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Heterogeneity in lipopolysaccharide responsiveness of endothelial cells identified by gene expression profiling: role of transcription factors

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Summary

Interindividual differences of endothelial cells in response to endotoxins might contribute to the diversity in clinical outcome among septic patients. The present study was conducted to test the hypothesis that endothelial cells (EC) with high and low proinflammatory potential exist and to dissect the molecular basis underlying this phenomenon. Thirty human umbilical vein endothelial cell (HUVEC) lines were stimulated for 24 h with lipopolysaccharide (LPS) and screened for interleukin (IL)-8 production. Based on IL-8 production five low and five high producers, tentatively called types I and II responders, respectively, were selected for genome-wide gene expression profiling. From the 74 genes that were modulated by LPS in all type II responders, 33 genes were not influenced in type I responders. Among the 41 genes that were increased in both responders, 17 were expressed significantly stronger in type II responders. Apart from IL-8, significant differences in the expression of proinflammatory related genes between types I and II responders were found for adhesion molecules [intercellular adhesion molecule (ICAM-1), E-selectin)], chemokines [monocyte chemoattractant protein (MCP-1), granulocyte chemotactic protein (GCP-2)], cytokines (IL-6) and the transcription factor CCAAT/enhancer binding protein-delta (C/EBP-δ). Type I responders also displayed a low response towards tumour necrosis factor (TNF)-α. In general, maximal activation of nuclear factor (NF)-κB was achieved in type I responders at higher concentrations of LPS compared to type II responders. In the present study we demonstrate that LPS-mediated gene expression differs quantitatively and qualitatively in types I and II responders. Our results suggest a pivotal role for common transcription factors as a low inflammatory response was also observed after TNF-α stimulation. Further studies are required to elucidate the relevance of these findings in terms of clinical outcome in septic patients.

Keywords: endothelial cells, gene expression profiling, heterogeneity, LPS

Introduction

Infections with gram-negative bacteria are considered to be the most frequent cause in the onset of organ dysfunction, although other mechanisms might also be involved [1]. Despite considerable effort that has been given over the past decade to understand the systemic inflammatory response and characteristics of severe sepsis, mortality in septic patients remains high. It is clear that mortality is highly dependent on individual patient factors, e.g. pre-existing disease, hospitalization and age [2,3]. However, the reason why the course of sepsis develops more vigorously in some patients than in others has not yet been solved. Apart from health status, age and hospitalization it is believed that genetic variations within promoter, intron or exon sequences of inflammatory genes may, in part, determine the clinical course in septic patients [4].

Genetic polymorphisms have been identified in genes encoding inflammatory molecules, e.g. interleukin (IL)-1, IL-1 receptor antagonist (IL-RA), tumour necrosis factor (TNF)-α or Toll-like receptors (TLR) [5,6]. In septic patients mortality seems to be associated only with the TNFB2 allele [7–10], which is also associated with high TNF-α production by mononuclear cells. This suggests

that TNF-α might play a pivotal role in mortality caused by sepsis. In clinical studies anti-TNF-α antibodies have nevertheless failed to demonstrate any significance on mortality in septic patients [11,12]. These findings therefore point towards the involvement of other factors that might, in concert with TNF-α, influence mortality in these patients.

There is compelling evidence that in addition to monocytes [13,14], endothelial cells also play an important role in the clinical outcome of sepsis. In septic patients a dysfunction in macro- and microcirculation is observed frequently. Moreover, endothelial cells have the propensity to produce high amounts of a variety of proinflammatory mediators, e.g. cytokines, chemokines and eicosanoids. As a consequence of increased expression of adhesion molecules on these cells, leukocytes of septic patients adhere much more strongly and migrate subsequently along a chemotactic gradient into the subendothelial interstitial tissue [15].

As the amount of inflammatory mediators, produced by endothelial cells, is highly heterogeneous [16,17], severity of inflammation and thus mortality in sepsis could be dependent on interindividual variations of endothelial cells to respond to bacterial toxins.

In the present study, we therefore tested the hypothesis that the response of endothelial cells to lipopolysaccharide (LPS) can be classified into general phenotypes of cells with a low and a high proinflammatory potential. Based on previous findings that serum IL-8 concentration is associated with severity of sepsis [16,18,19], and the fact that IL-8 is produced strongly by endothelial cells [20,21], endothelial cells were grouped according to their IL-8 production. The following questions were then raised: (1) is low IL-8 production associated with low production of other mediators, (2) if so, what are these mediators and (3) what is the molecular basis for this phenomenon?

Materials and methods

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords, as has been described previously [22]. The cells were cultured in essential growth medium for endothelial cells (Promocell, Heidelberg, Germany) in gelatine (1%) coated culture flasks (Greiner, Frickenhausen, Germany). Confluent monolayers were subcultured by trypsin 0025 vol%/ethylenediamine tetraacetic acid (EDTA) 0·01 vol% (Promocell, Heidelberg, Germany). Characterization of endothelial cells was performed on the basis of uptake of acetylated low-density lipoprotein (LDL), a positive staining for factor VIII-related antigen and platelet-endothelial cell adhesion molecule (PECAM), and a negative staining for alpha smooth muscle actin.

Chemokine production

HUVEC (1×10^5 cells/ml) were seeded in 24-well plates and grown until confluence. The cells were stimulated for 24 h with 1 μg/ml of LPS (Sigma, Deisenhofen, Germany). In each experiment control HUVEC were included to determine basal expression of chemokines. Supernatants were collected and assessed for IL-8 production by enzyme-linked immunosorbent assay (ELISA) (R&D Systems GmbH, Wiesbaden, Germany). ELISA was performed according to the manufacturer's instructions. Each experimental condition was performed in triplicate and each experiment was confirmed at least three times.

DNA-isolation and IL-8 genotyping

DNA was isolated from HUVEC using the Wizard genomic DNA purification Kit (Promega Corporation, Madison, WI, USA). IL-8 genotyping was performed by polymerase chain reaction (PCR), as described previously [23]. In brief, 40 ng of genomic DNA was added to a 25 μl reaction mixture containing 0·2 mM deoxyribonucleoside triphosphate (dNTPs) (Gibco BRL, Eggenstein, Germany), 12·5 pmol of primer (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0·5 units of *Taq*-DNA-polymerase (Invitrogen GmbH, Karlsruhe, Germany) and 1·5 mM $MgCl_2$. After 5 min of denaturation at 94°C, amplification was performed in 30 cycles, each consisting of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C. After the last cycle primer extension was performed for 5 min at 72°C. T/A alleles were assessed by Mfe I restriction enzyme digestion of the PCR products (Roche, Basel, Switzerland) and visualized by ethidium bromide in 2% agarose gels.

RNA isolation, cRNA and array hybridization

Sample preparation and processing was performed according to the Affymetrix GeneChip Expression Analysis Manual (http://www.Affymetrix.com). Briefly, endothelial cell monolayers were stimulated with LPS (1 μg/ml) or left in normal medium for 24 h. Total RNA was isolated from these cultures using Trizol®-Reagent (Gibco BRL). Hereafter DNase treatment was carried out, using RNase free DNase I (Ambion, Woodward, Austin, TX, USA). RNA concentration and quality were assessed by RNA 6000 nano assays on a Bioanalyser 2100 system (Agilent, Waldbronn, Germany). Five μg of RNA was converted into cDNA using T7-$(dT)_{24}$ primers and the SuperScript Choice system for cDNA synthesis (Life Technologies, Inc., Rockville, MD, USA). Biotin-labelled cRNA was prepared by *in vitro* transcription using the BioArray high yield RNA transcript labelling kit (Enzo Diagnostics, Farmingdale, NY, USA). The resulting cRNA was purified, fragmented and hybridized to U133A gene chips (Affymetrix, Santa Clara, CA, USA). After hybridization the chips were stained with streptavidin–phycoerythrin

(MoBiTec, Goettingen, Germany) and analysed on a GeneArray scanner (Hewlett Packard Corporation, Palo Alto, CA, USA).

Reverse transcription–polymerase chain reaction (RT-PCR)

One μg of total RNA was reverse transcribed into cDNA using the SuperScript TM II Preamplification System (Life Technologies, Karlsruhe Germany) according to the manufacturer's instructions.

The sequences of the primers used for amplification are listed in Table 1. Primers were purchased from Perkin Elmer. PCR was performed in a volume of 25 μl containing 10 mM Tris HCl, 75 mM KCl, 1·5 mM $MgCl_2$, 200 μM dNTPs, 20 pmol of each primer, 0·5 μl cDNA and 2·5 U of *Taq* DNA polymerase (Gibco BRL). A control containing no cDNA was always included. The PCR reactions were initiated at 94°C for 3 min and amplification was performed in 28–32 cycles, each consisting of 1 min at 94°C, 1 min at the annealing temperature and 2 min at 72°C, followed by final extension for 10 min at 72°C. PCR products were subjected to electrophoresis in 1% agarose (Serva, Boehringer Ingelheim, Heidelberg, Germany).

Flow cytometry

Confluent endothelial cell monolayers were harvested by T/E and washed three times in PBS. Hereafter, monoclonal antibodies directed against intercellular adhesion molecule (ICAM-1) and E-selectin, both from Dako, Glostrup, Denmark, were added to the cells in dilutions of 1 : 20. Isotype-matched anti-idiotypic antibodies were used as negative control. After 30 min at 4°C, the cells were washed and incubated with an anti-mouse F(ab′)2, fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 30 min at 4°C. Finally the cells were washed three times with PBS before analysis was performed on a FACScalibur (Becton Dickinson, Heidelberg, Germany).

Table 1. List of oligonucleotides used for cDNA amplification.

Primer	Sequence	Size of PCR product (bp)
GAPDH	5′-GTCTTCACCACCATGGAGAA-3′	268
	5′-ATCCACAGTCTTCTGGGTGG-3	
IL-8	5′-CGATGTCAGTGCATAAAGACA-3′	200
	5′-TGAATTCTCAGCCCTCTTCAAAAA-3′	
IL-6	5′-TCAATGAGGAGACTTGCCTGGT-3′	114
	5′-ACAGCTCTGGCTTGTTCCTCAC-3′	
MCP-1	5′-TGTGCCTGCTGCTCATAG-3′	326
	5′-GAATCCTGAACCCACTCCTG-3′	
ICAM-1	5′-GCAATGTGCAAGAAGATAGCCA-3′	105
	5′-ACCCGTTCTGGAGTCCAGTACA-3′	
E-selectin	5′-TGTGAAGCTCCCACTGAGT-3′	307
	5′-TCTGGCATAGTAGGCAAGAA-3′	

bp: Base pairs; GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; ICAM: intercellular adhesion molecule; IL: interleukin; MCP: monocyte chemoattractant protein; PCR: polymerase chain reaction.

Electrophorese mobility shift assay (EMSA)

Confluent monolayers of HUVEC were incubated overnight in 0·01% bovine serum albumin (BSA) containing culture medium without growth factors or fetal calf serum (FCS). The cells were stimulated for 4 h with different concentrations of LPS. Nuclear extracts and EMSA were performed essentially as described previously [24]. Briefly, nuclear factor-kappa B (NF-κB) consensus oligonucleotides (Promega, Mannheim, Germany) were labelled to a specific activity $> 5 \times 10^7$ counts per min (cpm)/μg DNA. The labelled oligonucleotide was added to 10 μg of nuclear extracts in a total volume of 20 μl containing 10 mM HEPES (pH = 7·5), 0·5 mM EDTA, 70 mM KCl, 2 mM dithiothreitol (DTT), 2% glycerol, 0·025% NP-40, 4% Ficoll, 0·1 M phenylmethylsulphonyl fluoride (PMSF), 1 mg/ml BSA and 0·1 μg/μl poly di/dc. In each experiment specificity of binding was demonstrated by adding cold consensus or mutated NF-κB oligonucluotides to the nuclear extracts. In addition supershifts were performed by adding anti-p65 and -p50 antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) to the samples. DNA–protein complexes were separated on 5% non-denaturating polyacrylamide gels in low ionic strength buffer and visualized by autoradiography.

Statistical analysis

Normalization and data analysis of the chips were performed according to instructions provided by Affymetrix. Pairwise comparisons were made by using unstimulated probe arrays as baseline and LPS-stimulated probe arrays of the same cell line as the experiment. The baseline corrected data were imported into the Affymetrix Data Mining tool (dmt version 4·0) using the publishing tool (mdb version 3·0). Subsequently, the genes were filtered using Affymetrix statistical data analysis software (Affymetrix Microarray Suite version 5·0). Probe sets were excluded when the detection call was absent, when the change call gave no change (NC) in comparison analysis, or when the signal log ratio (SLR) between unstimulated and stimulated cells was between −1 and 1. SLR was used to describe the change between a target and a reference array. The change was expressed as $\log_2$ ratio. Thus, a signal log ratio of 1 equals a fold change (FC) of 2. Only genes that fulfilled the filtering criteria were used for further analysis. Functional categorization of genes was based on ontological designations in the NetAffx Analysis Center (http://www.affymetrix.com), the AmiGO gene ontology database (http://www.godatabase.org) and gene descriptions in Online Mendelian Inheritance in Man (OMIM). Statistical analysis of SLRs was performed using Stata statistical software (Mann–Whitney test). A P-value < 0·05 was considered significant.

Results

Heterogeneity in IL-8 production

A total of 30 primary HUVEC cultures was analysed for IL-8 production. Among these, strong differences in basal and LPS-mediated IL-8 production were detected. In general, basal IL-8 production was low and varied from 0·225 to 4·13 ng/ml/10^6 cells (data not shown). Upon stimulation with LPS for 24 h IL-8 production was up-regulated, varying from 10 to 85 ng/ml/10^6 cells (Fig. 1a). Low basal IL-8 production was not associated with low IL-8 production after LPS stimulation. IL-8 production was not associated under basal nor under stimulatory conditions with the −251 T→A polymorphism in the IL-8 promoter (Fig. 1b).

Gene expression profiling

Based on LPS-mediated IL-8 production, 10 HUVEC cultures were selected for further analysis, i.e. low ($n = 5$) and high ($n = 5$) IL-8 producing HUVEC. These were tentatively called types I and II responders, respectively. All genotypes with respect to the −251 A→T polymorphism in the IL-8 promoter were present in our selection (Table 2).

In the next step we analysed if low IL-8 production was associated with low production of other mediators. Genome-wide gene expression profiling was performed and gene expression patterns were compared between types I and II responders using two algorithms, i.e. SLR and change *P*-value. From all genes present on the chip 74 genes were found to be modulated significantly by LPS in all type II responders (SLR > 1 or SLR < −1, change *P*-value increase ≤ 0·0045 < marginal increase ≤ 0·006; 0·006 < no change < 0·994; 0·94 ≤ marginal decrease ≤ 0·9955 ≤ decrease). Whereas the majority of these genes were up-regulated, only three were down-regulated. In contrast, only 41 genes were significantly up-regulated in all type I responders and these were also found up-regulated in type II responders. Thus 33 genes were modulated specifically only in all type II responders. Among these, genes encoding for cytoskeleton proteins, immune response genes and genes encoding for transcription factors were found (Table 3). The genes that were significantly down-regulated by LPS type II responders were thrombomodulin, the intercellular adhesion molecule connexin and bone morphogenetic protein (BMP)-4.

In order to compare whether the modulation of gene expression was quantitatively different for the 41 genes that

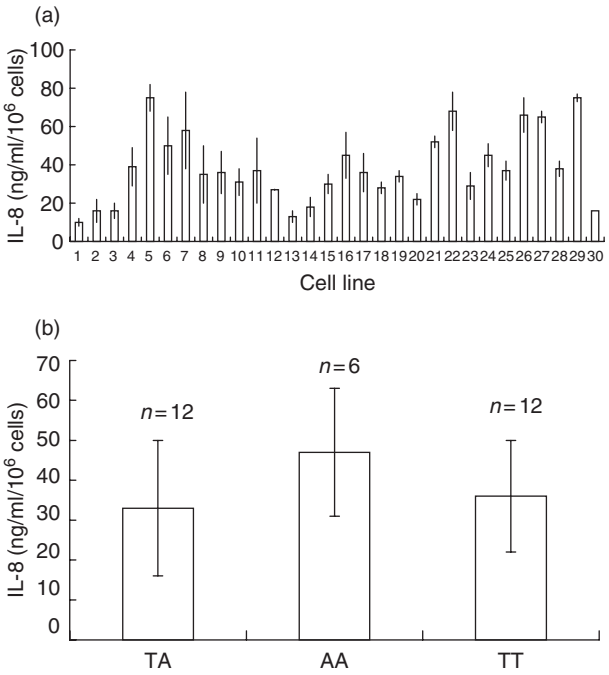

Fig. 1. Heterogeneity in lipopolysaccharide (LPS)-mediated interleukin (IL)-8 production among 30 human umbilical vein endothelial cell (HUVEC) lines. (a) Endothelial cells were stimulated for 24 h with 1 μg/ml of LPS. Supernatants were collected and assessed for IL-8 production. The results are expressed as mean IL-8 production ±s.d. of triplicate cultures. (b) Mean IL-8 production was calculated from the data set obtained in (a) for each of the different genotypes. The results are expressed as mean IL-8 production ± s.d.

Table 2. Characteristics of the selected cell lines.

HUVEC line	Responder type	Basal IL-8 production (ng/ml/10^6 cells)	LPS-mediated IL-8 production (ng/ml/10^6 cells)	IL-8 genotype
1	I	0·61 ± 0·12	10·87 ± 2·74	TA
2	I	0·92 ± 0·23	16·44 ± 2·67	TA
3	I	0·95 ± 0·19	16·38 ± 4·63	TA
13	I	0·54 ± 0·34	16·58 ± 2·09	TT
14	I	0·46 ± 0·33	18·70 ± 1·57	AA
5	II	0·38 ± 0·12	75·45 ± 7·98	AA
22	II	1·23 ± 0·33	58·45 ± 4·50	TT
26	II	1·12 ± 0·11	66·50 ± 9·76	TA
27	II	1·45 ± 0·33	64·87 ± 11·2	TA
29	II	1·88 ± 0·56	70·34 ± 12·0	AA

HUVEC: human umbilical vein endothelial cell; IL: interleukin; LPS: lipopolysaccharide.

Table 3. Comparison of lipopolysaccharide (LPS)-mediated gene expression between type I and type II responders. Genes are grouped based on known or putative function as determined by GeneOntology classifications.

Gene ID	GOMF	Type II SLR	Type I SLR	*P*-value
1	**Transcription factor binding/// activity**			
203973_s_at	***CCAAT/enhancer binding protein (C/EBP), delta***	***3·1***	***1·94***	***0·0068***
201502_s_at	**Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha**	**2·06**	**1·6**	0·24
209636_at	**Nuclear factor of kappa light polypeptide gene enhancer in B cells 2 (p49/p100)**	**1·93**	**1·65**	0·68
208436_s_at	Interferon regulatory factor 7	1·12		
2	**Cytoskeletal protein binding**			
202071_at	Syndecan 4 (amphiglycan, ryudocan)	1·1		
3	**GTP binding/// GTPase activity**			
202086_at	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	2·84		
4	**ATP binding/// ATPase activity**			
202307_s_at	Transporter 1, ATP-binding cassette B (MDR/TAP)	1·22		
204567_s_at	**ATP-binding cassette G (WHITE)**	**1·2**	**1·14**	0·83
205552_s_at	2′,5′-oligoadenylate synthetase 1, 40/46 kDa	2·1		
209545_s_at	Receptor-interacting serine–threonine kinase 2	1·27		
218943_s_at	DEAD (Asp–Glu–Ala–Asp) box polypeptide 58	1·31		
219209_at	**Interferon induced with helicase C domain 1**	**1·89**	**1·2**	0·21
5	**Complement activation**			
202357_s_at	**B-factor, properdin**	**2·25**	**1·,93**	0·5
208747_s_at	**Complement component 1, s**	**3·9**	**2·24**	0·24
6	**Immune response**			
202411_at	Interferon, alpha-inducible protein 27	1·55		
203153_at	Interferon-induced protein with tetratricopeptide repeats	3·85		
203828_s_at	**Natural killer cell transcript 4**	**2·02**	**2**	0·93
204279_at	Proteasome subunit, beta type, 9	1·28		
204415_at	Interferon, alpha-inducible protein	1·5		
207339_s_at	**Lymphotoxin beta**	**1·4**	**1·01**	0·14
208729_x_at	Major histocompatibility complex, class I, B	1·4		
209795_at	**CD69 antigen**	**2·04**	**2·71**	0·3
211911_x_at	Major histocompatibility complex, class IC/IB	1·85		
7	**Cathepsin K activity**			
202450_s_at	**Cathepsin K**	**1·41**	**1·28**	0·77
8	**Angiogenesis**			
202510_s_at	Tumour necrosis factor, alpha-induced protein 2	1·12		
9	**Protein binding/// transmembrane receptor activity**			
202637_s_at	***Intercellular adhesion molecule 1 (ICAM)***	***3·3***	***2·15***	***0·0005***
203868_s_at	**Vascular cell adhesion molecule 1 (VCAM)**	**3·05**	**2·1**	0·06
205483_s_at	Interferon, alpha-inducible protein	1·4		
206211_at	***Selectin E (endothelial adhesion molecule 1)***	***5·66***	***4·28***	***0·041***
207196_s_at	TNFAIP3 interacting protein 1	1·28		
209417_s_at	Interferon-induced protein 35	1·13		
214587_at	Collagen, type VIII, alpha 1	1·52		
204105_s_at	Neuronal cell adhesion molecule	1·16		
214022_s_at	Interferon induced transmembrane protein 1	1·36		
10	**DNA binding/// protein binding**			
202643_s_at	***Tumour necrosis factor,alpha-induced protein 3***	***2***	***1***	***0·0021***
206321_at	Regulatory factor X, 1 (influences HLA class II)	0·3		
214290_s_at	Histone 2, H2aa	0·92		
11	**Chemokine activity**			
202859_x_at	***Interleukin 8***	***4·1***	***2·4***	***0·0062***
203687_at	***MCP-1***	***2·6***	***1·12***	***0·006***
204470-at	**Melanoma growth stimulating activity-alpha**	**3·8**	**3**	0·177
205476_at	**Chemokine (C–C motif) ligand 20**	**4·46**	**3·56**	0·42

Table 3. *Continued*

Gene ID	GOMF	Type II SLR	Type I SLR	*P*-value
207850_at	***Chemokine (C–X–C motif) ligand 3***	***5***	***3·48***	***0·011***
209774_x_at	***Chemokine (C–X–C motif) ligand 2***	***3·95***	***2·78***	***0·017***
211122_s_at	**Fractalkine**	**2**	**1·3**	0·12
214974_x_at	**Chemokine (C–X–C motif) ligand 5**	**4·45**	**4·1**	0·43
215101_s_at	**ENA-78**	**5·28**	**3·84**	0·06
823_at	**Chemokine (C–X3–C motif) ligand 1**	**1·9**	**1·05**	0·08
204533_at	IP-10	1·7		
206336_at	***Granulocyte chemotactic protein 2***	***4·6***	***2·77***	***0·031***
210163_at	***Chemokine (C–X–C motif) ligand 11***	***2·39***	***1·54***	***0·01***
12	**Cathepsin S activity**			
202902_s_at	**Cathepsin S**	**1·87**	**1·2**	0·077
13	**Ion transporter activity/// ion binding**			
212067_s_at	Complement component 1, r	1·7		
203124_s_at	Solute carrier family 11, member 2	1		
204404_at	***Solute carrier family 12 member 2***	***1·5***	***2·8***	***0·04***
205680_at	**Stromelysin 2**	**1·72**	**1·34**	0·29
216841_s_at	***Superoxide dismutase 2, mitochondrial***	***3·9***	***2·6***	***0·033***
217873_at	Calcium binding protein 39	1·12		
220091_at	Solute carrier family 2	1·33		
14	**Blood coagulation**			
203887_s_at	Thrombomodulin	−1·90		
213506_at	***Thrombin receptor-like 1***	***1·64***	***0·98***	***0·01***
15	**Cell proliferation**			
33304_at	**Interferon stimulated gene 20 kDa**	**1·54**	**1**	0·07
16	**Phospholipase–cyclooxygenase–lipase pathway**			
204748_at	***Prostaglandin G/H synthase and cyclooxygenase***	***2·56***	***1·22***	***0·003***
209785_s_at	**Phospholipase A2, group IVC**	**2·08**	**2·15**	0·75
219181_at	***Lipase, endothelial***	***2·2***	***1·08***	***0·01***
17	**Transferase activity**			
221765_at	UDP-glucose ceramide glucosyltransferase	1·4		
18	**Intercellular junction assembly**			
204904_at	Connexin 37	−2·15		
19	**Cytokine activity**			
205207_at	***Interleukin 6***	***1·84***	***1·2***	***0·046***
207442_at	**Colony stimulating factor 3 (granulocyte)**	**5·5**	**2·6**	0·07
210229_s_at	**Colony stimulating factor 2 (granulocyte-macrophage)**	**4·68**	**3·4**	0·26
211518_s_at	Bone morphogenetic protein 4	−1·63		
219424_at	**Epstein–Barr virus induced gene 3**	**4·11**	**3·45**	0·09
20	**Proteolysis and peptidolysis**			
205890_s_at	***Ubiquitin D***	***6·12***	***4·3***	***0·05***
21	**G-protein coupled receptor protein signalling pathway**			
212977_at	Chemokine orphan receptor 1	1·8		
22	**Transferase activity**			
213988_s_at	Spermidine/spermine N1-acetyltransferase	1·27		

Bold type: enlisted are all the genes that were significantly up-regulated by lipolysaccharide (LPS) in all type I and all type II responders. Italic bold type: enlisted are all the genes that were expressed in both types I and II responders, but were expressed significantly more strongly in type II responders. Note that the *P*-value refers to the comparison between types I and II responders. Plain text: enlisted are genes that were significantly modulated by LPS in all type II responders only. The results are expressed as mean signal log ratio (SLR).

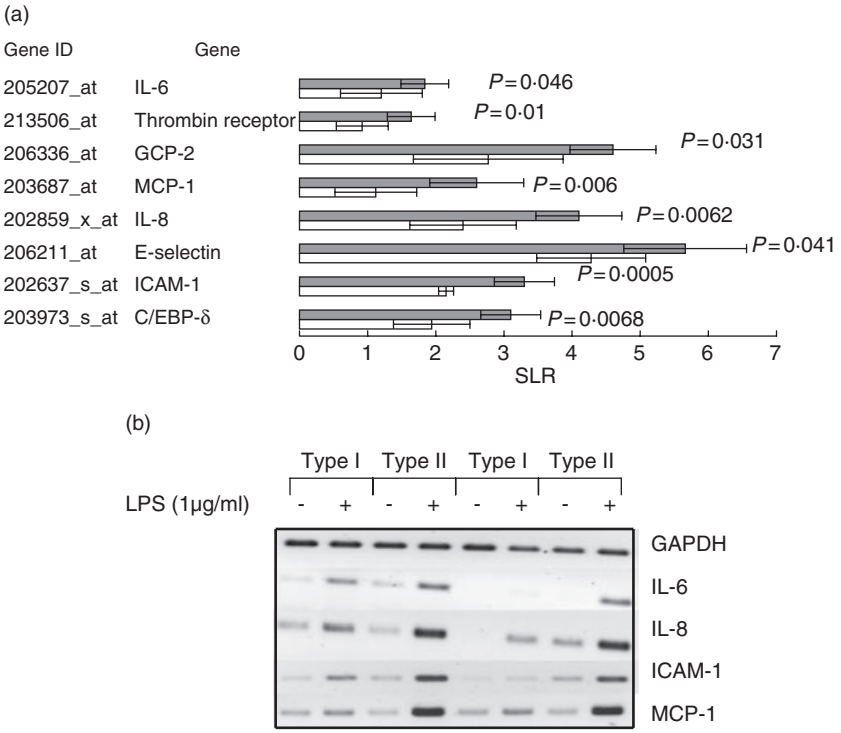

Fig. 2. Differences in gene expression between types I and II responders. (a) Significant differences in lipopolysaccharide (LPS)-mediated gene expression between type I and type II responders for genes implicated in inflammatory processes. Filled bars: type II responders, open bars: type I responders. The results are expressed as mean signal log ratio (SLR) ($n = 5$ for each responder type) ± s.d. (b) Validation of gene expression profiling was performed by reverse transcription–reverse polymerase chain reaction (RT-PCR) for interleukin (IL)-8, IL-6, monocyte chemoattractant protein (MCP)-1, E-selectin and intercellular adhesion molecule (ICAM)-1. Total RNA was isolated from unstimulated (–) or LPS (1 μg/ml, 24 h)-stimulated (+) cells. A representative experiment using two different types I and II responders is depicted. Similar findings were found in all types I and II responders.

were up-regulated in type I and type II responders the mean SLR for these genes was calculated in all five type I and type II responders. Comparisons between type I and type II responders revealed that 17 of 41 genes were more strongly up-regulated significantly in type II responders (Table 3). Significant differences between type I and type II responders for genes that might be implicated in inflammatory processes are depicted in Fig. 2a and include adhesion molecules (ICAM-1 and E-selectin), chemokines (MCP-1, IL-8, GCP-2), cytokines (IL-6), co-agglutination-related genes (thrombin receptor) and transcription factor (C/EBP-δ). Validation of the gene profiling data for IL-8, IL-6, MCP-1, E-selectin and ICAM-1 was performed by RT-PCR in all types I and II responders. Figure 2b shows the results of a representative experiment using two different types I and II responders.

Differences between types I and II responders are not restricted to LPS stimulation

In order to study if the differences between types I and II responders were specific for LPS stimulation, the production of IL-8 and the expression of ICAM was also investigated after TNF-α stimulation. As described for LPS, we found that TNF-α-mediated IL-8 production was significantly higher in type II than in type I responders. In fact, stimulation with 500 U/ml of TNF-α in type I compared to 5 U/ml in type II responders was required for the production of an equal amount of IL-8 (Fig. 3a). Similarly, TNF-α-mediated ICAM-1 expression was clearly higher in all type II responders (Fig. 3b).

To study if type I responders could be converted into type II responders, HUVEC were stimulated simultaneously with LPS and IFN-γ. This influenced neither the amount of IL-8 production (LPS *versus* LPS + IFN-γ) nor the type of responder (data not shown). Interestingly, however, pretreatment of HUVEC with IFN-γ 24 h before stimulation with LPS induced a nearly twofold up-regulation in IL-8 production in both responder types (Fig. 3c).

To gain more insight into the mechanism underlying the differences between types I and II responders, activation of NF-κB was investigated. In all types I and II responders NF-κB was activated by LPS in a dose- and time-dependent fashion. In all type II responders maximal activation was achieved with lower concentrations of LPS (0·02 μg/ml), while maximal activation of NF-κB in type I responders was observed at higher LPS concentrations (1 μg/ml) (Fig. 4a). No difference in the kinetic of NF-κB activation between types I and II responders was observed (Fig. 4b). In line with this, no difference in the kinetic of IL-8 production was found between both responders, although type II responders produced significantly more IL-8 (Fig. 4c). Activated NF-κB consisted of p65 and p50 subunits as demonstrated by supershift analysis (Fig. 4d). No differences between types I and II responders were found in this regard (data not shown). To demonstrate the functional relevance of NF-κB activation by low concentrations of LPS, the expression of ICAM-1 was determined in types I and II responders using suboptimal concentrations of LPS. While ICAM-1 was clearly

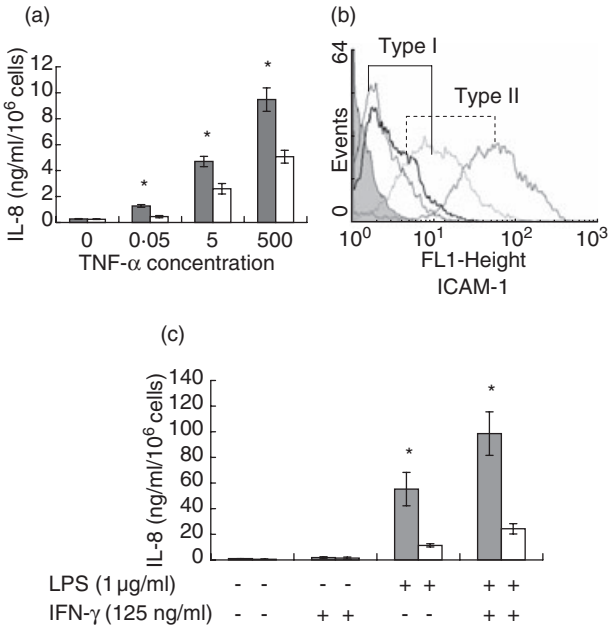

Fig. 3. Susceptibility to tumour necrosis factor (TNF)-α stimulation in type I and type II responders. (a) Type I and type II responder cell lines were stimulated for 24 h with different concentrations of TNF-α. The supernatants were collected and assessed for interleukin (IL)-8 production by enzyme-linked immunosorbent assay (ELISA). The results are expressed as mean IL-8 ± s.d. production for each responder type ($n = 5$ for both types I and II). Hatched bars: type II responders, open bars: type I responders, $^*P < 0{\cdot}05$ compared to type I. (b) Constitutive (thin lines) and TNF-α mediated (bold lines) intercellular adhesion molecule (ICAM)-1 expression in type I (histograms in black) and type II (histograms in grey) responders. The negative control is depicted as filled histogram. The results of a representative experiment of types I and II responders is depicted. Similar findings were found in all types I and II responders. (c) Endothelial cells of types I and II responders were pretreated with 125 ng/ml of interferon (IFN)-γ (+) or left untreated (–) for 24 h. Hereafter the cells were washed and stimulated with lipopolysaccharide (LPS) (1 μg/ml) (+) or not (–). Twenty-four h hereafter supernatants were collected and assessed for IL-8 production. The results are expressed as mean IL-8 ± s.d. production for each responder type ($n = 5$ for both types I and II). Hatched bars: type II responders, open bars: type I responders, $^*P < 0{\cdot}05$ compared to type I.

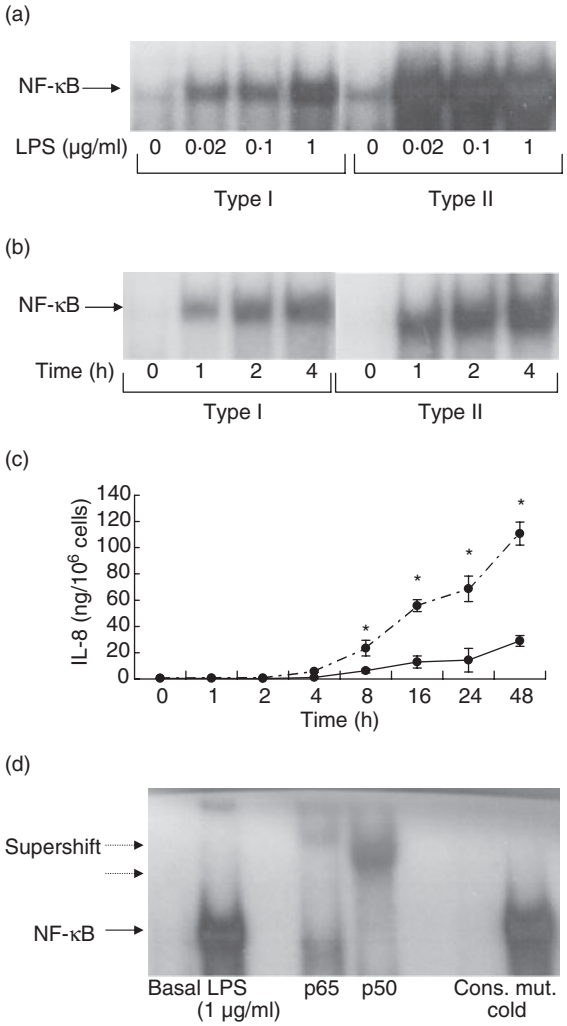

Fig. 4. Electrophoresis mobility shift assay (EMSA) for NF-κB. (a) Susceptibility towards lipopolysaccharide (LPS) stimulation as measured by NF-κB activation in types I and II responders. The data of representative experiment are depicted. Similar findings were observed in all types I and II responders. Endothelial cells were stimulated for 4 h using different concentrations of LPS. (b) Time response of NF-κB activation in types I and II responders. Endothelial cells were stimulated with 1 μg/ml of LPS. At different time-points the cells were harvested for preparation of nuclear extracts. The data of representative experiment are depicted. Similar findings were observed in all types I and II responders. Endothelial cells were stimulated for 4 h using different concentrations of LPS. (c) Kinetics of LPS-mediated interleukin (IL)-8 production in types I and II responders. Human umbilical vein endothelial cell (HUVEC) were stimulated for different time-periods with 1 μg/ml of LPS. Hereafter the supernatants were collected and assessed for IL-8 production. The results are expressed as mean IL-8 ± s.d. production for each responder type ($n = 5$ for both types I and II). Dotted line: type II responders, bold line: type I responders, $^*P < 0{\cdot}05$ compared to type I. (d) The specificity of the shifted bands was demonstrated by incubating a positive sample either with an excess of cold consensus (cons.) or mutated (mut.) NF-κB oligonucleotides before adding labelled consensus NF-κB oligonucleotides. Note that the NF-κB shifted band (arrow) consisted of both p50 and p65 as demonstrated by super-shift (dotted arrow).

up-regulated with 0·1 μg of LPS in all type II responders, this was not found in type I responders (Fig. 5).

Discussion

Sepsis and septic shock are major causes of morbidity and mortality in patients admitted to intensive care units. The systemic response to infection encompasses both pro- and anti-inflammatory phases that are marked by sequential generation of pro- and anti-inflammatory cytokines [25]. Among the proinflammatory cytokines TNF-α and IL-1β play a pivotal role [26]. These cytokines can, either alone or in conjunction with bacterial toxins, activate the vascular endothelium finally resulting in inflammation [27,28].

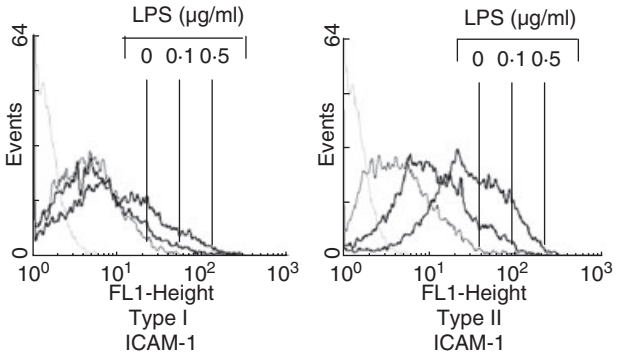

Fig. 5. Lipopolysaccharide (LPS) mediated intercellular adhesion molecule (ICAM)-1 expression in types I and II responders. Endothelial cells were stimulated for 24 h with different concentrations of LPS. ICAM-1 expression was measured by fluorescence activated cell sorter (FACS) analysis. The data of a representative experiment are depicted. Similar results were obtained for all types I and II responders. The negative control is depicted as grey dotted histogram.

Interindividual differences in the endothelial inflammatory response to bacterial toxins might be considered as an additional factor influencing the clinical course of septic patients. In the present study we investigated if and to what extent primary cultures of endothelial cells display a heterogeneous response to LPS stimulation, resulting in cells with low and high proinflammatory characteristics.

To test this hypothesis we have grouped endothelial cell cultures according to the amount of IL-8 produced upon LPS stimulation and questioned whether low IL-8 production was associated with low expression of other inflammatory genes. The main findings of this study are first, that endothelial cells with low and high proinflammatory characteristics as such do not exist, although some proinflammatory genes are expressed significantly lower in so-called type I responders; secondly, that this was not specific for LPS and also observed after TNF-α stimulation; thirdly, that gene expression after LPS stimulation in type I and type II responders differ qualitatively and quantitatively from each other. Fourthly, endothelial cells of type I responders required higher LPS concentrations for maximal NF-κB activation.

The search for predictors of mortality in septic patients is of crucial clinical relevance in order to treat these patients interindividually. Although a variety of factors has been suggested to be associated with mortality in sepsis [29–32], improvements in clinical outcome have been sporadic and, with few exceptions, are related to improvements in supportive care rather than to specific therapies. While in some studies soluble adhesion molecules, i.e. sICAM-1 and sVCAM-1 in serum of septic patients, were associated with mortality [29,30], this was not confirmed by others [33,34]. The relevance of circulating pro- and anti-inflammatory cytokines seems to be more consistent in this regard [31,32,35].

Apart from monocytes, the vascular endothelium is a large source from which circulating cytokines can be derived. This is particularly true for IL-8 and IL-6 [36,37]. Although it is difficult to estimate to what extent endothelial cells contribute to the amount of these cytokines in septic patients, the severity of sepsis is clearly associated with both [38,39]. It also remains to be elucidated whether low IL-8 and IL-6 production in Gram-negative sepsis is an intrinsic patient factor or whether this merely reflects bacterial load. Our data are in line with the former. LPS stimulation of some endothelial cells, i.e. type I responders, resulted in low IL-8 production while type II responders produced high amounts of IL-8. In addition low IL-8 producers also expressed low IL-6 mRNA. Interestingly, our data also revealed that in addition to LPS the up-regulation of IL-8 and ICAM-1 was less pronounced in type I responders when stimulated with TNF-α. Hence differences between types I and II responders were unlikely, due to functional differences in TLR or their level of expression. Moreover, no differences, either at mRNA or protein levels, for TLR4 and TLR2 between types I and II responders were identified (data not shown).

A more likely explanation could be the expression of transcription factors. Among the transcription factors that were influenced by LPS only the up-regulation of C/EBP-δ was reduced significantly in type I compared to type II responders. C/EBP-δ has been implicated in the regulation of proinflammatory cytokines such as IL-6, IL-8 and MCP-1 [40–42] and is activated by both TNF-α and LPS [43]. Because gene analysis was performed 24 h after LPS stimulation, differences in mRNA expression for other transcription factors may also exist between type I and type II responders, e.g. NF-κB [44] and activator protein (AP)-1 [45]. These transcription factors are known to be transcribed early after stimulation. In a study by Bohrer *et al.* [46] investigating 25 septic patients, non-survivors could be distinguished from survivors by an increased activation of NF-κB in mononuclear cells. This might be related to our findings using endothelial cells. Although no differences in NF-κB p65 or p50 mRNA expression were detected among the cell lines, activation of NF-κB was different between types I and II responders. Maximal activation of NF-κB was already obtained with low concentrations of LPS in type II responders. In contrast, type I responders required 500-fold higher concentrations of LPS for maximal NF-κB activation.

The clinical relevance of our findings with respect to types I and II responders remains to be elucidated and further studies are planned. Nevertheless, our study demonstrates that in addition to peripheral blood monocytes [47] heterogeneity in LPS responsiveness also occurs at the level of endothelial cells and thus might influence severity of the inflammatory response in septic patients.

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