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# Bacterial cell envelopes (ghosts) but not S-layers activate human endothelial cells (HUVECs) through sCD14 and LBP mechanism

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**Abstract**

Bacterial cell-envelopes (called ghosts) and surface layers (S-layers) are discussed to be used as vaccines and/or adjuvants, consequently it is necessary to find out which immunomodulatory mediators are induced in human cells. The present work focuses on the effects of ghosts (*Escherichia coli* O26:B6), S-layers (*Bacillus stearothermophilus*) in comparison with LPS and antibiotic-inactivated whole bacteria (*E. coli* O26:B6) on human umbilical vein endothelial cells (HUVEC) with regard to the release of interleukin 6 (IL-6) and the expression of surface E-selectin and the role of lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14) and serum for this activation.

Endothelial cells responded to ghosts, whole bacteria and LPS with IL-6 release up to 15000 pg/ml and surface E-selectin expression, while in contrast the response to S-layers with IL-6 release up to 500 pg/ml was very weak. Compared to LPS, 10–100-fold higher concentrations of bacterial ghosts and whole bacteria were required to induce the cytokine synthesis and E-selectin expression. IL-6 release and E-selectin expression of HUVECs were reduced in the absence of serum and equivalent to unstimulated samples. We have also studied the role of CD14 and LBP for the activation of endothelial cells using antiCD14 and antiLBP antibodies (Ab). AntiCD14 and antiLBP Ab both inhibited IL-6 release and E-selectin expression in a dose dependent manner after stimulation with ghosts, whole bacteria and LPS but had no effect on S-layers stimulated cells. AntiCD14 Ab inhibited more effectively than antiLBP Ab. These findings suggest that bacterial ghosts but not S-layers activate HUVECs through sCD14 and LBP dependent mechanisms. 

*Keywords:* Endotoxicity; Bacterial ghosts; S-layers; Cytokines; HUVEC

## 1. Introduction

Bacterial ghosts are cell envelopes preparations of gram-negative bacteria produced by controlled expression of the plasmid-encoded lysis gene E [1], encoding all antigenic structures of the living cell [2] and are an alternative to conventional nonliving vaccine preparations [3–5]. S-layers are crystalline surface layers in many prokaryotic organisms composed of protein or glycoprotein subunits [6,7]. These S-layers have many potential applications and have been used as a carrier/adjuvants for the development of model vaccines [8,9]. S-layers preparations have been shown to elicit a highly distinctive activation of the immune system, presumably influencing the response of TH1/TH2 [10,11].

Endothelial cells strongly respond to bacterial cell wall components like LPS by IL-6 release and de novo expression of E-selectin [12,13]. CD14, a membrane antigen expressed on the surface of monocytes and

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macrophages [14–16] but not on endothelial cells, acts as a receptor for LPS and LPS complexed with LPS binding protein (LBP) [14,15]. LBP is present at levels up to 10 μg/ml in plasma of healthy individuals and increases 10 fold during acute phase responses [17,18]. The soluble form of CD14 (sCD14) is present in plasma of healthy individuals in a concentration of 2–4 μg/ml [18,19] and several studies revealed that LPS/LBP complex binds to serum derived sCD14 and activates endothelial cells [20,21]. Therefore we studied the role of serum, sCD14 and LBP in the de novo expression of E-selectin and IL-6 release in HUVECs. We compared the activities of bacterial cell-envelopes (ghosts), antibiotics-inactivated whole bacteria and LPS (all from *Escherichia coli 026:B6*) and S-layers (from *Bacillus stearothermophilus*) using antihuman CD14 and antihuman LBP antibodies.

## 2. Materials and methods

### 2.1. Reagents

Phenol–water extracted lipopolysaccharide *E. coli 026:B6* was obtained from Sigma (St. Louis).

Purified antihuman CD14 monoclonal antibody 3C10 was kindly provided by Dr. Lobb (Biogen, Cambridge) and antihuman LBP antibody LH7-7–10 was a generous gift of Dr. Tobias (Scripps Research Inst., La Jolla, CA). We used both antibodies in a concentration of 10 μg/ml. Mouse monoclonal antihuman IL-6 antibody (5E1) as first antibody and rabbit polyclonal antihuman IL-6 as a second antibody were kindly provided by W.A. Buurman. E-selectin specific biotinylated antibody ENA-2 as a second antibody was used for surface E-selectin measurement. Recombinant human IL-6 was kindly provided by Dr. P. Mayer (Novartis Research Institute, Vienna). The Limulus amoebocyte lysate (LAL)-assay (Chromogenix, Mölndal) was used for detection of endotoxin contamination in the media and serum, which both were found to contain less than 1 endotoxin unit (EU)/ml.

### 2.2. Preparation of bacterial cell envelopes, ghosts

Bacterial ghosts were produced by expressing the cloned lysis gene E from bacteriophage PhiX174 as follows: 500 ml LB-medium containing 50 μg/ml kanamycin was inoculated with 5 ml of a fresh bacterial overnight culture harbouring the lysis plasmid pML1 [1,2]. The culture was grown in a 28°C water-bath shaker under aeration. At an optical density of 0.3 at 600 nm (OD 600) the culture was transferred to a 42°C water-bath shaker and the heat-induced plasmid-conferred lysis of the bacteria was followed by continuous reading of the OD600. At the end of lysis (i.e. no further decrease in OD600) ghosts were collected by centrifugation (10 min, 10,000 rpm, 40°C, Sorvall GSA), washed 3 times with physiological saline and lyophilized.

### 2.3. Bacterial cell wall preparation and S-layer self-assembly products

*Bacillus stearothermophilus* PV72/p2 was grown aerobically by continuous culture at 57°C. Preparation of cell walls and S-layers self-assembly products followed published methods [22,23].

Briefly, to disrupt the cells frozen biomass was suspended in buffer (50 mM Tris–HCl, pH 7.2), sonicated, centrifuged several times and the cytoplasmic membrane was removed by treatment with 0.5% Triton X-100 in 50 mM Tris–HCl buffer, pH 7.2. The cell walls were washed with buffer and distilled water extensively and S-layers were isolated by repeated extraction with 5 M guanidine hydrochloride (GHCl). After dialysis against water to remove GHCl, the self-assembly products were collected by centrifugation. The precipated S-layers after dialysis were removed by centrifugation and the resulting supernatant fluid was dialysed against 2 mM $CaCl_2$ (2× for 1 h, room temperature and subsequently overnight at 4°C). This procedure resulted in self-assembly products of appropriate sizes (approximately 0.5–5 μm). The S-layers were lyophilized and used for the experiment.

### 2.4. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained by treatment with 50 U/ml collagenase (Gibco, Paisly) for 5 min at 37°C. The cells were cultured in fibronectin coated (30 μg/ml) tissue culture plates (Corning, NY) in M199 (Gibco, Life technology) supplemented with 20% 0.2 μm filtered heat inactivated human serum HS (derived from the Red-Cross bloodbank, Vienna), 40 μg/ml endothelial cell growth supplement ECGS (Biomedica, Vienna) and 40 μg/ml gentamycin (Refobacin, Merck, Darmstadt). The confluent cells were trypsinized with 0.025% trypsin/EDTA, collected with 30% HS-medium and centrifuged (800 rpm). The cell pellets were resuspended in 20% HS–M199 medium with ECGS and antibiotics and seeded at $1 \times 10^3$ cells/100 μl/well in fibronectin coated 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA) and cultured for 2 days to achieve confluence.

### 2.5. Induction of cytokine release

The confluent HUVECs were rinsed twice with MEM 199 without HS, endothelial cell growth sup-

plement and gentamycin. The media were replaced by the solution containing the stimuli of interest in presence or absence of 10% HS, in presence or absence of 10 μg/ml antiLBP and antiCD14 antibodies in 6 replicates 6 h for E-Selectin and 6 and 20 h for the IL-6 assay. We used 4 stimuli in varying concentrations between 0.001 and 50 μg/ml.

Supernatants were harvested and kept at −20°C until use in the IL-6 ELISA. Endothelial cell monolayers were fixed with 0.025% glutaraldehyde for 10 min at room temperature and washed twice with PBS and kept at 4°C until use in the E-selectin immunoassay.

### 2.6. IL-6 ELISA

The IL-6 concentration in the culture supernatant was determined using an ELISA for IL-6. Briefly, 96-well immuno maxisorp plates (Nunc, Roskilde) were coated with a mouse monoclonal antihuman IL-6 antibody (5E1) overnight at 4°C. The plates were blocked for 1 h with PBS–1% BSA (Sigma, St. Louis) at room temperature. The samples and recombinant human IL-6 as standard for standard titration curves were added in duplicates and incubated for 1 h at room temperature. Plates were incubated with a rabbit polyclonal antihuman IL-6 as second antibody (antibodies kindly provided by W.A. Buurman) for another 1 h, followed by streptavidin-peroxidase (Boehringer Mannheim) as conjugate for 1 h. *O*-phenyl-diamine (Sigma, St. Louis) was added as a substrate and the colour reaction was stopped with 4 N $H_2SO_4$. The plates were measured at 492–620 nm by photometer (SLT, Salzburg) using Biolise program for calculation.

Table 1
Endotoxin activity[a]

| | EU/μg |
|---|---|
| S-layers (*Bacillus stearothermophilus*) | 0.057 |
| Ghosts (*E. coli* O26:B6) | 316 |
| Whole cells (*E. coli* O26:B6) | 118 |
| LPS (*E. coli* O26:B6) | 2273 |

[a] Stimuli were prepared in medium (M199) containing 10% human serum and endotoxin activity was measured using a LAL-test. Endotoxin activity by medium and human serum were under 0.01 EU/ml.

### 2.7. E-selectin

E-selectin expression was determined by ELISA. Briefly, plates with endothelial cells were blocked with PBS–1% BSA (Sigma) for 1 h at room temperature. The plates were incubated with the E-selectin specific biotinylated antibody ENA-2 as second antibody for 1 h at room temperature. As conjugate peroxidase-conjugated streptavidin–POD (Boehringer Mannheim) was used. *O*-phenyl-diamine (Sigma, St. Louis) was

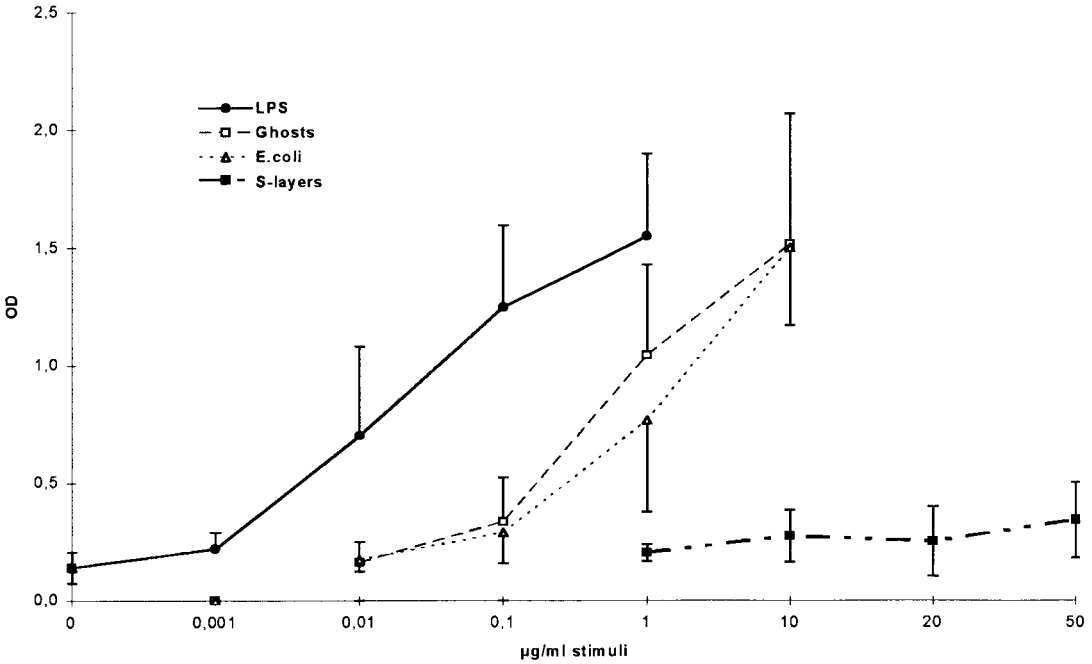


Fig. 1. Surface E-selectin expression on HUVECs. HUVEC monolayers were incubated with stimuli in several concentration between 0,001–50 μg/ml in medium M199 containing 10% human serum for 6 h. Data are expressed as mean ± S.D. of three measurements.

Table 2
Effects of serum, CD14 and LBP on LPS- (a) and *E. coli*-induced (b) E-selectin expression on HUVECs[a]

| | No serum | 10% serum | 10% serum + antiCD14 Ab | 10% serum + antiLBP Ab |
|---|---|---|---|---|
| *(a) LPS (μg/ml)* | | | | |
| 0 | 0,11 ± 0,03 | 0,14 ± 0,06 | 0,17 ± 0,01 | 0,17 ± 0,03 |
| 0,001 | 0,11 ± 0,03 | 0,22 ± 0,06 | 0,14 ± 0,02 | 0,19 ± 0,06 |
| 0,01 | 0,10 ± 0,03 | 0,70 ± 0,37 | 0,17 ± 0,04 | 0,50 ± 0,20 |
| 0,1 | 0,11 ± 0,03 | 1,24 ± 0,34 | 0,70 ± 0,32 | 1,50 ± 0,37 |
| 1 | 0,14 ± 0,05 | 1,54 ± 0,34 | 1,82 ± 0,39 | 2,20 ± 0,22 |
| *(b) E. coli (μg/ml)* | | | | |
| 0 | 0,11 ± 0,03 | 0,14 ± 0,06 | 0,17 ± 0,01 | 0,17 ± 0,03 |
| 0,01 | 0,12 ± 0,02 | 0,17 ± 0,05 | 0,15 ± 0,02 | 0,18 ± 0,05 |
| 0,1 | 0,11 ± 0,02 | 0,28 ± 0,13 | 0,14 ± 0,03 | 0,20 ± 0,06 |
| 1 | 0,11 ± 0,03 | 0,76 ± 0,38 | 0,21 ± 0,07 | 0,65 ± 0,21 |
| 10 | 0,12 ± 0,02 | 1,49 ± 0,32 | 0,60 ± 0,43 | 1,70 ± 0,22 |

[a] HUVEC were incubated with various concentration of either LPS from *E. coli* O26:B6 (a) or antibiotics-inactivated whole *E. coli* O26:B6 (b) for 6 h in medium M199 in presence or absence of 10% human serum. Further HUVEC were also incubated with 10 μg/ml of either antiCD14 or antiLBP antibody in presence of 10% human serum. Surface E-selectin expression was measured as OD. Data are mean ± S.D. of three measurements.

added as substrate and the colour reaction was stopped with 4 N $H_2SO_4$. The plates were measured at 492–620 nm by photometer (SLT, Salzburg) using Biolise program for calculation.

## 3. Results

### 3.1. Endotoxin

Preparations of stimuli were examined for endotoxic activity using a standard amoebocyte lysate (LAL) assay. This assay was performed for all tested preparations to exclude endotoxic contamination. Data are shown in Table 1. We measured 3-times higher endotoxin activity in ghosts than in antibiotics inactivated-wholecell *E. coli O26:B6* calculated on μg dry weight.

### 3.2. Role of serum in E-selectin expression

HUVEC monolayers were rinsed intensively with serum-free medium before the addition of stimuli to ensure maximal removal of soluble serum components like sCD14 and LBP. Monolayers were subsequently incubated in the presence or absence of 10% HS with different stimuli as described in concentrations between 0,001–50 μg/ml and incubated for 6 h.

At least a 10 times higher concentration of *E. coli* and ghosts than LPS was needed to reach the maximum of E-selectin expression in a concentration dependent manner. The E-selectin expression correlated with stimuli concentration. In contrast, endothelial cells responded to S-layers even at highest concentration of 50 μg/ml with fewer expression of E-selectin than to LPS at 0.01 μg/ml. Further it was no significant increase of E-selectin levels comparing the cells stimulated with S-layers to unstimulated cells (Fig. 1).

The E-selectin expression of LPS as well as *E. coli* and ghosts-stimulated cells in presence of serum was highly dose dependent and was strongly reduced in absence of serum and reached the values of unstimulated cells. In contrast, the serum did not play a role for E-selectin expression when the cells were stimulated with S-layers (Tables 2).

Table 3
Effects of serum and time on IL-6 release on HUVECs caused by different stimuli[a]

| | No serum 6 h | No serum 20 h | 10% serum 6 h | 10% serum 20 h |
|---|---|---|---|---|
| 0 μg/ml stimuli | 47 ± 16 | 174 ± 83 | 89 ± 18 | 297 ± 61 |
| 1 μg/ml LPS | 131 ± 65 | 566 ± 269 | 4200 ± 1161 | 13002 ± 1229 |
| 10 μg/ml ghosts | 74 ± 31 | 216 ± 125 | 2450 ± 1044 | 14390 ± 3861 |
| 10 μg/ml whole cell *E. coli* | 61 ± 31 | 201 ± 29 | 2419 ± 609 | 10036 ± 2132 |
| 50 μg/ml S-layers | 42 ± 4 | 271 ± 63 | 100 ± 14 | 479 ± 238 |

[a] HUVECs were incubated with LPS, ghosts, antibiotics-inactivated whole bacteria (all from *E. coli O26:B6*) and S-layers (from *Bacillus stearothermophilus*) in concentration between 1–50 μg/ml in medium M199 in presence or absence of 10% human serum for 6 and 20 h, respectively. IL-6 level was measured in the supernatants. Data are mean ± S.D. of three measurements.

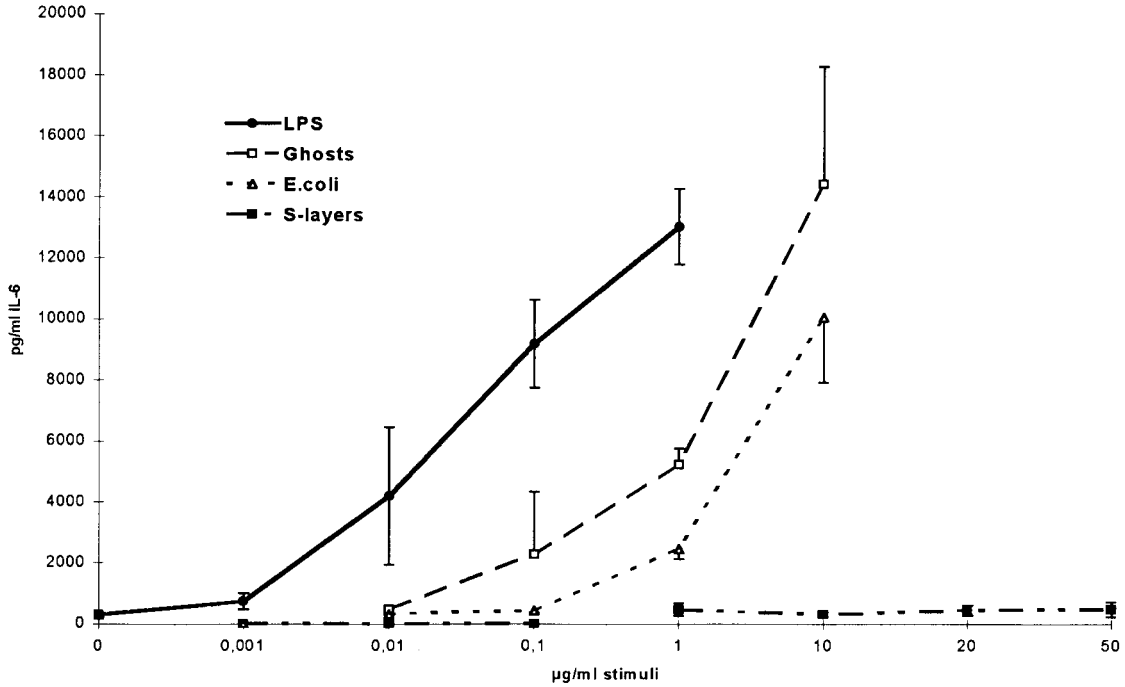


Fig. 2. IL-6 release from HUVECs after 20 h incubation. HUVEC monolayers were incubated with stimuli in several concentration between 0,001–50 µg/ml in medium M199 containing 10% human serum for 20 h. Data are expressed as mean ± S.D. of three measurements.

### 3.3. Role of serum and time on IL-6 release

The IL-6 release level correlated with concentration of ghosts, whole cells and LPS but not of S-layers and in presence of 10% human serum was 100% higher after 20 h than after 6 h of incubation (Table 3, Fig. 2).

The IL-6 release of LPS as well as *E. coli* and Ghosts-stimulated cells in presence of serum was highly dose dependent and was strongly reduced in absence of serum and reached the values of control cells. In contrast the serum did not play a role for IL-6 release when the cells were stimulated with S-layers (Table 4).

### 3.4. Inhibition of IL-6 release and E-selectin expression by antiCD14 and antiLBP antibodies

The 3C10 antiCD14 antibody inhibited LPS, *E. coli* and ghosts-induced IL-6 release and E-selectin ex-

Table 4
Effects of CD14 and LBP on LPS- (a) and *E. coli*-induced (b) IL-6 release of HUVEC after 20 h of incubation[a]

| | No serum | 10% serum | 10% serum + antiCD14 Ab | 10% serum + antiLBP Ab |
|---|---|---|---|---|
| *(a) LPS (µg/ml)* | | | | |
| 0 | 174 ± 83 | 297 ± 61 | 0 | 0 |
| 0,001 | 169 ± 45 | 743 ± 264 | 0 | 172 ± 122 |
| 0,01 | 148 ± 29 | 4193 ± 2265 | 219 ± 149 | 356 ± 85 |
| 0,1 | 178 ± 40 | 9173 ± 1447 | 1963 ± 1174 | 4003 ± 80 |
| 1 | 566 ± 269 | 13002 ± 1229 | 8581 ± 2285 | 9142 ± 30 |
| *(b) E. coli (µg/ml)* | | | | |
| 0 | 174 ± 83 | 297 ± 61 | 0 | 0 |
| 0,01 | 176 ± 34 | 325 ± 16 | 94 ± 67 | 126 ± 89 |
| 0,1 | 151 ± 14 | 430 ± 57 | 114 ± 81 | 160 ± 114 |
| 1 | 154 ± 24 | 2452 ± 340 | 288 ± 204 | 915 ± 651 |
| 10 | 201 ± 29 | 10036 ± 2132 | 471 ± 287 | 7796 ± 1477 |

[a] HUVEC were incubated with various concentration of either LPS from *E. coli O26:B6* (a) or antibiotics-inactivated whole *E. coli O26:B6* (b) for 20 h in medium M199 containing 10% human serum in presence or absence of either 10 µg/ml antiCD14 or antiLBP. IL-6 level was measured in the supernatants. Data are mean ± S.D. of three measurements.

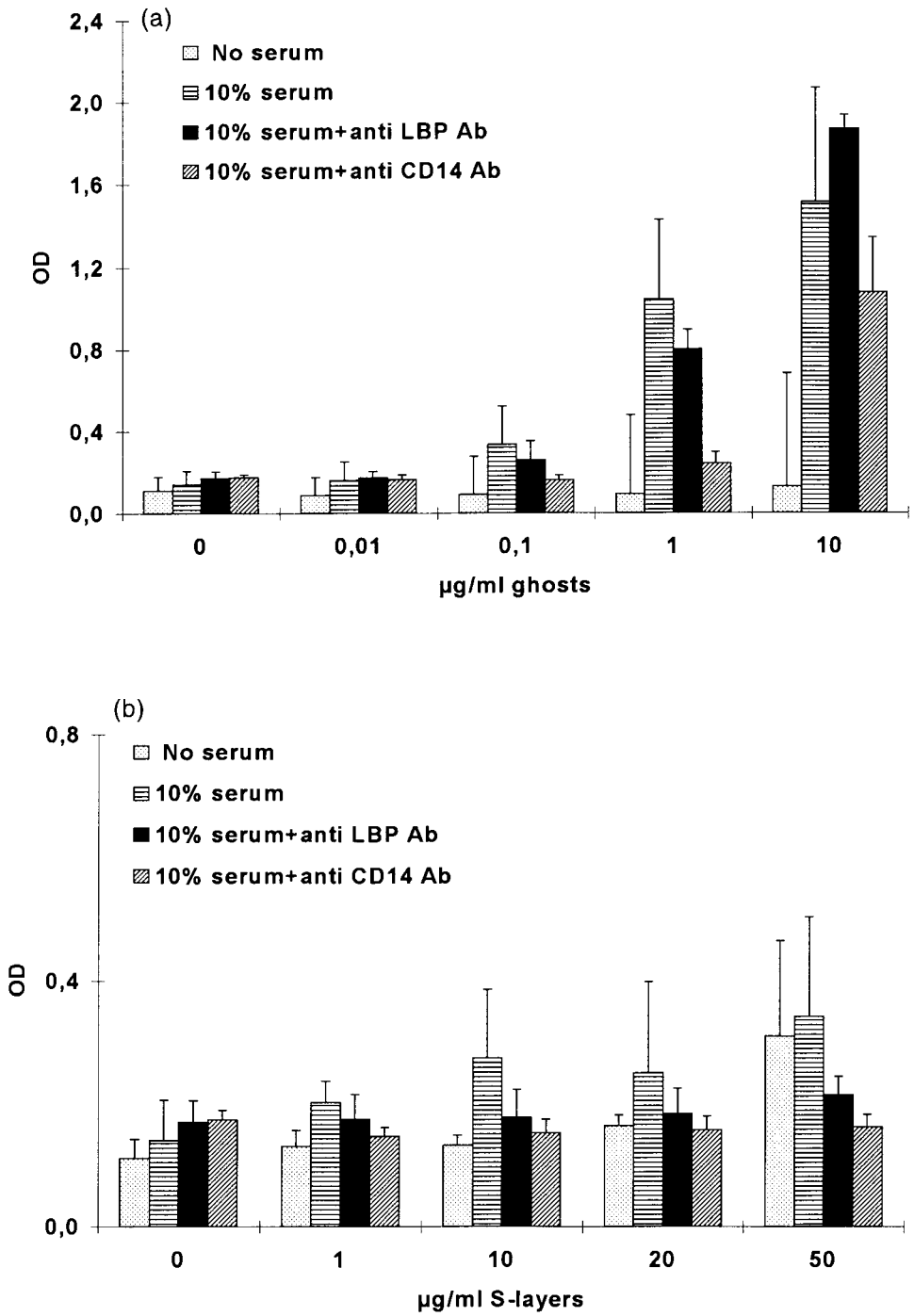


Fig. 3. HUVECs E-selectin expression after 6 h incubation. HUVEC monolayers were incubated with ghosts (a) or S-layers (b) in several concentration between 0,01–50 µg/ml in medium M199 in presence or absence of 10% human serum. Further HUVECs were also incubated with 10 µg/ml either antiCD14 or antiLBP antibody in presence of 10% human serum. Surface E-selectin expression of the cells were measured using an ELISA. Data are means ± S.D. of three measurements.

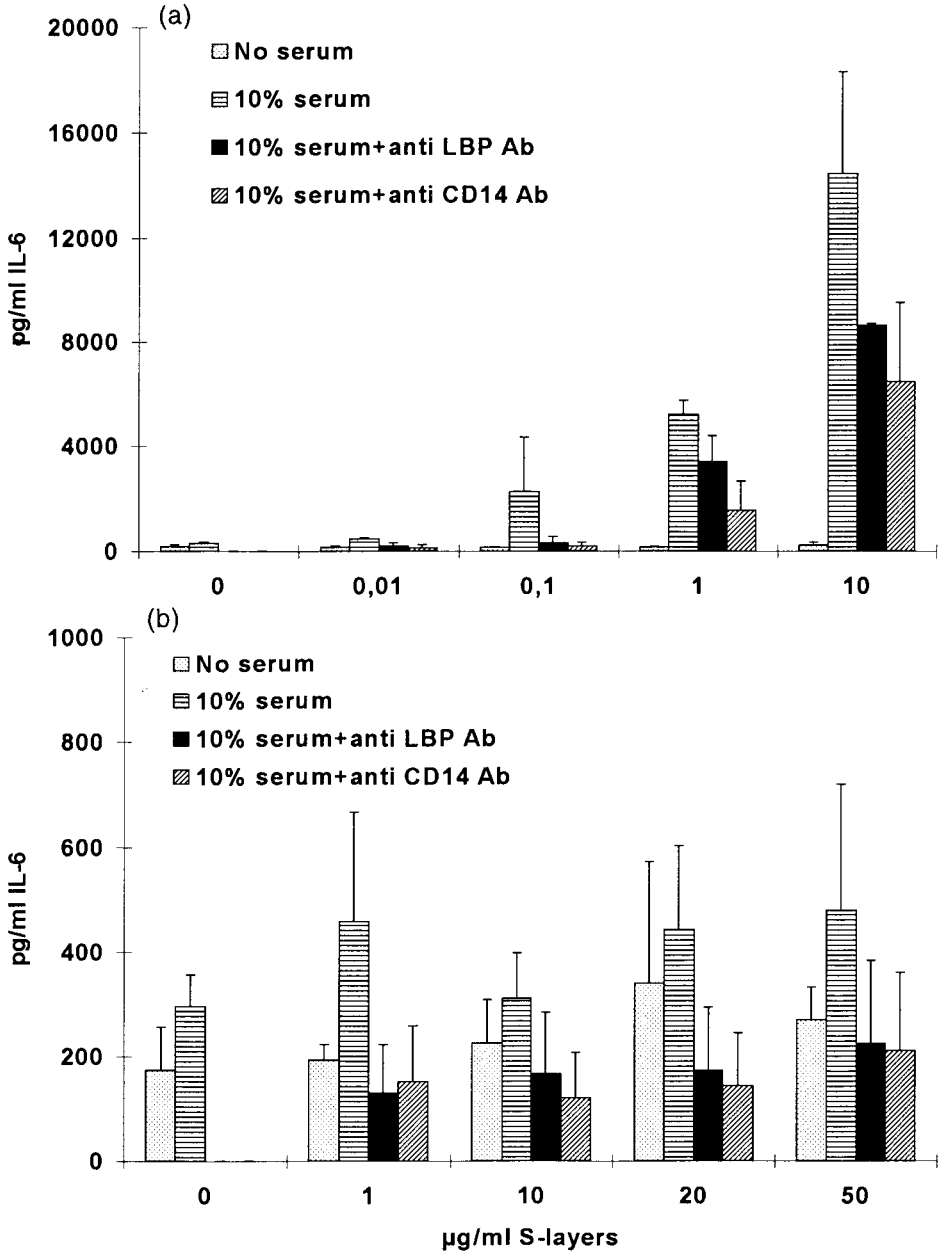


Fig. 4. IL-6 release from HUVECs after 20 h incubation. HUVEC monolayers were incubated with ghosts (a) or S-layers (b) in several concentration between 0,01–50 µg/ml in medium M199 in presence or absence of 10% human serum. Further HUVECs were also incubated with 10 µg/ml of either antiCD14 or antiLBP-antibody in presence of 10% human serum. IL-6 release were measured in supernatants. Data are average ± S.D. for three measurements.

pression by 70% or more in a dose dependent manner, most effectively with lower stimuli concentration and less with highest stimuli concentration. In contrast antiCD14 antibody had no inhibition effect on the minimal IL-6 release and E-selectin expression induced by S-layers.

The LH7-10 antiLBP antibody inhibited also LPS, *E. coli* and ghosts induced IL-6 release, but in the higher concentration range (1–10 µg/ml) of stimuli had even an amplification effect on IL-6 release and E-selectin expression. AntiLBP antibody like antiCD14 antibody had no inhibition effect on the minimal IL-6 release and E-selectin expression induced by S-layers (Figs. 3 and 4).

## 4. Discussion

Bacterial cell envelope, ghosts, contain all bacterial cell surface structures like LPS and receptors that are

present in live bacteria. This is due to the fact that bacteria are inactivated without any physically or chemically alteration of the bacterial surface through the expression of the plasmid-encoded lysis gene E. A tunnel through the inner and outer bacterial membranes is built and cytoplasma releases into the surrounding medium and the cell envelope is preserved. These envelopes are termed 'ghosts' in the literature and are under investigation as candidate vaccines and adjuvants [3–5]. As an alternative, bacterial S-layers, which are present on the cell surface of many bacteria have been investigated for use as adjuvants [8,9].

While the immune response of LPS is well known in vitro and in vivo in human and animal models, we investigated, which immune responses are induced by ghosts and S-layers. Some knowledge is available for ghosts and S-layers from several animal models and from a mouse macrophage cell-line model [24], while little is known about the effect on human cells in vitro. We compared the activations of HUVECs with regard to cytokine production and upregulation of adherence molecule induced by different vaccine candidates like bacterial cell envelope, ghosts (*E. coli O26:B6*) and S-layers (*B. stearothermophilus*) with those by LPS and antibiotic inactivated whole cells (*E. coli O26:B6*).

The role of LBP and CD14 in human monocyte stimulation with LPS are well investigated. CD14, a membrane antigen expressed on the surface of monocytes and macrophages acts as a kind of receptor for LPS and LPS complexed with LPS binding protein (LBP) [14,15]. Endothelial cells lack the membrane CD14, but several studies revealed that soluble CD14 (sCD14) which is present in a concentration of 2–4 μg/ml in plasma of healthy individuals, binds the LPS/LBP complex and activates endothelial cells [20,21]. This shows the importance of sCD14 in LPS-induced activation of nonhemopoetic cells like endothelial cells. Asmuth et al. [25] demonstrated that antiCD14 mAB inhibits only the LPS-induced activation but does not inhibit the endothelial cells responses to cytokines and PMA. This would indicate that CD14 (or sCD14) is only involved in the LPS-induced pathway. Ishii at al. [26] could show that bovine pulmonary arterial endothelial cells treated with LPS had an increase of endothelial permeability and that only sCD14 but not LBP is important and necessary for this effect.

We have investigated whether sCD14, LBP or serum are necessary for ghosts and S-layers to activate HUVECs similar to LPS. We studied the influence of CD14 and LBP using antibodies against CD14 and LBP to eventually block the signal transduction activated by LPS, ghosts, S-layers and antibiotics inactivated whole *E. coli* and measured the expression of membrane E-selectin and release of IL-6.

According to the endotoxin activity of our stimuli (using a Limulus-assay) we found that compared on weight base, LPS (*E. coli O26:B6*) expressed endotoxic activity values 100-times higher than bacterial ghosts and 300-times higher than antibiotics inactivated whole *E. coli*. Bacterial ghosts had higher endotoxin Limulus activity compared to antibiotics-inactivated whole cells, presumably because of a higher cell numbers when compared on a dry-weight basis. In contrast S-layers (*B. stearothermophilus*) preparation, exhibited an extremely low endotoxin Limulus activity. Compared to LPS, however 100-fold higher concentrations of bacterial ghosts and whole cells were required to induce the same cytokine synthesis and E-selectin expression. IL-6 release and E-selectin expression of HUVECs were also strongly reduced in the absence of serum and were similar to unstimulated samples.

Antibodies against CD14 and LBP inhibited LPS, *E. coli* and ghosts-induced activation of endothelial cells measured by IL-6 release and E-selectin expression in a dose dependent manner. Whereas antibodies had no inhibitory effect on weak IL-6 release and E-selectin expression induced by S-layers.

We could see more profound inhibitory effects mediated by the 10 μg/ml antiCD14 Ab than 10 μg/ml antiLBP Ab. This could be because the antiCD14 Ab was in contrast to antiLBP Ab a monoclonal Ab, or that sCD14 has a more important role in activation than LBP.

We have also investigated the cytokine release (TNF, IL-6) induced by the different stimuli in a whole blood system using antiCD14 and antiLBP Ab (data not shown) and could see similar inhibitory effects on IL-6 release. In contrast to endothelial cells, S-layers induced a strong TNF and IL-6 release by whole blood, but similar to endothelial cells antiCD14 and antiLBP antibodies had no inhibitory effect.

In conclusion we could demonstrate that endothelial cells responded much stronger to bacterial ghosts than to S-layers. Bacterial ghosts but not S-layers activated the cells via a LBP-CD14 pathway.

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