Amplification of Lipopolysaccharide-Induced Cytokine Synthesis in Non–Small Cell Lung Cancer/Neutrophil Cocultures

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Abstract
Proinflammatory cytokines are centrally involved in tumor progression and survival in non–small cell lung cancer, and both the presence of infiltrating neutrophils and bacterial infection in the lung may indicate a poor prognosis. Against this background, we investigated the effect of the bacterial cell wall component lipopolysaccharide (LPS) on interleukin (IL)-6 and IL-8 synthesis in the non–small cell lung cancer line A549 and in A549-neutrophil cocultures. The LPS induced a dose-dependent and time-dependent release of IL-8 from A549 cells, whereas IL-6 could not be detected. Interestingly, in A549-neutrophil cocultures, IL-8 synthesis was massively amplified and IL-6 was also released, compared with the respective monocultures. The A549 cells were identified as the primary cellular source of these cytokines, as enhanced cytokine mRNA transcription was detected in this cell type, although not in neutrophils in the coculture system. Experiments done in transwells indicated that direct cell-cell contact was a prerequisite for the increased cytokine generation. Inhibition of tumor necrosis factor-α bioactivity by neutralizing antibodies and blocking cyclooxygenase-2 activity blunted the enhanced cytokine generation in the coculture system. Amplification of LPS-induced cytokine secretion could be reproduced when the small cell lung cancer cell line H69 was cocultured with neutrophils. When the Gram-positive cell wall component lipoteichoic acid was used instead of LPS, cytokine synthesis was also amplified in A549-neutrophil cocultures, to a similar extent to that observed with LPS. These data indicate that interaction between bacterial pathogens, neutrophils, and tumor cells might amplify the release of proinflammatory cytokines which may promote tumor growth in vivo. (Mol Cancer Res 2009;7(10):1729–35)

Introduction
Lung cancer is the leading cause of cancer deaths in the Western world, with non–small cell lung cancer (NSCLC) histology predominating over small cell lung cancer (SCLC). Regardless of the histologic type, patients with lung cancer frequently suffer from pulmonary infections which are associated with a reduction in median survival (1). The most frequent pathogens found in patients with lung cancer are Gram-negative bacteria such as 

\textit{Haemophilus influenzae} and \textit{Escherichia coli}, and an increase in Gram-positive infections, especially with \textit{Streptococcus pneumoniae} and \textit{Staphylococcus aureus} has been observed recently (2, 3). The use of antibiotics, especially clarithromycin, has profitable effects on survival and tumor progression in NSCLC (4). It is not clear whether bacterial infections are only a simple epiphenomenon of advanced lung cancer, or whether they promote cancer growth and metastasis formation (5). However, \textit{in vivo} and \textit{in vitro} observations have indicated that bacterial pathogens promote malignant transformation of cells and cancer growth. Chronic \textit{Helicobacter pylori} infection is known to cause gastric cancer (6), and in lung cancer, an association of \textit{Chlamydia pneumoniae} infection and tumorigenesis has been postulated (7).

In general, bacterial pathogenicity is mediated by bacterial toxins. The most prominent virulence factor of Gram-negative bacteria is endotoxin, which consists of lipopolysaccharides (LPS) from the bacterial cell wall. Gram-positive bacteria generate endotoxin-like substances such as lipoteichoic acid (LTA) and peptidoglycan. Both LPS and LTA activate a variety of inflammatory reactions in multiple target cells (8-10).

During the course of bacterial infections in the lung, neutrophils are recruited into the alveolar space and this mechanism is driven by chemotactic cytokines, in particular, interleukin (IL)-8 (11). Apart from attracting and activating neutrophils, IL-8 is a potent proangiogenic and proliferative agent in NSCLC. In animal models, IL-8 facilitates tumorigenesis \textit{in vivo} (12), whereas \textit{in vitro}, IL-8 induces the proliferation of NSCLC cells (13, 14). Moreover, in patients with NSCLC, elevated levels of IL-8 are associated with a poor prognosis (15). Another predictor of survival in patients with NSCLC are elevated levels of IL-6 (16) and a strong association between tumor cell proliferation and IL-6 production has been found \textit{in vitro} (17, 18). These findings suggest a decisive role for IL-6 and IL-8 in the progression of NSCLC (19). In adenocarcinoma of the lung, tumor cells themselves may be the primary cellular source of IL-8 (20, 21). \textit{In vitro}, IL-8 and IL-6 mRNA and protein are induced in response to proinflammatory cytokines, such as.

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COX-2 expression has been detected both in vivo and in vitro (23, 24). Furthermore, in adenocarcinoma of the lung, marked induction of cyclooxygenase (COX-2) seems to play a decisive role in the development and progression of NSCLC (22). Apart from cytokines, prostanoids derived from the inducible isoform of cyclooxygenase (COX-2) seem to play a key role in the amplification of cytokine generation in these cocultures. We identified LPS-induced IL-8 generation in A549-PMN cocultures, and IL-6 was also detected. The amplification of LPS-induced IL-8 synthesis was not observed when neutrophils and A549 cells were cocultured in a transwell system, preventing direct cell-cell contact. Interestingly, the amplification of LPS-induced IL-8 synthesis was observed in the coculture system.}

**Results**

**LPS Induces a Time-Dependent and Dose-Dependent Release of IL-8 in A549 Cells**

The A549 monolayers were incubated with different concentrations of LPS (0.1, 1, and 10 μg/mL) for various time periods. Application of LPS stimulated the release of IL-8 from A549 cells in a time-dependent and dose-dependent manner, with significant amounts of IL-8 already accumulating 8 hours after stimulation with LPS (Fig. 1). In contrast to the IL-8 response, only trace amounts of IL-6 were liberated from A549 cells in response to LPS (Table 1).

**LPS-Induced IL-8 Formation Is Massively Amplified in A549-PMN Cocultures**

When A549 cells were cocultured with isolated human neutrophils (PMN, 5 × 10⁶/mL), a massive amplification of LPS-induced IL-8 formation was observed (Fig. 2). As depicted for stimulation with 0.1 μg/mL of LPS for 24 hours, coculturing of these two cell types resulted in an ~10-fold increase in LPS-induced IL-8 formation compared with LPS-exposed monocultures of neutrophils or A549 cells (PMN, 3.4 ± 0.7 ng/mL IL-8; A549, 0.2 ± 0.06 ng/mL IL-8; PMN + A549, 49.6 ± 8.7 ng/mL IL-8). In the absence of LPS, no amplification of IL-8 synthesis was observed in the coculture system. Interestingly, the amplification of LPS-induced IL-8 synthesis was not observed when neutrophils and A549 cells were cocultured in a transwell system, preventing direct cell-cell contact (Fig. 2).

**IL-6 Is Induced in A549-PMN Cocultures**

When A549 cells were stimulated with LPS, only trace amounts of IL-6 could be detected (Table 1). In neutrophils, LPS also failed to activate IL-6 synthesis. However, in A549-neutrophil cocultures, large amounts of IL-6 were detected in the cell culture supernatants after endotoxin stimulation (Fig. 3). In contrast, in cocultures of A549 cells and neutrophils in the transwell system, in which direct cell-cell contact was prevented, no IL-6 was detected in response to LPS (Fig. 3).

**A549 Cells Are the Cellular Origin of the Amplified Cytokine Generation Cocultures**

In order to determine the cellular origin of the enhanced cytokine response in supernatants of A549 and neutrophil cocultures, we coinoculated neutrophils with A549 cells for 8 hours, then harvested neutrophils and A549 cells separately, and quantified IL-8 and IL-6 mRNA by reverse transcription-PCR. By this experimental approach, A549 cells were clearly identified as the cellular source of the enhanced cytokine generation. In cocultures, transcription of the IL-8 mRNA in A549 cells was ~30-fold increased compared with monocultures of A549 cells (Fig. 4). In contrast, no significant regulation was evident in PMN in either system (data not shown). In A549 cells cocultured with neutrophils, an impressive upregulation of IL-6 mRNA synthesis was also observed (Fig. 4).

**Table 1. IL-6 Release of A549 Cells in Response to LPS**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>LPS 0.1 μg/mL</th>
<th>LPS 1 μg/mL</th>
<th>LPS 10 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>12.2 ± 2.8</td>
<td>13.5 ± 4.5</td>
<td>30.0 ± 10.2</td>
<td>52.1 ± 11.9</td>
</tr>
<tr>
<td>12</td>
<td>17.74 ± 9.29</td>
<td>15.4 ± 3.5</td>
<td>30.6 ± 10.8</td>
<td>61.6 ± 18.5</td>
</tr>
<tr>
<td>24</td>
<td>28.66 ± 9.26</td>
<td>19.2 ± 2.9</td>
<td>35.7 ± 12.3</td>
<td>101.1 ± 15.4*</td>
</tr>
</tbody>
</table>

*Values which showed significant differences from controls.

NOTE: A549 cells were plated on 24-well culture plates and incubated with LPS at various concentrations or sham-incubation was done. At the indicated time points, cell supernatants were harvested and IL-6 release is expressed in pg/mL. Data represent the mean ± SEM of at least four independent experiments, each done with duplicate values.
Neutrophils Amplify Cytokine Synthesis in Lung Cancer Cells

Amplification of Cytokine Synthesis Was Dependent on TNF-α and COX-2 Activity

Although direct cell-to-cell contact was a prerequisite for the observed amplification of IL-8 and IL-6 synthesis in A549-PMN cocultures, we tried to identify soluble mediators possibly involved in this response. As depicted for LPS-activated IL-8 release in cocultures, blocking of TNF-α bioactivity by a neutralizing polyclonal anti–TNF-α antibody (1 μg/mL) resulted in an inhibition of IL-8 generation by ∼50% (Fig. 5). Inhibition of COX was even more effective in this context: nonspecific inhibition of COX with indomethacin (100 μmol/L) resulted in the reduction of IL-8 synthesis in the coculture system to 27% and specific inhibition of COX-2 by NS-398 (10 μmol/L) was equally effective.

LPS-Induced IL-8 Synthesis Is Also Amplified in PMN-SCLC (H69) Cocultures

When neutrophils were cocultured with the SCLC-derived cell line H69, the amplification of IL-8 generation could be reproduced. As depicted in Fig. 6, significant amounts of IL-8 (5.2 ± 0.2 ng/mL) accumulated in the cell supernatant of this cell type. When cocultured with neutrophils, the LPS-induced IL-8 response was massively amplified (89.2 ± 35.6 ng/mL IL-8; Fig. 6). This ∼10-fold amplification of LPS-induced IL-8 synthesis was comparable to the response observed in A549-PMN cocultures.

LTA Mimics the Effects of LPS in A549-PMN Cocultures

When LTA was used instead of LPS, amplified IL-8 synthesis was also detected in A549-PMN cocultures (Fig. 7). When A549 cells were incubated with LTA in the absence of PMN only trace amounts of IL-8 were detected (0.2 ± 0.1 ng/mL). Likewise, LTA was not a strong inducer of IL-8 synthesis in purified PMN (1.6 ± 0.4 ng/mL). However, when A549 cells were cocultured with PMN LTA induced marked liberation of IL-8 (8.1 ± 2.7 ng/mL).

Discussion

Frequent bacterial infections of the lung are associated with a poor prognosis in patients with lung cancer (1). Secretion of proinflammatory cytokines in response to bacterial toxins is part of the host response to bacterial infections, but has also been suggested to promote tumor cell proliferation and may thereby contribute to the poor prognosis of patients with lung cancer. In the present study, we showed that the capacity of lung cancer cells to release the proangiogenic and proproliferative cytokines IL-8 and IL-6 in response to the clinically relevant bacterial pathogens E. coli LPS and S. aureus LTA is critically determined by the presence of neutrophils. Moreover, we provide evidence that direct cell-cell contact is a prerequisite for enhanced cytokine generation, and TNF-α and COX-2 activity are major regulator molecules in this context.

The key finding of the present study is that massive amplification of cytokine synthesis in response to LPS was found in cocultures of A549 cells and neutrophils but not in monocultures. In monocultures of A549 cells or neutrophils, LPS induced a modest release of IL-8. However, in coculture, a massive (10-fold) amplification of IL-8 secretion was observed in response to LPS. To test whether this phenomenon was restricted to IL-8 synthesis, we also analyzed the production of IL-6. In A549 cells and in PMN in monocultures, no IL-6 was detected. In cocultures exposed to LPS, a massive induction of IL-6 was noted. This finding supports the notion that amplified cytokine synthesis is not limited to IL-8 production but may extend to other pathophysiologically relevant cytokines.
Analysis of gene transcription clearly identified A549 cells as the cellular origin of enhanced cytokine generation in this coculture model. When A549 cells and neutrophils were separated at the end of the incubation period, a 30-fold upregulation of expression of IL-8 and IL-6 transcripts was observed in A549 cells. In contrast, expression of IL-8 mRNA was not enhanced, and no IL-6 mRNA was detected in neutrophils derived from these cocultures. This corroborates previous studies which reported on the lack of IL-6 gene expression in this cell type (30).

We have not elucidated the precise mechanisms of IL-8 amplification in our cocultures. However, direct cell-cell contact of neutrophils and A549 cells was a prerequisite for amplified IL-8 release in the LPS-stimulated cocultures. When A549 cells and neutrophils were cultured in transwells, which prevented direct cell-cell contact between the two cell types, the amplification of IL-8 release in response to LPS was abrogated. We do not have an explanation for this phenomenon. However, neutrophils have been shown to interact with A549 cells via binding of CD11/CD18 to intercellular adhesion molecule-1 (31), and ligation of intercellular adhesion molecule-1 on A549 cells might activate the mitogen-activated protein kinases ERK and JNK with subsequent secretion of chemokines such as IL-8 (32).

Apart from direct cell-cell interaction, TNF-α bioactivity was another prerequisite for amplified IL-8 release in our coculture model. When TNF-α was blocked by a neutralizing antibody, the amplified IL-8 synthesis was substantially reduced. Paracrine stimulation of A549 cells by TNF-α may offer an attractive explanation. First, TNF-α is known to stimulate the generation of IL-8 and IL-6 in NSCLC cells by paracrine pathways (19, 22, 33, 34). Second, although A549 cells do not produce TNF-α (35), they are known to express TNF receptor 1 (36). Third, the cellular source of TNF-α are most likely neutrophils which released TNF-α in response to LPS (151 ± 5 pg/mL upon stimulation with LPS, 0.1 μg after 24 hours) in the current study.

Apart from proinflammatory cytokines, COX-2 has been implicated as an important factor in lung cancer development and progression (23, 24). Against this background, we investigated whether amplified release of cytokines is modulated by COX-2 activity in our experimental setup. Interestingly, nonspecific inhibition of COX by indomethacin as well as specific inhibition of COX-2 activity by NS-398 equally blunted amplified IL-8 secretion in the coculture model. This is well in line with a recent report showing that COX-2 activity correlates with chemokine synthesis in NSCLC cell lines (37), and with another report showing that inhibition of COX-2 is associated with reduced activity of nuclear factor-kB activation and inhibition of JNK, p38MAPK, and ERK activity in NSCLC as a molecular prerequisite for cytokine generation (38). The particular importance of COX-2 in a coculture system challenged with LPS may be explained by the fact that LPS is known to activate phospholipase A2 with subsequent release of free arachidonic acid from neutrophils (39). Adjacent A549 cells may internalize and metabolize AA by COX-2, thus synthesizing prostanooids such as prostaglandin E2, which are relevant to tumor progression (40).

Interestingly, the amplification of cytokine release was not restricted to the NSCLC-derived A549 cells. In cocultures of SCLC-derived H69 cells, the release of IL-8 in response to LPS was also dramatically enhanced compared with neutrophils or H69 cells in monoculture. This is in line with a previous report which shows that elevated levels of IL-8 are actually found in patients with SCLC (41). At least in vitro, IL-8 may contribute to the rapid growth of SCLC cells (14).

Because pulmonary infections with Gram-positive bacteria are of increasing clinical relevance (2, 3), we further tested...
whether amplification of IL-8 release also occurs in A549-PMN cocultures exposed to the Gram-positive pathogenic factor LTA, because LTA is probably the most important pathogen of Gram-positive germs, and is found in all clinically relevant strains. When incubated with LTA, a comparable upregulation of IL-8 release was observed in the cocultures. This effect is clearly attributable to LTA and not contaminating LPS because the isolation procedure of LTA renders a purity of >99% (42). The fact that higher concentrations of LTA compared with LPS were necessary to amplify IL-8 synthesis might reflect the fact that LTA requires costimulatory molecules to evoke full cellular activation (43).

In conclusion, we showed in the current study that cytokine generation of human lung cancer cells is strongly potentiated in the presence of neutrophils. Direct cell-to-cell contact is a prerequisite for this response, and key regulators of this amplified cytokine generation are TNF-α and COX activity. These findings further support the concept that the host response can promote tumor progression under conditions of inflammation and infection and may be taken into consideration when developing new therapies for lung cancer.

Materials and Methods
Materials
Ficoll-Paque was purchased from Pharmacia and FCS was from Greiner. All other media and supplements were purchased from Life Technologies unless otherwise indicated. The LPS (E. coli, 0111:B4) and indomethacin were purchased from Sigma, whereas NS-398 was from Calbiochem. All antibodies and recombinant cytokines used for cytokine ELISAs were purchased from R&D Systems: monoclonal antibodies against IL-6 (MAB 206) and IL-8 (MAB 208), biotinylated anti-human antibodies against IL-6 (BAF 260) and IL-8 (BAF 208), recombinant human IL-6 (206-IL-010) and IL-8 (208-TA-010), as well as the neutralizing anti-TNF antibody (AF-210-NA). Peroxidase-conjugated streptavidin (HRP) and ABTS were purchased from Zymed Laboratories. The RNeasy Mini Kit and QIAshredder columns were from Qiagen. The Platinum SYBR Green qPCR SuperMix UDG was purchased from Invitrogen. Cell culture plasticware was purchased from Falcon.

Isolation of Human Neutrophils
Polymorphonuclear cells (neutrophils, PMN) were isolated from venous blood from healthy donors by centrifugation over a Ficoll-Paque gradient as previously described (44). In brief, EDTA-anticoagulated blood was layered over Ficoll-Paque and centrifuged at 400 × g for 35 min. After removal of mononuclear cells, erythrocytes were sedimented in 1% polyvinyl alcohol. Residual erythrocytes were removed by hypotonic lysis, cells were washed twice in Ca2+/Mg2+-free PBS, and resuspended in RPMI containing 10% FCS, at 107 PMN/mL. Cell purity was >97%, as quantified by flow cytometry, and cell viability was >96%, as assessed by trypan blue dye exclusion.

Purification of LTA
LTA was isolated from S. aureus as previously described in detail, which yields a purity of >99% (42).

Cell Lines
The A549 human lung adenocarcinoma cell line and the H69 human small cell lung cancer cell line were obtained from the American Type Culture Collection and cultured at 37°C in a humidified atmosphere (95% air and 5% CO2). The A549 cells were kept in DMEM/F12 supplemented with 10% FCS, 2 mmol/L of L-glutamine, 105 units/L of penicillin, and 100 mg/L of streptomycin. The H69 cells were maintained in RPMI 1640 supplemented with 2 mmol/L of L-glutamine, 10 mmol/L of HEPES, 1 mmol/L of sodium pyruvate, 4.5 g/L of glucose, 1.5 g/L of NaHCO3, and 10% FCS. Cells were grown to

![FIGURE 6](image-url) Effect of coculturing H69 cells and neutrophils on LPS-induced IL-8 release. Neutrophils (PMN, 5 x 10⁶/mL) and cells from the SCLC line H69 (2 x 10⁵/mL) were either cultured separately and stimulated with LPS (0.1 μg/mL) for 24 h or both cell types were cultured together and activated with LPS (H69 + PMN + LPS). After 24 h, cell supernatants were harvested and IL-8 synthesis was analyzed by ELISA. Columns, mean of at least five independent experiments, each done in duplicate; bars, SEM. *, P < 0.05, significantly different values.

![FIGURE 7](image-url) Effect of LTA on cytokine release in A549-PMN cocultures. Neutrophils (PMN, 5 x 10⁶/mL) and A549 cells were either cultured separately and stimulated with LTA (10 μg/mL) for 24 h or both cell types were cultured together and activated with LTA (10 μg/mL, A549 + PMN + LTA). After 24 h, cell supernatants were harvested and IL-8 synthesis was analyzed by ELISA. Columns, mean of at least five independent experiments, each done in duplicate; bars, SEM. *, P < 0.05, significantly different values.
confluence and subcultured every 2 to 3 d, and split at a ratio of 1:10 for A549 cells and 1:4 for H69 cells.

Neutrophil-Tumor Cell Cocultures
The coculture experiments were done in 24-well cell culture plates (1 mL/well). The A549 cells were plated at a density of 10^4/mL in modified DMEM/F12 and H69 cells were resuspended at 2 × 10^5/mL in modified RPMI. After 24 h, medium was discarded, and cells were incubated in 1 mL of RPMI supplemented with 10% FCS. When indicated, neutrophils were directly added to the tumor cells at a final density of 5 × 10^6 PMN/mL. Cocultures were continuously shaken to prevent the aggregation of neutrophils. In selected experiments, neutrophils were not placed directly onto the tumor cells, but cocultured with A549 in a transwell system [700:300 μL (lower/upper) compartment; pore size, 0.4 μm]. For stimulation of cytokine synthesis, cells were activated with LPS or LTA at various concentrations and cell supernatants were harvested after 24 h of incubation, centrifuged at 13,000 × g, and stored at −80°C for further processing. In neutralization studies, a mouse monoclonal anti–TNF-α antibody which neutralizes TNF-α bioactivity (1 μg/mL), a nonspecific COX inhibitor, indomethacin (100 μmol/L), and the selective COX-2 inhibitor, NS-398 (10 μmol/L) were used.

Cytokine ELISAs
The release of IL-6 and IL-8 was determined in a direct sandwich ELISA. In brief, immunoassay plates were coated with mouse monoclonal anti-human IL-6 or IL-8 antibodies sandwich ELISA. In brief, immunoassay plates were coated with mouse monoclonal anti-human IL-6 or IL-8 antibodies at a concentration of 50 ng/mL of anti–standard titration curves. To sandwich the antigen, biotinylated with mouse monoclonal anti-human IL-6 or IL-8 antibodies and amounted to 0.95 ± 0.02 (95 ± 2%). The constant, K_0, is assumed to be equal within a definite primer system and thus does not influence the comparison of calculated relative ratios. Due to the nonselective dsDNA binding of the SYBR Green, gel electrophoresis was done to confirm the exclusive amplification of the expected PCR product.

Relative mRNA Quantitation
Relative mRNA quantitation was done by the Sequence Detection System 7900 (Applied Biosystems) and real-time PCR.

\[ T_e = K \times (1 + E)^{(C_T,R - C_T)} \]

where \( T_e \) initial number of target gene mRNA copies; \( R_e \) initial number of standard gene mRNA copies; \( E \), efficiency of amplification; \( C_T \), threshold cycle of target gene; \( C_T,R \), threshold cycle of standard gene; and \( K \), constant.

Based on the given equation, we used comparative quantitation (ΔC_T) normalizing IL-8 and IL-6 to two unregulated internal standard genes (45). Therefore, mRNA transcribed from the genes encoding hypoxanthine guanine phosphoribosyltransferase (HPRT) and glycerdehyde-3-phosphate dehydrogenase (GAPDH) was used. In preliminary experiments, we could show that the amplification efficiencies of HPRT, GAPDH, IL-8, and IL-6 primer sets were approximately equal and amounted to 0.95 ± 0.02 (95 ± 2%). The constant, \( K \), is assumed to be equal within a definite primer system and thus does not influence the comparison of calculated relative ratios.

mRNA Extraction
For quantitation of cytokine mRNA, neutrophils and A549 cells were cocultured as described. In pilot experiments, assessing IL-8 and IL-6 mRNA after various time points (1, 4, 8, and 24 h), expression of cytokine mRNA plateaued after 8 h in both cell types, therefore, this incubation period was selected for further experiments. After an incubation period of 8 h, the neutrophil-containing supernatants were harvested and washed twice at 300 × g, whereas the A549 monolayer was washed twice, cells were digested with trypsin, washed again, and pelleted. Neutrophil contamination of A549 cells, as analyzed routinely by flow cytometry, was consistently <1%.

Aliquots of 5 × 10^6 neutrophils and 1 × 10^5 A549 cells were transferred into 1.5 mL reaction tubes. After centrifugation at 300 × g, the supernatant was removed and the pellet was lysed in 350 μL of lysis buffer of the RNasey Minikit, containing 3.5 μL of β-mercaptoethanol. After homogenization using QIAshredder columns, RNA extraction was done according to protocols. A DNase digestion of 20 min at 30°C was included. RNA was eluted with 30 μL of RNase-free water.

cDNA Synthesis and Real-time PCR
For cDNA synthesis, reagents and incubation steps were applied as described (45). An aliquot of 10 μL of each of the isolated mRNA were applied for reverse transcription. The reactions were set up with the Platinum SYBR Green qPCR SuperMix UGD, according to the protocols of the manufacturer. Using the oligonucleotide primer pairs given in Table 2, 0.5 μL of each primer (10 μmol/L) and 2 μL of cDNA were added to a final volume of 25 μL. Cycling conditions were adapted to 95°C for 6 min, followed by 45 cycles of 95°C for 5 s, 59°C for 5 s, and 72°C for 10 s. PCR products were routinely identified by 2.5% agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>HPRT amplicon size (94 bp)</td>
<td>5′-AGGAAGGCAAGTGCTGCAATGTT-3′</td>
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<tr>
<td></td>
<td>5′-GGCTTTGATTTTGCTTTTCCA-3′</td>
</tr>
<tr>
<td>HPRT forward</td>
<td>5′-CCACATCGCTGACACCATC-3′</td>
</tr>
<tr>
<td>HPRT reverse</td>
<td>5′-AAAAAGGACTCGCGAC-3′</td>
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<tr>
<td>GAPDH amplicon size (87 bp)</td>
<td>5′-GCCCTCGATTCTGCTAGGAC-3′</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>5′-GGCAGTGGCCACTCTCT-3′</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5′-GGCAGTGGCCACTCTCT-3′</td>
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<tr>
<td>IL-8 amplicon size (151 bp)</td>
<td>5′-GCCCTGATTCTGCTAGGAC-3′</td>
</tr>
<tr>
<td>IL-8 forward</td>
<td>5′-GCCAGTGGCCACTCTCT-3′</td>
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<tr>
<td>IL-8 reverse</td>
<td>5′-GCCAGTGGCCACTCTCT-3′</td>
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<tr>
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</tr>
<tr>
<td>IL-6 reverse</td>
<td>5′-GCCAGTGGCCACTCTCT-3′</td>
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</table>

NOTE: Selecting suitable intron-spanning primers, cDNA amplicon was much shorter than genomic DNA amplicon. Excluding falsification by amplification of possible pseudogene sequences, both primer sets were shown to detect no genomic DNA with cDNA sequence. The primer sets were selected to work under identical PCR cycling conditions, so that simultaneous amplification of HPRT, GAPDH, IL-6, and IL-8 was obtained in the same run.
Statistics

For statistical comparison, one-way ANOVA was done, followed by Tukey's honestly significant difference test when appropriate. \( P < 0.05 \) levels were considered to be significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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