaCl (pH 8.3; coupling buffer). The beads were pelleted by centrifugation, resuspended in 3330 µl coupling buffer containing 6.7 mg Infliximab and the mixture was incubated overnight with gentle shaking at 4°C. After incubation, the adsorbent was extensively washed with coupling buffer and remaining active groups were blocked with 2 ml 0.1 M Tris-HCl, (pH 8.0) at 4°C for 2 h. The adsorbent was washed alternately with 12 ml high (0.1 M Tris-HCl, pH 8.0, containing 0.5 M NaCl) and low pH buffer (0.1 M sodium acetate, pH 4.0, containing 0.5 M NaCl), three times each, to remove excess uncoupled ligand. Finally, the TNF-α adsorbent was washed with 0.9% (w/v) NaCl containing 0.2% NaN_3 and stored at 4°C until further use. To produce a control adsorbent, human serum albumin was immobilized on CNBr-activated Sepharose using the same protocol.

Adsorption of TNF-α from conditioned medium

Aliquots of 10 mg TNF-α adsorbent (or control adsorbent coated with human serum albumin) were washed

with 0.9% (w/v) NaCl three times, added to 1 ml conditioned medium and incubated for 1 h at 37°C with gentle rolling. The adsorbent was pelleted by centrifugation and the supernatant was immediately used for HUVEC stimulation. Aliquots of the supernatant were kept at –70°C until quantification of TNF-α and IL-6.

Stimulation of HUVECs with TNF-α depleted conditioned medium

For this series of experiments, three different HUVEC batches were stimulated using the same pool of conditioned medium pretreated with TNF-α adsorbent.

Aliquots of 2×10^5 HUVECs/well were seeded into 12-well plates (NUNC, Langenselbold, Germany) and cultured overnight in M199/HEPES/PS containing 20% FBS, 15 IU/ml heparin and 10 µg/ml ECGS. Before stimulation, the culture medium was aspirated, the cells were washed with M199/HEPES/PS and stimulated using 1 ml/well conditioned medium pretreated with TNF-α or control adsorbent. After 1, 3, 5, 7, and 15 h, respectively, media were aspirated, centrifuged at 2300 *g* and stored at –70°C until cytokine detection. Human umbilical vein endothelial cells were harvested for determination of NF-κB activity and surface expression of ICAM-1 and E-selectin after 1 and 15 h of stimulation, respectively.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS Statistics v.17.0 for Windows (SPSS Inc., Chicago, IL, USA). When comparing two groups, data were analyzed by the non-parametric Wilcoxon rank sum test. Data are expressed as mean ± SD. Significance was accepted at $P \leq 0.05$.

RESULTS

Stimulation of THP-1 with LPS

The monocytic cell line THP-1 was stimulated with LPS in medium M199 both in the presence and absence of 10% human plasma, recombinant human sCD14, or recombinant human LBP (Table 1). Upon stimulation with 10 ng/ml LPS in M199/FBS, THP-1 (1×10^6 cells/ml) secreted 18 ± 1 pg/ml TNF-α after 4 h, as compared to 101 ± 8 pg/ml when sCD14 (1 µg/ml) and LBP (0.5 µg/ml) were added (Fig. 1). Tumour-necrosis factor-α levels were highest (177 ± 2 pg/ml after 4 h) with M199/plasma. Addition of sCD14 (1 µg/ml) and LBP (0.5 µg/ml) to M199/plasma did not further increase TNF-α secretion (171 ± 31 pg/ml after 4 h). After 4 h, TNF-α levels started to decrease (41 ± 4 pg/ml after 24 h). The influence of LPS concentration on TNF-α secretion by THP-1 is shown in Figure 2. Stimulation of THP-1 (1×10^6 cells/ml) with 1 ng/ml LPS for 4 h in M199/plasma did not result in detectable TNF-α levels, compared to stimulation with 10 ng/ml (177 ± 2 pg/ml TNF-α) or 100 ng/ml (2265 ± 106 pg/ml TNF-α). Stimulation of THP-1 with 10 ng/ml LPS in medium M199/plasma was used for all further experiments.

Release of cytokines and adhesion molecules from stimulated THP-1 and HUVECs

As shown in Figure 3, THP-1 (1×10^6 cells/ml) stimulated for 4 h with 10 ng/ml LPS in M199/plasma secreted high levels of TNF-α (854 ± 472 pg/ml), IL-8 (2069 ± 710 pg/ml), and IL-18 (305 ± 124 pg/ml), as well as low amounts of IL-10 (14 ± 5 pg/ml) and IL-1β (24 ± 11 pg/ml) (t_0 in Fig. 3). For each of these factors, except for IL-18, the secretion from stimulated THP-1 cells was significantly increased as compared to the unstimulated control ($P \leq 0.05$). Incubation of HUVECs with the conditioned media resulted in significantly increased secretion of IL-6 (3249 ± 288 pg/ml) and IL-8 (57922 ± 21867 pg/ml) after 16 h as compared to HUVECs treated with control medium ($P \leq 0.05$). In addition, soluble adhesion molecules sICAM-1 (54 ± 2 pg/ml) and sE-selectin (39 ± 7 pg/ml) were significantly increased after 16 h of HUVEC stimulation ($P \leq 0.05$).

Expression of ICAM-1 and E-selectin on HUVECs

Expression of ICAM-1 and E-selectin on HUVECs was increased after 16 h of stimulation with conditioned medium as compared to the unstimulated control (Fig. 4). Under culture conditions, HUVECs expressed the same basal levels of ICAM-1 as control HUVECs in stimulation experiments and hardly any E-selectin.

Nuclear factor-κB p65 activity in HUVECs

As shown in Figure 5, nuclear translocation of NF-κB in HUVECs was increased from 15 min to 16 h of stimulation with conditioned media as compared to basal NF-κB activity in the control ($P \leq 0.05$). Removal of conditioned medium after 16 h of stimulation resulted in a decrease of NF-κB activity to baseline levels within 1 h. Restimulation of HUVEC with conditioned medium lead to an increase of NF-κB activity to levels comparable to the activity in HUVECs that were under control conditions before restimulation.

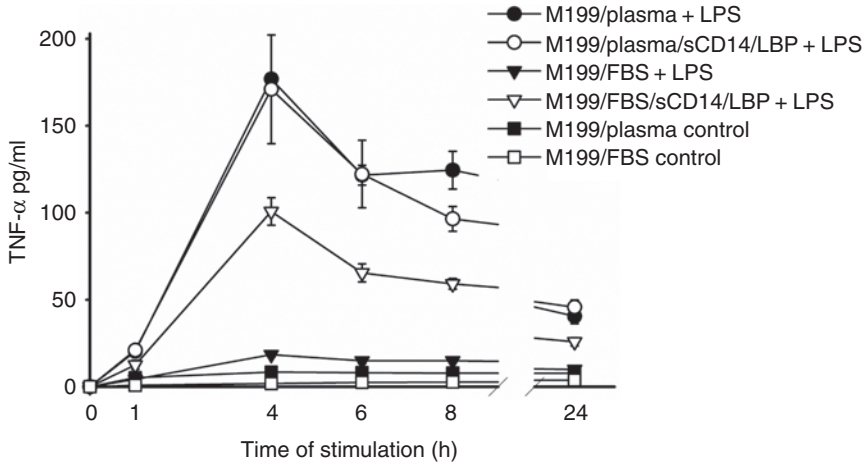

Fig. 1. Secretion of TNF-α from THP-1 after stimulation with 10 ng/ml LPS in different media. Cultures of THP-1 were stimulated in medium M199 containing 10% FBS or 10% human plasma with or without addition of sCD14 (1 μg/ml) and LBP (0.5 μg/ml). The concentrations of TNF-α are expressed as mean ± SD ($n = 3$).

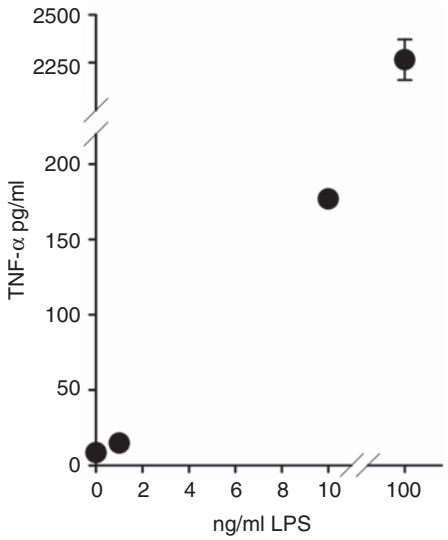

Fig. 2. Secretion of TNF-α from THP-1 after stimulation in M199/plasma for 4 h using different LPS concentrations. Concentrations of TNF-α are expressed as mean ± SD ($n = 3$).

Stimulation of HUVECs with TNF-α depleted conditioned medium

To assess whether the cell culture model was sensitive enough to respond to single mediator removal (in contrast to removal of conditioned medium containing a range of stimulating factors as described above), TNF-α was adsorbed from the conditioned medium and HUVECs were exposed to the TNF-α depleted conditioned medium. As shown in Figure 6, TNF-α was completely removed from the conditioned medium treated with the TNF-α adsorbent, while use of the control adsorbent had no effect on TNF-α concentrations. The reduction in TNF-α concentrations seen at later time points in the medium treated with control adsorbent is most likely due to degradation of TNF-α over time. Human umbilical vein endothelial cell stimulation with the TNF-α depleted conditioned medium resulted in significantly decreased secretion of IL-6 ($P \leq 0.05$ for 5, 7, 15 h of stimulation), significantly decreased surface expression of ICAM-1 ($P \leq 0.05$) and E-selectin (P ? 0.05) after 15 h of stimulation, as well as significantly decreased NF-κB activity already after 1 h of stimulation ($P \leq 0.05$).

DISCUSSION

The endothelium fulfils many vital functions, such as control of vasomotor tone, vascular permeability, and haemostatic balance. Together with monocytes, macrophages, and other myeloid-derived cells, endothelial cells are also part of the innate immune response, since these cells serve as a first line of defence and recognize invading pathogens.[12,23] Thus, the endothelium acts as a kind of sensor for alterations in the extracellular environment, and endothelial activation is part of a normal physiological response to infection. However, this physiological response may become dysregulated, resulting in massively disturbed haemostatic balance, increased cell adhesion and leukocyte migration, apoptosis, as well as loss of barrier function.

Since sustained endothelial activation represents one of the central events in sepsis, we aimed to develop and characterize a cell culture model as a tool to monitor endothelial activation caused by inflammatory mediators released from THP-1, a monocytic cell line, after stimulation with LPS. While such a model might serve to identify new potential targets for the therapy of Gram-negative sepsis, it can also be used to monitor the effect of modulation or adsorption of defined factors or of

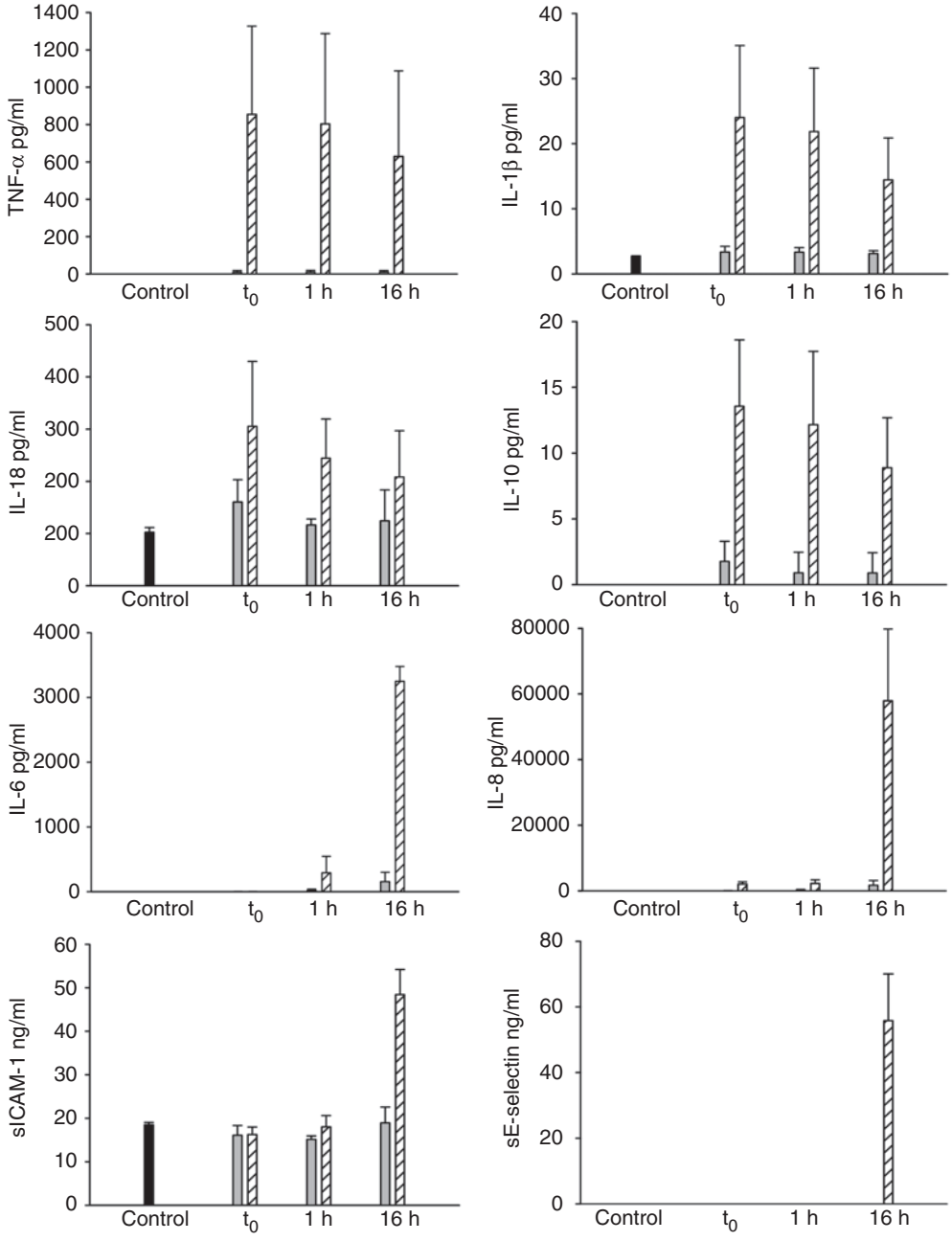

Fig. 3. Release of cytokines and soluble adhesion molecules from stimulated THP-1 and HUVECs. Black bars: control (medium M199 containing 10% human plasma). Cultures of THP-1 were stimulated for 4 h with media containing LPS (hatched bars) or with control media without LPS (grey bars). The cytokine concentrations in the supernatants after 4 h of THP-1 treatment are indicated as t_0. Subsequently, HUVECs were incubated with these supernatants and cytokine concentrations were determined after 1 h and 16 h. Concentrations are expressed as mean ± SD ($n = 3$).

combinations of factors on endothelial activation. The model is based on stimulation of THP-1 cells with LPS in the presence of 10% human plasma and subsequent exposure of HUVECs to the culture supernatants of the stimulated THP-1 cells. Human plasma was added as a source of sCD14 and LBP, which are co-factors for LPS signalling. Thus, relatively low LPS concentrations (10 ng/ml) were sufficient for monocyte stimulation, resulting in typical TNF-α levels in the range of 200 pg/ml TNF-α after 4 h of stimulation. In comparison, published studies used at least 50–100 ng/ml LPS to stimulate monocytes or endothelial cells.[24–26] Another feature characteristic of our model is the use of conditioned medium, which contains the physiological proportions of mediators released from stimulated THP-1 cells. This approach is closer to the *in vivo* situation than stimulation of endothelial cells with isolated factors such as LPS or TNF-α alone.

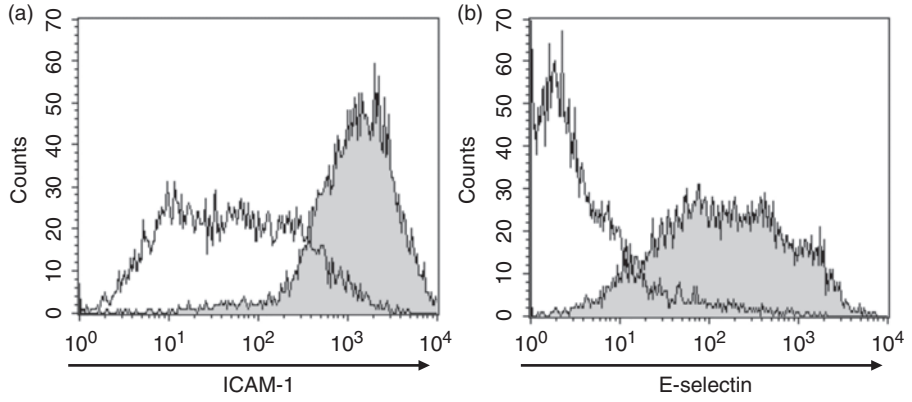

Fig. 4. Expression of ICAM-1 (a) and E-selectin (b) on HUVECs after 16 h of stimulation with conditioned media (grey) or control (black line). The figures show one representative FACS analysis ($n = 3$).

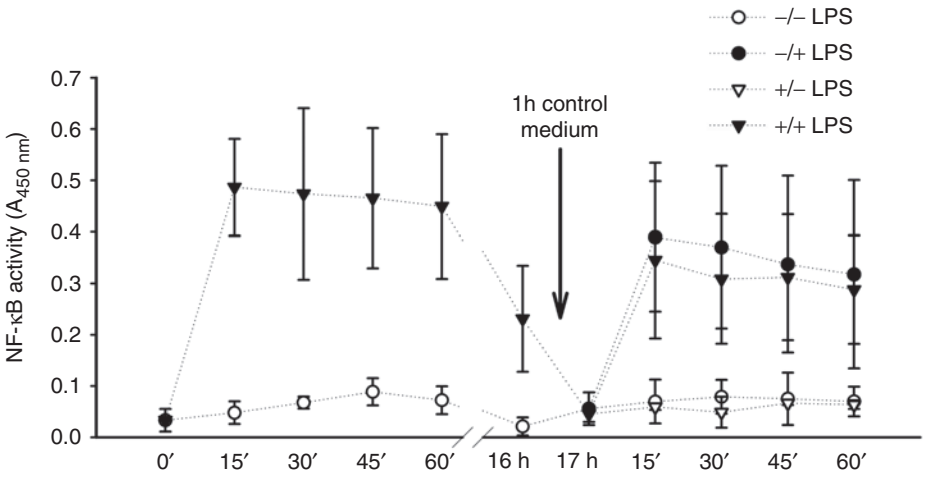

Fig. 5. Activity of NF-κB in HUVECs stimulated with conditioned media. Activity of NF-κB was significantly increased from 15 min to 16 h of HUVEC stimulation with conditioned media. The 1-h removal of conditioned media after 16 h of stimulation resulted in a decrease of NF-κB activity to baseline levels. A re-stimulation of HUVECs lead to a re-increase in NF-κB activity. Activity of NF-κB is given as mean ± SD ($n = 3$).

To avoid high donor-to-donor variation, we chose to use an immortalized human monocyte cell line instead of peripheral blood mononuclear cells. THP-1 cells have been shown to react in a very similar way to peripheral blood mononuclear cells and thus to be suited as a model system.[27]

Upon stimulation with LPS in the presence of 10% human plasma, THP-1 cells secreted a range of cytokines also known from literature,[27] including high amounts of TNF-α, IL-8 and IL-18, as well as low amounts of IL-1β. Human umbilical vein endothelial cell activation upon exposure to the conditioned medium harvested from the THP-1 cells was monitored by the release of cytokines as well as the expression of surface-bound and soluble adhesion molecules. Human umbilical vein endothelial cells secreted high amounts of IL-6 and IL-8 after 16 h of stimulation with conditioned medium. In addition, both the secretion of soluble ICAM-1 and E-selectin and the surface expression of these adhesion molecules were enhanced, compared to the unstimulated control. These factors have been described to be elevated in sepsis patients and are discussed to serve as predictors for mortality at different time courses of the disease.[28]

Since NF-κB is centrally involved in the regulation of the transcription of many of the immunomodulatory mediators in sepsis, NF-κB activity (translocation of p65 to the nucleus) was assessed for stimulated HUVECs in our model. Nuclear translocation of NF-κB increased quickly (within 15 min) after exposure of HUVECs to conditioned medium and remained elevated for at least 16 h. After 16 h of HUVEC stimulation, the conditioned medium was removed from the endothelial cells and replaced with fresh control medium without LPS. This step, which is basically equivalent to the simultaneous removal of all inflammatory mediators, resulted in a decrease of NF-κB activity to baseline levels. Re-stimulation of HUVEC after 1 h was followed by a

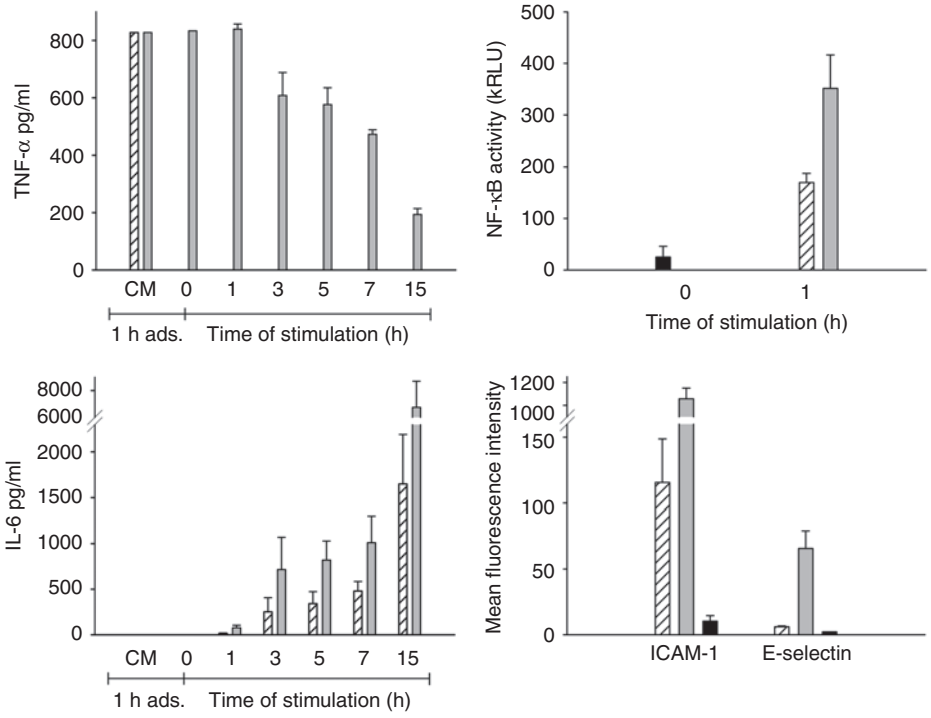

Fig. 6. Effect of TNF-α depletion on HUVEC stimulation. Activity of NF-κB as well as IL-6, ICAM-1 and E-selectin expression were significantly decreased after 1 h (NF-κB) and 15 h (IL-6, ICAM-1 and E-selectin), respectively, for HUVECs stimulated with TNF-α depleted medium (hatched bars) as compared to stimulation with conditioned medium pretreated with control adsorbent (grey bars). Black bars represent HUVECs under culture conditions. Data are given as mean ± SD ($n = 3$).

re-increase in NF-κB activity to levels comparable to those during the first stimulation, demonstrating that HUVECs could be re-activated. Thus, simultaneous removal of all stimuli in our model resulted in reduction of NF-κB translocation to baseline levels. To assess whether the model was sensitive enough to monitor the effect of a single mediator removal, TNF-α was adsorbed from the conditioned medium using Sepharose beads functionalized with the chimeric human–mouse monoclonal anti-TNF-α antibody Infliximab. Stimulation of HUVECs with the TNF-α depleted conditioned medium resulted in significant reduction of NF-κB activity, demonstrating the strong influence of TNF-α on NF-κB activation. Additionally, the secretion of IL-6 and the surface expression of ICAM-1 and E-selectin was significantly reduced after 15 h of HUVEC stimulation with TNF-α depleted conditioned medium as compared to medium treated with a control adsorbent. Together, these findings demonstrate that the cell culture model responds to depletion of a single mediator in the stimulation medium.

Conclusions

The model developed and characterized in this study allows for the monitoring of LPS-induced endothelial activation, which plays a central role in sepsis. It may serve to assess the effect of mediator modulation by methods such as extracorporeal blood purification. Studies to monitor the effect of removal of individual mediators or of combinations of mediators at different time points are underway.

Acknowledgements

The authors thank Kaisa Hellevuo who initiated the project to set up the cell culture model. This work was supported by the Austrian Research Promotion Agency (FFG; Bridge Project No. 812987) and by Fresenius Medical Care, Bad Homburg, Germany.

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