RESEARCH ARTICLE

Upregulation of cytokine mRNA in circulating leukocytes during human endotoxemia

Petra Jilma-Stohlawetz[1], Tuende Kliegel[2], Irene Kantner-Schlifke[2], Claudia Strasser-Marsik[2], Florian B. Mayr[2], Bernd Jilma[2]

[1] Dept. of Laboratory Medicine, Medical University of Vienna, Vienna, Austria
[2] Department of Clinical Pharmacology, Division of Immunology and Hematology, Medical University of Vienna, Austria

Correspondence: Jilma B. Department of Clinical Pharmacology, Division of Immunology and Hematology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Wien, Austria
<Bernd.Jilma@meduniwien.ac.at>

Accepted for publication March 22, 2017

To cite this article: Jilma-Stohlawetz P, Kliegel T, Schlifke I, Marsik C, Mayr FB, Jilma B. Upregulation of cytokine mRNA in circulating leukocytes during human endotoxemia. *Eur. Cytokine Netw.* 2017; 28(1): 19-26 doi:10.1684/ecn.2017.0389

ABSTRACT. ***Background:*** **Endotoxemia induces pronounced changes in leukocyte count and enhances the release of many cytokines. However, the molecular regulation of this cytokine release is poorly characterized in humans.** ***Methods:*** **The time course of mRNA expression of 24 cytokines in circulating leukocytes was studied in a well-standardized model of human endotoxemia (2 ng/kg). Real-time polymerase chain reaction (RT-PCR) was used to quantify the lipopolysaccharide (LPS)-inducible mRNA levels of leukocytes from 16 healthy volunteers in a randomized, placebo-controlled trial.** ***Results:*** **Baseline mRNA levels of interleukins including IL-1α, IL-3, IL-5, IL-6, IL-12p40, IL-13, IL-15, IL-17, granulocyte colony-stimulating factor (G-CSF) and granulocyte monocyte CSF (GM-CSF) were below detectable levels in normal blood of the healthy participants. After 2 h, LPS infusion increased median mRNA levels of IL-1α by >1100-fold and IL-1β and IL-8 by 33-fold and 46-fold, respectively. In contrast, levels of tumor necrosis factor (TNF-α) and IL-10 mRNA increased by only 7-fold, whereas changes in mRNA expression of other cytokines showed either a more than two fold increase or were undetectable.** ***In vitro*** **incubation of whole blood with 50 pg/mL LPS for 2 h enhanced transcription levels of IL-1α mRNA by >10,000-fold, IL-6 and IL-12p40 by >1000-fold, IL-1β by 400-fold, TNF-α by 100-fold, IL-8, IL-18, interferon γ (IFN-γ) and G-CSF by >10-25-fold, and IL-10, IL-12p35, TNF-β, and IL-13 by 10-25-fold.** ***Conclusion:*** **Only half of the 24 evaluated cytokines were expressed at the mRNA level in circulating leukocytes under basal conditions and after an LPS challenge. Only IL-1α, IL-1β, IL-10, IL-8, and TNF-α were upregulated in the circulating leukocytes, whereas several other cytokines (including IL-6 and G-CSF), were expressed on the mRNA level following *in vitro* incubation of blood with LPS. In addition, IL-1α and IL-1β might be potential diagnostic targets in inflammatory diseases.**

Key words: randomized controlled trial, endotoxemia, mRNA, cytokines, RT-PCR

More than three decades ago, endotoxin derived from *Escherichia coli* (*E. coli* group O113:H10:K negative) was processed to serve as a reference standard in the assays of pyrogenicity using Limulus amebocyte lysate [1].

Because the endotoxin derived from *E. coli* was already in use as a standard, investigators infused small amounts (1-4 ng/kg) of this reference endotoxin to study experimental inflammation in humans. This model has proved to be a unique means to study the mechanisms of inflammation in humans and has generated valuable insights regarding the interactions among inflammatory mediators and cell activation [2-7]. Insights in the regulatory mechanisms of acute inflammation have been delineated by the concomitant administration of drugs that modify inflammatory responses, including endotoxin antagonists [8-11], cytokines [12, 13], cytokine antagonists [14, 15], and inhibitors of the signaling cascade [16, 17].

Numerous clinical studies have described the regulation of cytokines at the protein level, that is, by measuring the amount of cytokines released into the circulation. Moreover, the induction of endotoxemia in a human volunteers has also been widely investigated by using transcriptomic analysis. De Kleijn *et al.* characterized the transcriptome profiles of circulating neutrophils in the human experimental endotoxemia model and demonstrated early proinflammatory activation of neutrophils, which was reflected by expression of the corresponding signaling parthway [18]. The effects of lipopolysaccharide (LPS)-induced inflammation on whole body metabolism were studied by Kamisoglu *et al.*, and the temporal changes in lipid and protein metabolism were identified, which peaked at 6 h after LPS infusion [19]. Haimovich *et al.* compared more than 400 genes that exhibited similar expression trends in the peripheral blood leukocytes derived from either LPS-challenged participants or trauma patients [20]. Another study also found similarities between the increased expression of genes involved in the systemic inflammatory, innate immune, and compensatory

doi: 10.1684/ecn.2017.0389

anti-inflammatory responses in severe trauma patients and those in healthy volunteers after LPS infusion [21].

Although gene arrays provide information regarding a wide variety of markers, microarray studies are often characterized by a high background and a relatively limited dynamic range [22]. In contrast, real-time polymerase chain reaction (RT-PCR) measurements provide more quantitative data.

Hence, we tested the hypotheses that (i) mRNA upregulation may contribute to cytokine release during endotoxemia and that (ii) mRNA levels may be differentially regulated among cytokines.

For this purpose, we used the well-standardized model of human endotoxemia and compared the *in vivo* and *in vitro* effects of LPS on the regulation of cytokine mRNA using RT-PCR, which allows the quantification of even minute quantities of constitutively expressed mRNA levels.

METHODS AND MATERIALS

The study was approved by the Institutional Ethics Committee of the Vienna University Medical School, and written consent was obtained from all the participants before their enrollment in the study. As age and sex may contribute to the variation of immune gene expression [23], 16 healthy male volunteers were included in a randomized, placebo-controlled trial in parallel groups.

Study participants

The mean age of the healthy study participants was 29 ± 7 years, and mean body mass index was 23.4 ± 2.2 kg/m^2. Determination of the health status included medical history, physical examination, laboratory parameters, and serological screening, as described in previous trials [24, 25]. The study participants who were having drug abuse issue were identified by analyzing their urinary samples, and such participants were excluded. Additional exclusion criterion was regular or recent intake of medication including nonprescription medication.

Study protocol

The participants were admitted to the study ward at 8:00 AM after an overnight fast, in order to exclude a possible effect of food intake on cytokine secretion patterns [26] and the induction of endotoxin-induced nausea. Throughout the study period, the participants were confined to bed and fasted for 8.5 h, followed by LPS or placebo infusion. The vital parameters (electrocardiogram, heart rate and oxygen saturation, and blood pressure) were monitored on an automated monitoring system (Care View System, Hewlett Packard, Böblingen, Germany). The participants received either an intravenous bolus of 2 ng/kg LPS (lot g1, National Reference Endotoxin, *Escherichia coli*; USP Convention, Rockville, MD) or an equal volume of 0.9% saline (placebo) and were concomitantly hydrated with 80 mL/h NaCl (0.9%) iv.

Sampling

The blood samples were collected through intravenous catheters into EDTA Vacutainer tubes before drug administration and at 45 min, 2, 4, 6, 8, and 24 h after LPS administration; the blood samples were collected by applying minimal venostasis. The blood samples were processed immediately and stored at –80 °C.

In vitro incubation experiments

To mimic *in vivo* situations as closely as possible, whole EDTA anticoagulated blood was incubated at 37 °C with phosphate-buffered saline or 50 pg/mL LPS [27], corresponding to the estimated peak plasma levels reached after injection of 2 ng/kg LPS in humans (considering a plasma volume of approx. 3 L in a 70-kg individual). Samples for RNA extraction were taken before and after 2 h of incubation.

Extraction of total cellular RNA from human whole blood

To avoid artificial activation of the samples [28, 29], all the blood samples were placed on ice and processed immediately after blood drawing. QIAamp RNA Mini Kits were used for the preparation of total cellular RNA from human whole blood according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). With this method, contaminants and enzyme inhibitors such as hemoglobin and heparin are completely removed, leaving purified RNA ready for use in downstream applications.

Reverse transcription

mRNA was directly transcribed into cDNA using the RT reagent kit (Applied Biosystems, Foster City, CA, USA) and stored at –80 °C until further analysis. Only random hexamers were used to reverse transcribe the total RNA samples for cytokine gene expression assays. Two μg of RNA was used for reverse transcription.

The TaqMan Human Cytokine Card

For the analysis of cDNA samples, we used the TaqMan Human Cytokine Card (according to the Protocol supplied by the manufacturer), a research tool for profiling human cytokine gene expression using the comparative CT method of relative quantification. The card evaluates a single cDNA sample generated from human total RNA (1 μg in our setup) in a two-step RT-PCR experiment in quadruplicate. The card functions as the reaction vessel for the PCR/sequence detection step. The wells of the card contain multiplexed fluorogenic 5′ nuclease assays that detect amplification of 24 cytokine targets as quadruplicates and an 18S rRNA endogenous control. The relative levels of cytokine gene expression are determined from the fluorescence data generated during PCR using ABI PRISM 7700 Relative Quantification Software that uses the comparative CT method for relative quantification. In the comparative CT method, the quantity of gene expression is expressed relative to a calibrator sample (baseline or 0 h sample) that is used as the basis for comparison. During analysis, the ABI PRISM 7700 Relative Quantification Software calculates the levels of cytokine gene expression in samples relative to the level of expression in the calibrator.

The TaqMan Human Cytokine Card consists of a specially developed 96-well consumable divided into 24 sets of replicates, one set for each cytokine assay.

Each well contains TaqMan® probes and primers for a single human cytokine mRNA target and an 18S rRNA endogenous control.

To avoid false-positive amplifications, we used AmpErase® uracil-N-glycosylase (UNG), a pure, nuclease-free, 26-kDa recombinant enzyme encoded by the *E. coli* uracil-N-glycosylase gene. UNG acts on single- and double-stranded dU-containing DNAs by hydrolyzing uracil–glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. Besides, the TaqMan Human Cytokine assays have been experimentally proven incapable of detecting up to 10,000 copies of contaminating genomic DNA per card. All measurements that provided either a fluorescence signal after 36 cycles or no signal were set to 36 cycles for further conservative statistical comparisons, because quadruplicate determinations mostly resulted in relatively high variance in such cases.

Quantification of plasma levels of the circulating cytokines

Plasma levels of IL-6, IL-8, IL-10, and tumor necrosis factor α (TNF-α) were measured by enzyme immunoassays purchased from R&D Systems (Oxon, UK); plasma levels of IL-1β were measured by an immunoassay purchased from CytImmune Sciences Inc. (Rockville, MD, USA) [5, 6, 15].

Statistical analysis

In case of highly skewed distribution, data are expressed as median and interquartile ranges (IQR) or as mean and SEM. All statistical comparisons of continuous variables were made with nonparametric tests. After a two-way ANOVA of log-transformed data, Friedman's ANOVA and the Wilcoxon test for post hoc comparisons were used to assess the time-dependent chances in outcome variables within groups. The differences between groups were confirmed by the *U*-test. Spearman's correlation was used to measure correlation among the parameters. To account for multiple comparisons of the five cytokines with major regulation, in *figures 3 and 4*, a two-tailed P-value < 0.01 was considered significant for comparisons between groups. All statistical calculations were performed using commercially available statistical software (Statistica Vers. 6.0, Tulsa, OK).

RESULTS

Leukocyte counts and circulating cytokines

Infusion of LPS produced the expected changes in leukocyte counts: neutrophil counts increased by >2.5-fold, profound monocytopenia was induced after 2 h, and lymphocyte counts dropped to 25% of baseline after 4-6 h (*figure 1*). LPS also increased the plasma levels of circulating TNF-α, IL-6, IL-8, and IL-10 by several orders of magnitude (*figure 2*), whereas IL-1β only increased from a median level of 0.2 pg/mL to 2.4 pg/mL in 4 h (data not shown). Infusion of placebo did not significantly alter any of these parameters.

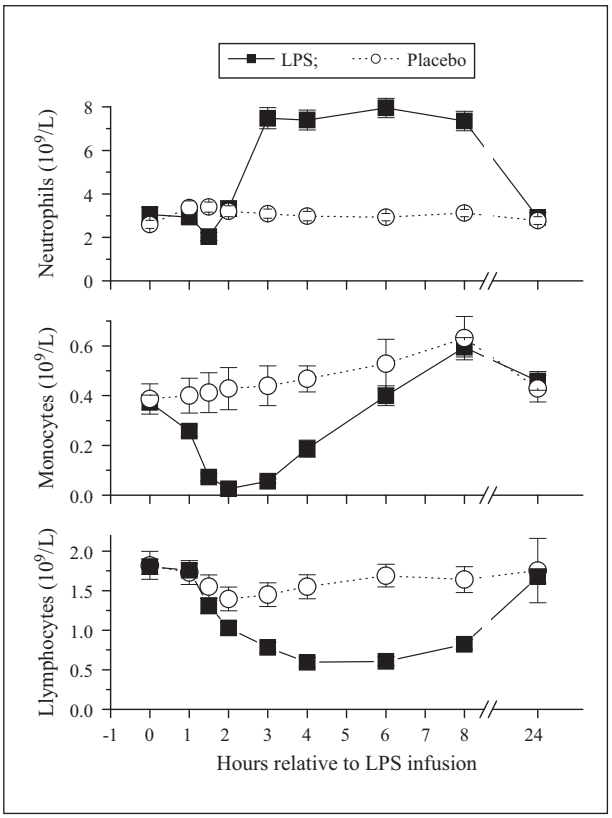

Figure 1
Neutrophilia, monocytopenia, and lymphopenia following LPS infusion. Sixteen healthy male volunteers received 2 ng/kg endotoxin or placebo. Short-lasting neutropenia was followed by neutrophilia (top), profound monocytopenia (middle), and lymphopenia (bottom). Data are presented as mean and SEM; $p < 0.01$, two-way ANOVA for all parameters between groups. The increase in monocyte counts in the placebo group could reflect a finding due to chance or a diurnal variation [24].

Baseline values of cytokine mRNA

We used 18S RNA as a housekeeping gene, which did not show significant variation after incubation with LPS *in vitro* or on being challenged with LPS *in vivo* (data not shown).

Baseline levels of mRNA for interleukins IL-1α, IL-3, IL-5, IL-6, IL-12p40, IL-13, IL-15, IL-17, G-CSF, and GM-CSF were below detectable levels in normal leukocytes from the majority of healthy volunteers, that is, even after 36 cycles of amplification, no fluorescence signal was detectable. Baseline mRNA levels of detectable cytokines are shown in *table1* and are ranked by their abundance relative to 18S RNA. The dCT numbers indicate the difference in amplification cycles between target and housekeeping (18S) genes, which is most abundant and thus results in an earlier fluorescence signal: 11.9 cycles indicate that 2E11.9 (3822-fold) more 18S RNA is present as compared to TGF-β mRNA. Transforming growth factor β, IL-8, LT-β, and TNF-α mRNA were most abundantly expressed under basal conditions (*table 1*).

Cytokine mRNA expression patterns after LPS infusion

The median level changes in the mRNA expression of cytokines following LPS challenge are depicted in *figures 3 and 4*. Most impressively, LPS infusion increased IL-1α mRNA levels on average by 1100-fold after 2 h.

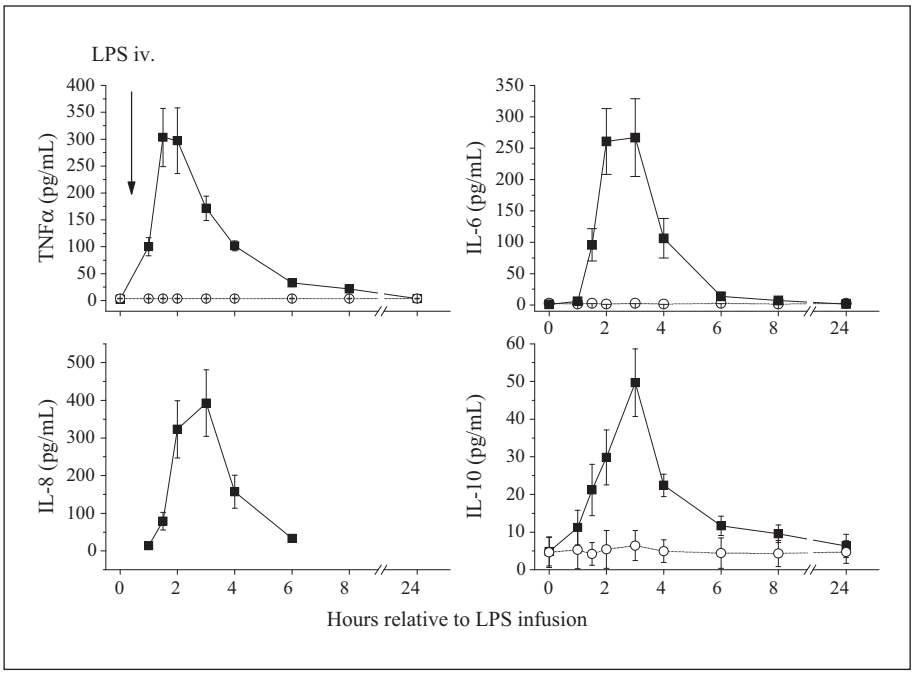

Figure 2
Cytokine release following LPS infusion. Sixteen healthy male volunteers received 2 ng/kg endotoxin or placebo. As expected, TNF-α (upper left), IL-6 (upper right), IL-8 (lower left), and IL-10 (lower right) increased by 1-2 orders of magnitude. IL-8 levels were below detectable limit at the beginning and the end of the LPS-treatment period as well as in placebo-treated patients. Data are presented as mean and SEM; $p < 0.01$, two-way ANOVA for all parameters between groups.

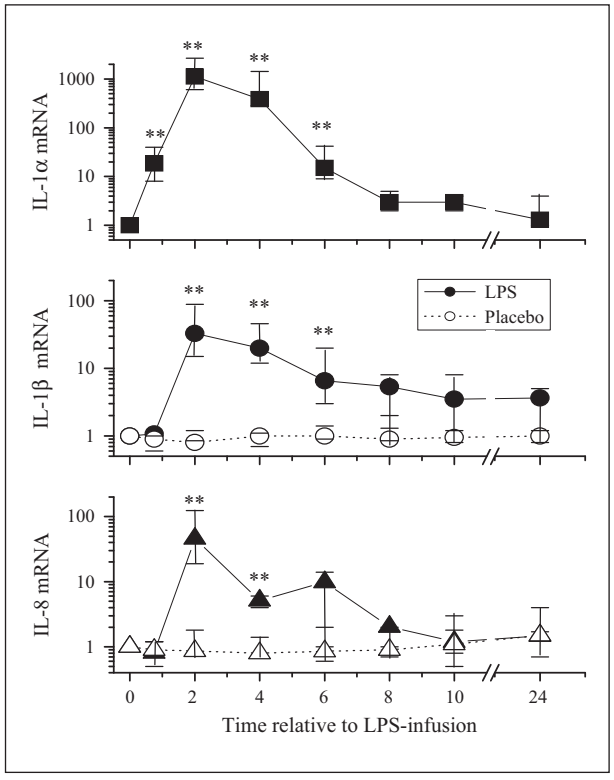

Figure 3
Fold increase in messenger RNA (mRNA) levels of IL-1α, IL-1β, and IL-8 in circulating blood leukocytes after in vivo challenge with 2 ng/kg *E. coli* endotoxin (solid symbols) or placebo (open symbols). As IL-1α mRNA was not detectable at baseline or in the placebo arm (thus not depicted), the time course in the top layer may underestimate the true increase in IL-1α. Data are presented as median and interquartile range; **$p < 0.01$ between groups.

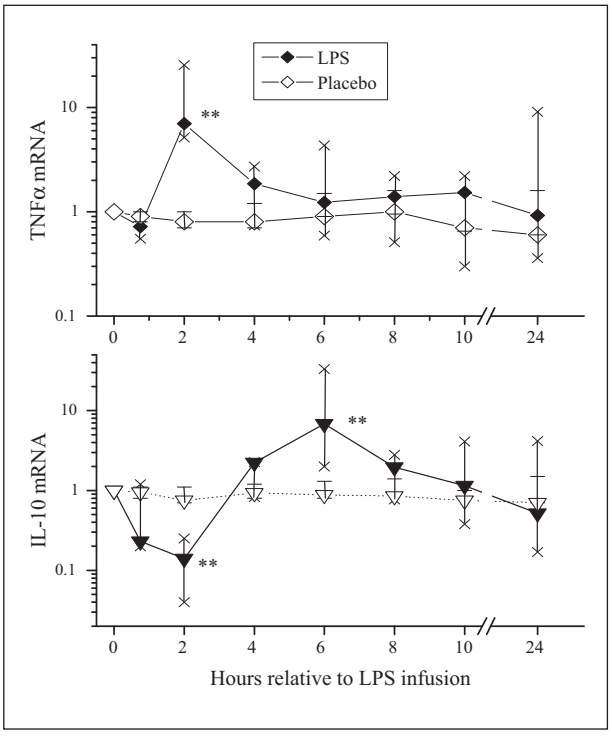

Figure 4
Fold increase in messenger RNA (mRNA) levels of TNF-α (top) and IL-10 (bottom) in circulating blood leukocytes after in vivo challenge with 2 ng/kg *E. coli* endotoxin (solid symbols) or placebo (open symbols). Data are presented as median and interquartile range; **$p < 0.01$, two-way ANOVA for parameters between groups.

Furthermore, after 2 h, LPS enhanced the mRNA levels of IL-1β and IL-8 on average by 33-fold and 46-fold, respectively. In contrast, TNF-α and IL-10 mRNA increased only 7-fold, after 2 h and 6 h, respectively ($p < 0.01$ between groups). In contrast, infusion of placebo did not enhance mRNA levels of any of these cytokines.

Interestingly, IL-10 levels initially decreased to reach nadir levels 2 h after LPS infusion, when monocytopenia was

Table 1
Basal cytokine mRNA expression in circulating leukocytes of healthy men.

	dCT values	Absolute CT-values
Cytokine mRNA: detectable		
Transforming growth factor β (TGF-β)	11.9	21.6 (1.2)
Interleukin-8 (IL-8)	14.5	24.5 (1.1)
Lymphotoxin-β (LT-β)	15.1	25.6 (1.5)
Macrophage colony-stimulating factor (M-CSF)	16.1	26.3 (1.0)
Tumor necrosis factor α (TNF-α)	16.3	26.8 (1.4)
Lymphotoxin-α (LT-α)	18.6	29.1 (1.7)
Interleukin-1β (IL-1β)	18.9	29.0 (2.7)
Interleukin-10 (IL-10)	22.1	33.2 (1.0)
Interleukin-7 (IL-7)	22.2	32.6 (1.2)
Interleukin-18 (IL-18)	22.5	32.5 (1.4)
Interferon γ (IFN-γ)	23.4	31.8 (1.5)
Borderline or undetectable		
Interleukin-12 subunit p35 (IL-12p35)	24.3	35.1 (1.3)
Granulocyte monocyte colony-stimulating factor (GM-CSF)	24.8	>36 (0.7)
Interleukin-2 (IL-2)	24.9	35.1 (0.8)
Interleukin-5 (IL-5)	25.0	>36 (0.3)
Interleukin-4 (IL-4)	25.1	>36 (0.2)
Interleukin-15 (IL-15)	25.2	>36 (0.6)
Interleukin-6 (IL-6)	25.5	>36 (0.3)
Interleukin-17 (IL-17)	25.5	>36 (0.1)
Interleukin-13 (IL-13)	25.6	>36 (0.4)
Interleukin-12 subunit p40 (IL-12p40)	25.7	>36 (0.3)
Interleukin-3 (IL-3)	25.8	>36 (0.0)
Interleukin-1 α (IL-1α)	25.8	>36 (0.6)
Granulocyte colony-stimulating factor (G-CSF)	25.9	>36 (0.0)

Basal cytokine mRNA expression is ordered by relative abundance as compared to the housekeeping gene 18S: dCT, difference in amplification cycles between the fluorescence signal of the target gene and 18S.
Data is expressed as medians (and SD in parentheses for basal CTs).

also most pronounced (0.02×10^9/L as compared to baseline levels of 0.42×10^9/L). Later, when the number of monocytes increased again, IL-10 mRNA levels also increased above baseline after 6 h (*figure 4*). Changes in the mRNA expression of other cytokines were <2-fold after LPS infusion (data not shown).

In vitro incubation experiments

Two hours after *in vitro* incubation with 50 pg/mL LPS, a more than 10,000-fold increase in mRNA was observed for IL-1α, a >1000-fold increase for IL-6 and IL-12p40, a 400-fold increase for IL-1β, a 100-fold increase for TNF-α, a 10-25-fold increase for IL-8, IL-18, IFN-γ, and G-CSF, and a 4-7-fold increase for IL-10, IL-12p35, TNF-β, and IL-13. These relative changes in response to LPS may underestimate the true increase for any cytokine, especially when mRNA levels are not detectable at baseline (*table 1*).
To further address the variability in response among individuals as compared to the variability of the assay, we sampled the blood two times at 1-h interval, and once again after 1 week (n = 9 volunteers). Determination of the *in vitro* mRNA response to LPS was repeated for IL-1α mRNA and IL-1β mRNA and compared to the variability in TNF-α release. Interindividual variability on the 2 days of study (coefficient of variation (CV%): IL-1α: 40-81%; IL-1β: 29-36%; TNF-α: 54-72%) was much greater than intraindividual variability within 1 h (CV%: IL-1α and IL-1β: 19-36%; TNF-α: 17-23%) or the day-to-day variability (CV%: IL-1α and IL-1β: 13-18%; TNF-α: 6%). This demonstrates excellent reproducibility of the method.

DISCUSSION

While ample data exist on the release of cytokines into circulation during human endotoxemia, little is known about their molecular regulation, that is, mRNA levels of cytokines during endotoxemia. This is the first systematic RT-PCR study on the LPS-induced regulation of the mRNA levels of 24 cytokines in circulating leukocytes.
Basal expression of mRNA was detectable for only half of the cytokines measured (*table 1*). It is interesting that cytokines such as IL-6 and G-CSF were not detectable on mRNA level in circulating leukocytes, although basal levels of these proteins can easily be quantified with

high-sensitivity assays [24, 30]. Thus, it is conceivable that these cytokines are generated by cells other than circulating blood leukocytes under basal conditions. Even during low-grade endotoxemia, plasma levels of interleukin-6 (*figure 2*) and G-CSF increase 100-fold at protein level [24, 31], but no elevation in cytokine mRNA levels was seen in circulating leukocytes after an LPS challenge *in vivo*. In contrast, the *in vitro* studies clearly showed a more than 1000-fold increase in IL-6 mRNA and 25-fold increase in GCSF mRNA.

The mechanism underlying this phenomenon could be related to the endotoxin-induced profound monocytopenia (1% of baseline counts) and possibly lymphopenia (*figure 1*): up to 98% of the cell population of interest (monocytes) has been removed from the circulation. One may speculate that those monocytes remaining in the circulation may not even be the most activated subset. Consistent with leukocyte trapping are radiolabeling studies that indicated leukocyte accumulation in the lung after iv injection of LPS to humans [13] and in lung and hepatic microvasculature in animals [32]. Alternatively, monocytic cells residing or pooling in the reticuloendothelial system may significantly contribute to systemic cytokine release. This is supported by a high-quality study, which showed that the splanchnic circulation is a relevant source of systemic cytokine generation during endotoxemia [33].

Although 24 cytokines have been quantified at the mRNA level, the remainder of the discussion will focus on the regulation of those cytokines that demonstrate an upregulation on mRNA level *in vivo*. The first cytokine that is released after an LPS challenge into circulation is TNF-α. Peak levels are seen within 90 min after endotoxin challenge (*figure 2*). TNF does not exist as a preformed molecule in human circulating leukocytes, but is first induced upon activation as a membrane form released by the TNF-α converting enzyme (ADAM17) [34]. Accordingly, we now show relatively high constitutive TNF-α mRNA expression in peripheral blood leukocytes even in healthy participants, and possibly as a consequence only, a relatively small upregulation of TNF-α mRNA in response to LPS infusion. The peak in TNF-α mRNA at 2 h coincides with peak plasma levels of TNF-α (*figure 2*).

The release of TNF-α is followed by the release of several other cytokines. Although the maximum increase in IL-10 levels usually occurred at 2-3 h (*figure 2*), we observed an early decrease in IL-10 mRNA levels (concomitantly with the nadir in monocyte counts (*figure 1*)) and a later (5-fold) increase in IL-10 mRNA levels after 6 h (*figure 4*). However, the late IL-10 mRNA upregulation is consistent with the anti-inflammatory response following initial proinflammatory changes in systemic inflammation. Alternatively, demargination and reentry of IL-10-expressing monocytes into the circulation could occur.

The increase in IL-8 mRNA level (46-fold at 2 h; *figure 3*) was greater than that of TNF-α or IL-10 and even slightly preceded peak plasma levels of IL-8 (*figure 2*). Acute mRNA upregulation could thus contribute to enhanced generation of TNF-α and IL-8 at protein level during endotoxemia.

Although it has been controversial whether IL-1β expression occurs constitutively [35], our RT-PCR data unequivocally demonstrate that this is the case. In contrast to the manifold increase in IL-1β mRNA levels after an LPS challenge, protein levels of IL-1β are hardly detectable even with sensitive enzyme immunoassays after endotoxin infusion [29]. The failure to detect circulating IL-1β may rather reflect technology limitation. Alternatively, it may also be due to post-transcriptional degradation of IL-1β. Our RT-PCR data provide a more accurate measure of the increase in IL-1β mRNA levels than a previous publication using a multiplex ligation-dependent probe amplification (MLPA) [8]. This MLPA revealed only a 25-fold increase in IL-1β mRNA levels, and failed to detect IL-1α, TNF-α, and IFN-γ at baseline or after injection of even a 2-fold higher LPS dose (4 ng/kg). In another study, Spek *et al.* [8] determined the role of circulating leukocytes in CD14-dependent gene expression. The LPS-induced rise of IL-8 and IL-1β was delayed and reduced after administration of the CD14 antibody IC14, suggesting that LPS may activate genes also via a CD14-independent, slower, and less-efficient pathway.

In contrast, our RT-PCR method demonstrated undetectable mRNA levels of IL-1α at baseline and a very high (>1100-fold) upregulation of IL-1α following LPS infusion and also after *in vitro* incubation with LPS. Hence, IL-1α and IL-1β mRNA determination can provide useful information complementing measurements at the protein level (*figure 3*). However, as the half-life appears to be short for mRNA levels, serial assessments are likely to provide better data as compared to a single measurement at one point in time. Our data are in line with the findings of De Kleijn *et al.* [18] who characterized the transcriptome profiles of circulating neutrophils after LPS infusion in healthy volunteers and found a 6-fold upregulation of TNF-α mRNA after 2 h. This compares well to the observed effect size (7-fold increase) in our trial. In addition, our trial increases these data by demonstrating upregulation of the mRNA levels of several other cytokines including IL-1α and β, IL-8, and IL-10. The >1100-fold increase in IL-1α level is remarkable because it substantially exceeds the rise in the most strongly regulated CD177 in the transcriptome study (200-fold in the more sensitive TaqMan q-PCR but only 20-fold in the array). Our whole blood analysis does not allow differentiating which leukocyte population contributed most to the observed changes. However, the severe monocytopenia after 2 h likely excludes monocytes as contributors to early changes in most cytokines. Yet, the biphasic IL-10 mRNA changes appear to mirror the changes in monocytes over time and could be derived from monocytes.

Performing an mRNA expression profiling *in vitro* was very instructive: *in vitro* experiments did not closely parallel those seen in the human volunteers. Thus, it may be impossible to mimic the complex *in vivo* interaction *in vitro*. Alternatively, an important subpopulation of leukocytes may be removed from circulation during endotoxemia and will no longer be assessable by peripheral blood sampling. Hence, an important limitation of this study is that we are unable to extrapolate our data obtained from circulating leukocytes to marginated leukocytes or those pooled in the reticuloendothelial system, or those homing to lymph nodes. Another limitation is that we have measured mRNA from whole blood rather than from isolated cell fractions. However, the cell isolation procedure may lead to artificial cell activation *in vitro*, and is technically difficult during times of profound monocytopenia

(*figure 1*). As already discussed, it is conceivable and supported by our *in vitro* LPS incubation experiments that we are only able to measure the tip of the iceberg by our approach. However, our data providing extensive information on the constitutive and LPS-inducible mRNA levels of a multitude of cytokines can be important for the cross-validation of newer techniques including microarrays, as already discussed for the MPLA method.

In summary, this is the first comprehensive investigation of the mRNA regulation of cytokines during endotoxemia in humans belonging to the male sex. Only half of the 24 evaluated cytokines are expressed at the mRNA level in circulating leukocytes under basal conditions and after an LPS challenge. Measurement of the *in vivo* expression of cytokine mRNA revealed upregulation only for IL-1α, IL-1β, IL-10, IL-8, and TNF-α in circulating leukocytes, whereas transcripts for several other cytokines (including IL-6 and G-CSF) are expressed after *in vitro* incubation of blood with LPS. In addition, investigation of mRNA levels of IL-1α and IL-1β are potential diagnostic interest in inflammatory diseases, because IL-1 protein levels are hardly detectable in the circulation using conventional enzyme immunoassays.

Disclosure. *Financial support: This work was in part supported by the Austrian Science Funds SFB54-P04. Conflict of interest: none.*

REFERENCES

1. Hochstein HD, Mills DF, Outschoorn AS, Rastogi SC. The processing and collaborative assay of a reference endotoxin. *J Biol Stand* 1983; 11: 251.
2. Fiuza C, Suffredini AF. Human models of innate immunity: local and systemic inflammatory responses. *J Endotoxin Res* 2001; 7: 385.
3. Martich GD, Boujoukos AJ, Suffredini AF. Response of man to endotoxin. *Immunobiology* 1993; 187: 403.
4. Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP. New insights into the biology of the acute phase response. *J Clin Immunol* 1999; 19: 203.
5. Thaler B, Hohensinner PJ, Krychtiuk KA, *et al*. Differential in vivo activation of monocyte subsets during low-grade inflammation through experimental endotoxemia in humans. *Sci Rep* 2016; 6: 30162.
6. Leitner JM, Firbas C, Mayr FB, Reiter RA, Steinlechner B, Jilma B. Recombinant human antithrombin inhibits thrombin formation and interleukin 6 release in human endotoxemia. *Clin Pharmacol Ther* 2006; 79: 23.
7. Suffredini AF, Noveck RJ. Human endotoxin administration as an experimental model in drug development. *Clin Pharmacol Ther* 2014; 96: 418.
8. Spek CA, Verbon A, Aberson H, *et al*. Treatment with an anti-CD14 monoclonal antibody delays and inhibits lipopolysaccharide-induced gene expression in humans in vivo. *J Clin Immunol* 2003; 23: 132.
9. Olszyna DP, Verbon A, Pribble JP, *et al*. Effect of IC14, an anti-CD14 antibody, on plasma and cell-associated chemokines during human endotoxemia. *Eur Cytokine Netw* 2003; 14: 158.
10. Jansen J, van der Poll T, Levi M, *et al*. Inhibition of the release of soluble tumor necrosis factor receptors in experimental endotoxemia by an anti-tumor necrosis factor-alpha antibody. *J Clin Immunol* 1995; 15: 45.
11. Matzneller P, Strommer S, Drucker C, *et al*. Colistin reduces LPS-triggered inflammation in a human sepsis model in vivo: a randomized controlled trial. *Clin Pharmacol Ther* 2016. doi: 10.1002/cpt.582 [Epub ahead of print].
12. Kumar A, Zanotti S, Bunnell G, *et al*. Interleukin-10 blunts the human inflammatory response to lipopolysaccharide without affecting the cardiovascular response. *Crit Care Med* 2005; 33: 331.
13. Pajkrt D, Manten A, van der Poll T, *et al*. Modulation of cytokine release and neutrophil function by granulocyte colony-stimulating factor during endotoxemia in humans. *Blood* 1997; 90: 1415.
14. Preas H.L. 2nd, Reda D, Tropea M, *et al*. Effects of recombinant soluble type I interleukin-1 receptor on human inflammatory responses to endotoxin. *Blood* 1996; 88: 2465.
15. Derhaschnig U, Bergmair D, Marsik C, Schlifke I, Wijdenes J, Jilma B. Effect of interleukin-6 blockade on tissue factor-induced coagulation in human endotoxemia. *Crit Care Med* 2004; 32: 1136.
16. van den Blink B, Branger J, Weijer S, *et al*. P38 mitogen activated protein kinase is involved in the down regulation of granulocyte CXC chemokine receptors 1 and 2 during human endotoxemia. *J Clin Immunol* 2004; 24: 37.
17. De Vries F, Leuschner J, Jilma B, Derhaschnig U. Establishment of a low dose canine endotoxemia model to test anti-inflammatory drugs: effects of prednisolone. *Int J Immunopathol Pharmacol* 2013; 26: 861.
18. De Kleijn P, Odent T, Berntorp E, *et al*. Differences between developed and developing countries in paediatric care in haemophilia. *Haemophilia* 2012; 18: 94.
19. Kamisoglu K, Calvano SE, Coyle SM, Corbett SA, Androulakis IP. Integrated transcriptional and metabolic profiling in human endotoxemia. *Shock* 2014; 42: 499.
20. Haimovich B, Reddell MT, Calvano JE, *et al*. A novel model of common Toll-like receptor 4- and injury-induced transcriptional themes in human leukocytes. *Crit Care* 2010; 14: R177.
21. Xiao W, Mindrinos MN, Seok J, *et al*. A genomic storm in critically injured humans. *J Exp Med* 2011; 208: 2581.
22. Jabbari A, Suarez-Farinas M, Dewell S, Krueger JG. Transcriptional profiling of psoriasis using RNA-seq reveals previously unidentified differentially expressed genes. *J Invest Dermatol* 2012; 132: 246.
23. McLoughlin K, Turteltaub K, Bankaitis-Davis D, *et al*. Limited dynamic range of immune response gene expression observed in healthy blood donors using RT-PCR. *Mol Med* 2006; 12: 185.
24. Hollenstein U, Homoncik M, Knobl P, *et al*. Acenocoumarol decreases tissue factor-dependent coagulation during systemic inflammation in humans. *Clin Pharmacol Ther* 2002; 71: 368.
25. Jilma B, Marsik C, Mayr F, *et al*. Pharmacodynamics of active site-inhibited factor VIIa in endotoxin-induced coagulation in humans. *Clin Pharmacol Ther* 2002; 72: 403.
26. van Oostrom AJ, Sijmonsma TP, Verseyden C, *et al*. Postprandial recruitment of neutrophils may contribute to endothelial dysfunction. *J Lipid Res* 2003; 44: 576.
27. Marsik C, Mayr F, Cardona F, Schaller G, Wagner OF, Jilma B. Endotoxin down-modulates P-selectin glycoprotein ligand-1 (PSGL-1 CD162) on neutrophils in humans. *J Clin Immunol* 2004; 24: 62-5.
28. Pahl A, Brune K. Stabilization of gene expression profiles in blood after phlebotomy. *Clin Chem* 2002; 48: 2251.
29. Copeland S, Warren HS, Lowry SF, *et al*. Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol* 2005; 12: 60.

30. Jilma B, Hergovich N, Homoncik M, *et al*. Granulocyte colony-stimulating factor (G-CSF) down regulates its receptor (CD114) on neutrophils and induces gelatinase B release in humans. *Br J Haematol* 2000; 111: 314.

31. Hollenstein U, Homoncik M, Stohlawetz PJ, *et al*. Endotoxin down-modulates granulocyte colony-stimulating factor receptor (CD114) on human neutrophils. *J Infect Dis* 2000; 182: 343.

32. Wagner JG, Roth RA. Neutrophil migration during endotoxemia. *J Leukoc Biol* 1999; 66: 10.

33. Fong YM, Marano MA, Moldawer LL, *et al*. The acute splanchnic and peripheral tissue metabolic response to endotoxin in humans. *J Clin Invest* 1990; 85: 1896.

34. Dekkers PE, Lauw FN, ten Hove T, *et al*. The effect of a metalloproteinase inhibitor (GI5402) on tumor necrosis factor-alpha (TNF-alpha) and TNF-alpha receptors during human endotoxemia. *Blood* 1999; 94: 2252.

35. Watkins LR, Hansen MK, Nguyen KT, Lee JE, Maier SF. Dynamic regulation of the proinflammatory cytokine, interleukin-1beta: molecular biology for non-molecular biologists. *Life Sci* 1999; 65: 449.