Colistin Reduces LPS-Triggered Inflammation in a Human Sepsis Model *In Vivo:* A Randomized Controlled Trial

P Matzneller[1], S Strommer[1], C Drucker[1], K Petroczi[1], C Schörgenhofer[1], E Lackner[1], B Jilma[1] and M Zeitlinger[1]

The previously described anti-endotoxin effect of colistin has not been investigated in humans yet. We performed a randomized, double-blind, placebo-controlled crossover trial to determine the degree of colistin-driven modulation of inflammatory response in blood of lipopolysaccharide (LPS)-challenged healthy volunteers in a human endotoxemia model. After a single intravenous dose of 2.5 million IU colistin methanesulfonate, interleukin (IL)-6, IL-8, tumor necrosis factor alpha (TNF-α), and IL-1β concentrations as well as other biomarkers of inflammation such as C-reactive protein, differential leukocyte counts, and body temperature were measured up to 24 h postdose. Colistin significantly decreased the inflammatory cytokine response to LPS in blood of healthy volunteers. This effect was most evident for IL-6, IL-8, and TNF-α. This study is the first to confirm the anti-endotoxin effect of colistin in humans *in vivo*. Further studies might increase our knowledge on the interaction between colistin and the effectors of the immune system.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Preclinical data indicate that the antibiotic drug colistin has endotoxin-neutralizing properties which, however, have not been investigated in humans yet.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This randomized, double-blind, placebo-controlled two-way crossover trial was designed to investigate whether the anti-endotoxin effect of colistin can be described in a human endotoxemia model in healthy volunteers *in vivo*.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ Colistin markedly reduced key markers of inflammatory response in LPS-treated healthy volunteers.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

☑ With increasing knowledge of the interaction between colistin and the effectors of the immune system, the anti-endotoxin properties of the drug might be more specifically capitalized in clinical practice e.g., in patients with sepsis.

The polymyxin antibiotic colistin has been long abandoned due to concerns relating to its neurological and renal toxicity. Nowadays, however, it is increasingly used as a last-line therapy option to treat infections caused by multidrug resistant (MDR) Gram-negative bacteria, including most notably *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Colistin is a polycationic molecule and interacts with the lipid A portion of negatively charged lipopolysaccharide (LPS) groups of the outer membrane of its target pathogens. This leads to competitive displacement of divalent cations (Ca^{2+} and Mg^{2+}), which in turn causes disruption of membrane integrity and ultimately bacterial cell death.[1]

Apart from its direct antibacterial effect, the above-mentioned interaction of colistin with bacterial LPS has led to the hypothesis of an additional immunomodulatory mode of action. Indeed, colistin dose-dependently decreased inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-8, and C-reactive protein (CRP) both in *in vitro* tests and in animal infection models.[2–5] The observed decrease in cytokine levels correlated with a reduction in circulatory LPS levels, suggesting that LPS might be bound and/or neutralized by colistin[2,6] as described for other polymyxins.[7–9] Based on the same hypothesis, polymyxin B-coated adsorption cartridges for extracorporeal removal of circulating endotoxin in patients with sepsis are under evaluation in clinical trials.[10,11]

If confirmed, this immunomodulatory effect might counteract cytokine dysregulation in critical illness, e.g., in acute sepsis, and thus contribute to the overall beneficial effects of the drug.

However, the anti-endotoxin effect of colistin has not been investigated in humans yet, and it is unclear whether the available evidence from preclinical studies can be translated to the clinical setting.

Human endotoxemia model

Reports on the intravenous administration of endotoxin to humans date back to the late 1970s.[12,13] In the following decades,

[1]Department of Clinical Pharmacology, Medical University of Vienna, Austria. Correspondence: M Zeitlinger (markus.zeitlinger@meduniwien.ac.at).

Received 7 October 2016; accepted 15 November 2016; advance online publication 3 February 2017. doi:10.1002/cpt.582

the human endotoxemia model was further expanded by several research groups to investigate an extensive number of research questions including the endotoxin scavenging action of proteins and therapeutics.[14–19] It offers a well-established and safe means to study immunological responses in the early phases of human sepsis. In brief, the model consists of the intravenous infusion of standardized doses of bacterial endotoxin, which elicit a transient inflammatory response involving tachycardia, hypotension, and a series of flu-like symptoms like fever, chills, and headache. Importantly, these clinical features have been shown to correlate with an increase of both pro- and antiinflammatory cytokines. All features are self-limiting and volunteers are usually free of symptoms within ~6 h postinfusion. The duration and extent of LPS-induced inflammatory response are dose-dependent.[14] While administration of high doses of LPS (40 mg/kg) leads to lethal septic shock in mice, infusion of low doses (2–4 ng/kg body weight) of LPS in humans provides a unique model to evaluate the pathophysiological responses to endotoxin and their potential therapeutic modulation.[14,17]

Study rationale and research question

Based on available data from *in vitro* experiments and animal models, we hypothesized that the anti-endotoxin effect of colistin might be measured as well in human subjects. The present trial investigated the effect of colistin on LPS-induced inflammatory cytokine levels in the blood of healthy volunteers in a combined *in vitro* / *in vivo* approach. In both parts of the study, inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were chosen as the main outcome parameters. First, we tested the impact of different concentrations of colistin on LPS and cytokine concentrations in an *in vitro* model of endotoxemia. We assessed colistin-induced changes in levels of selected pro- and antiinflammatory cytokines in LPS-spiked blood of healthy volunteers, in order to explore and, if necessary, optimize the experimental setting for comparison with findings of the *in vivo* study.

Subsequently, we evaluated the impact of colistin on LPS-triggered inflammation in a human endotoxemia model in healthy volunteers *in vivo*. We investigated the inflammatory response elicited by infusion of a low dose of LPS and its possible modulation by a single intravenous dose of 2.5 million IU of colistin methanesulfonate (CMS). Some of the results of these studies have been previously reported in the form of abstracts.[20,21]

RESULTS

In vitro study

LPS triggered a marked increase of cytokine levels in colistin-free samples. Augmentation of cytokine concentrations compared to negative control samples (blood and saline solution only) after 2 h of incubation ranged from 3-fold (IL-1β) to ~30-fold (IL-8). All cytokines showed a marked further increase from 2 to 4 h except for TNF-α, which showed a slight decline after the initial peak at 2 h. Colistin reduced the expression of inflammatory cytokines IL-6, IL-8, and TNF-α by up to 10-fold both after 2 and 4 h of incubation (**Figure 1a–c**). The colistin-induced reduction of IL-1β was less marked and did not reach statistical significance (**Figure 1d**). Of note, no statistically significant difference in the magnitude of colistin-induced reduction of cytokine release could be observed between different colistin concentrations. Remarkably, while the mean overall relative increase of cytokine levels in LPS-spiked samples compared to negative controls was highly variable between subjects (coefficient of variation, COV, 90.1%), the impact of colistin, denoted as relative reduction vs. LPS-only samples, was more reproducible (COV 36.8%). If IL-1β, which showed particularly high variability, is not taken into account, the COVs of the overall relative increase and relative reduction are 98.8% and 19.4%, respectively.

In vivo study

Fifteen healthy volunteers (mean ± SD age, 28.5 ± 4.5 years, mean ± SD body weight, 78.9 ± 6.4 kg, mean ± SD body mass index, 23.5 ± 2.0 kg/m^2) completed the study. One subject missed the second study period due to unforeseen unavailability and was excluded from analysis.

Adverse events

CMS was well tolerated. No relevant increase of serum creatinine was noted at 24 h postdose. Following LPS infusion, many subjects experienced flu-like symptoms. In the group of CMS-treated subjects, five (33.3%) cases of shivering, three (20%) cases of headache, and four (26.7%) cases of joint pain were documented. In the placebo period, 10 subjects (66.7%) reported shivering, eight (53.3%) reported headache, and five (33.3%) reported joint pain. Two placebo-treated subjects (13.3%) had a self-limiting asystole over 4 to 5 sec ~1 h after LPS administration with concurrent reversible loss of consciousness. In both cases, restart of regular heart rhythm and full recovery of consciousness occurred spontaneously within few seconds without sequelae of any kind. Between-period differences in adverse events were of a merely descriptive character and did not reach statistical significance.

Comedication

Four of 15 CMS-treated subjects (27%) received a single oral 500 mg dose of paracetamol. In the placebo period, nine subjects (60%) needed one or more single doses of paracetamol 500 mg. Overall, 14 single doses (0.93 doses per subject) were administered in the placebo-treated group compared to four single doses (0.27 doses per subject) in the CMS-treated group.

Cytokines

Concentration–time curves of IL-6, IL-8, TNF-α, and IL-1β in blood of LPS-challenged healthy volunteers after treatment with CMS or placebo, respectively, are shown in **Figure 2**. The concentration–time profile of formed colistin in plasma of healthy volunteers after intravenous infusion of 2.5 million IU CMS is displayed in **Figure 3**.

As shown in **Table 1**, peak concentrations (c_{max}) and area under the concentration–time curves ($AUC_{0\text{-}n}$) of IL-6, IL-8, and TNF-α were significantly reduced in the CMS treatment period compared to the placebo treatment period. Mean ± SD between-period ratios of c_{max} and $AUC_{0\text{-}n}$ were 6.4 ± 3.7 and 8.0 ± 14.1 for IL-6, 8.7 ± 12.8 and 5.2 ± 4.9 for IL-8, as well as 7.5 ± 4.4 and 4.7 ± 2.3 for TNF-α.

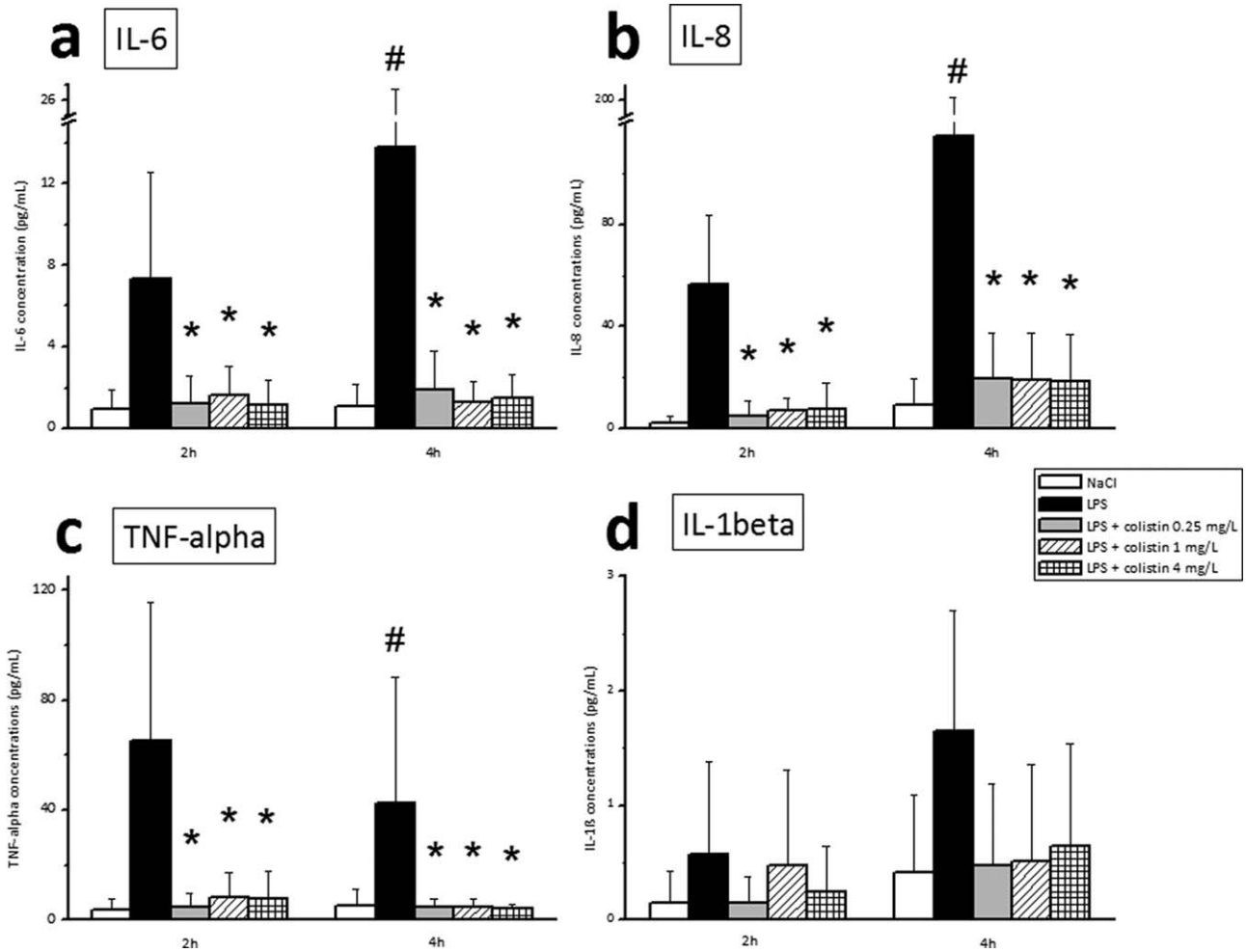

Figure 1 Concentrations of IL-6 (**a**), IL-8 (**b**), TNF-α (**c**), and IL-1β (**d**) in blood of healthy volunteers spiked with 0.9% saline solution (NaCl, white columns), 50 pg/mL of LPS (black columns), and LPS with different concentrations (0.25, 1, and 4 mg/L) of colistin sulfate (light gray, striped and checkered columns, respectively). Diagrams show values after 2 and 4 h of incubation. *Statistically significant difference to LPS column of the same timepoint. #Statistically significant difference to LPS column at 2 h.

Differential blood count

Mean ± SD baseline values of total leukocyte counts (6.1 ± 1.9 and 5.9 ± 0.9 G/L) and neutrophils (3.4 ±1.9 and 3.0 ± 0.6 G/L for CMS and placebo treatment, respectively) were similar between periods. During the first 2–3 h after CMS/placebo infusion, all values showed a slight decline, which was unaffected by LPS infusion and might be attributable to simple dilution by continuous 0.9% saline infusion. Total leukocyte and neutrophil counts markedly increased by ∼50% compared to baseline values. Peak values of leukocytes (9.9 ± 1.9 G/L in CMS-treated subjects, 10.0 ± 2.3 G/L in placebo-treated subjects) and neutrophils (8.3 ± 1.8 G/L in CMS-treated subjects, 9.0 ± 2.2 G/L in placebo-treated subjects) were reached between 5 and 6 h after LPS infusion and showed no significant difference between CMS and placebo treatment periods. $AUC_{0\text{-}n}$ values of both total leukocytes and neutrophils were higher in the placebo period. Lymphocyte and monocyte counts as well as basophilic and eosinophilic granulocyte counts all showed a temporary decline in response to treatment but returned to baseline after 24 h. As displayed in **Figure 4**, this decline was generally less pronounced in CMS-treated individuals. However, these numerical differences did not reach statistical significance.

Temperature

CMS delayed the LPS-triggered increase of sublingual body temperature compared to placebo (**Figure 5**). Peak values were comparable between periods (37.1 ± 0.5°C vs. 37.3 ± 0.4°C in the CMS and placebo treatment periods, respectively) but time to peak temperature was longer in colistin-exposed subjects (4.6 ± 0.8 h after LPS challenge) compared to placebo (3.7 ± 1.1 h). As presented in **Table 1**, body temperature deviation from baseline was also slightly lower in the CMS group.

CRP

Colistin significantly attenuated the LPS-driven, IL-6-mediated[22] elevation of CRP after 24 h (**Table 1**). Both absolute mean ± SD values (1.43 ± 0.54 mg/dL) and 24h/baseline ratios (32.4 ± 26.9)

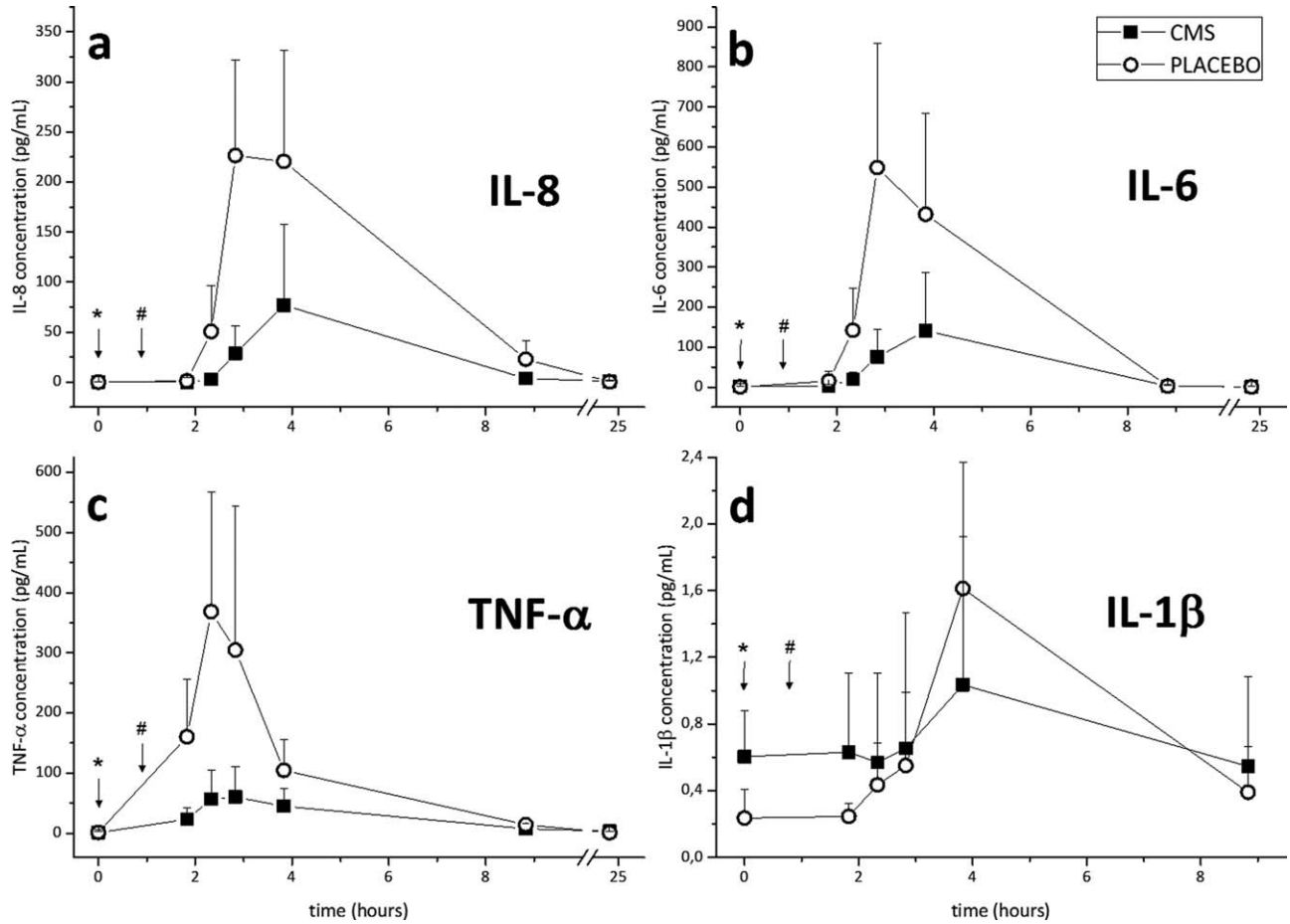

Figure 2 Concentration–time profiles of IL-8 (**a**), IL-6 (**b**), TNF-α (**c**), and IL-1β (**d**) in blood of LPS-treated healthy volunteers after administration of 2.5 million IU of CMS (closed squares) or placebo (open circles). °Timepoint of CMS/placebo administration. #Timepoint of LPS administration.

in the CMS group were markedly lower than in placebo-treated individuals (2.38 ± 0.62 mg/dL and 49.8 ± 26.2, respectively).

DISCUSSION

The present randomized controlled trial demonstrated the ability of colistin to significantly decrease the inflammatory cytokine response to LPS in blood of healthy volunteers both *ex vivo* and *in vivo*. This effect was most evident for inflammatory cytokines IL-6, IL-8, and TNF-α but was also reflected by an attenuated response of CRP, differential blood count, and even clinical parameters such as body temperature.

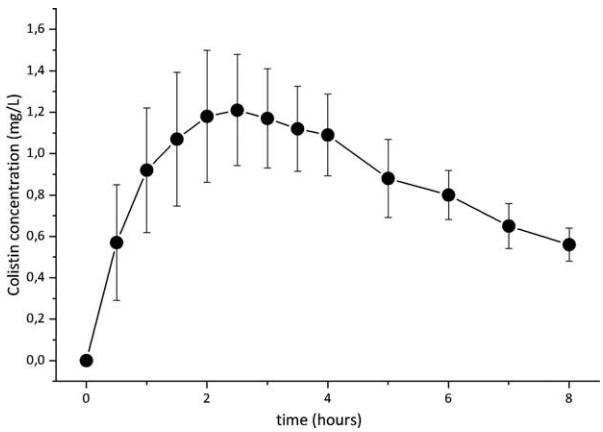

Figure 3 Concentration–time profile of formed colistin in plasma of healthy volunteers after a single dose of 2.5 million IU of CMS as an intravenous infusion over 60 min.[34]

In the *ex vivo* experiments, where cytokine concentrations following exposure to multiple colistin concentrations were tested, there was no clear concentration-dependence of colistin-induced reduction of cytokine levels. Among the reasons for this, high between-subject and within-subject variability of cytokine concentrations seems the most likely factor, but also the drug concentrations chosen for the experiments might play a role. Although the range of tested concentrations reflected clinically relevant values, a wider range of colistin levels (including lower concentrations) might have helped to further differentiate various degrees of cytokine modulation.

The *in vivo* study extended the investigation of the immune reaction to LPS from cytokines to other markers of inflammation such as CRP, body temperature, and differential leukocyte count. Overall, both the increase of inflammatory markers and clinical symptoms in individuals who received placebo after LPS challenge were in good agreement with previous reports,[14,17] highlighting the reproducibility of the human endotoxemia model.

Table 1 Key outcome parameters of inflammatory response in LPS-challenged healthy volunteers after treatment with CMS (Colistin) and 0.9% saline solution (Placebo), respectively

	Placebo	Colistin	*P* value
IL-1β			
c_{max} (pg/mL)	1.6 ± 0.8	1.2 ± 0.8	0.968
t_{max} (hours)	3.8 ± 0.0	4.2 ± 2.6	0.977
$AUC_{0\text{-}n}$ (pg*h/mL)	6.5 ± 4.2	6.3 ± 5.2	0.083
IL-6			
c_{max} (pg/mL)	595.6 ± 306.4	144.9 ± 143.5	<0.001[a]
t_{max} (hours)	3.1 ± 0.5	3.6 ± 0.5	0.001[b]
$AUC_{0\text{-}n}$ (pg*h/mL)	1,789.3 ± 2,326.8	334.9 ± 233.7	<0.001[a]
IL-8			
c_{max} (pg/mL)	259.6 ± 112.1	77.5 ± 80.0	<0.001[a]
t_{max} (hours)	3.2 ± 0.5	3.7 ± 0.4	0.007[b]
$AUC_{0\text{-}n}$ (pg*h/mL)	1,183.9 ± 745.3	468.8 ± 645.5	0.001[a]
TNF-α			
c_{max} (pg/mL)	390.2 ± 253.8	63.7 ± 49.8	<0.001[a]
t_{max} (hours)	2.4 ± 0.3	2.8 ± 0.5	0.006[b]
$AUC_{0\text{-}n}$ (pg*h/mL)	1,323.3 ± 577.1	324.5 ± 193.8	<0.001[a]
CRP 24h	2.38 ± 0.62	1.43 ± 0.54	<0.001[a]
CRP 24h/BL ratio	49.8 ± 26.2	32.4 ± 26.9	0.013[b]
$Temp_{max}$	37.3 ± 0.4	37.1 ± 0.5	0.257
ΔTemp	1.3 ± 0.4	1.0 ± 0.4	0.039[b]
Time to $Temp_{max}$ (hours)	3.7 ± 1.1	4.6 ± 0.8	0.016[b]
WBC			
c_{max} (G/L)	10.0 ± 2.3	9.9 ± 1.9	0.908
$AUC_{0\text{-}24}$ (G*h/L)	171.0 ± 48.2	151.0 ± 24.3	0.175
Neutrophils			
c_{max} (G/L)	9.0 ± 2.2	8.3 ± 1.8	0.345
$AUC_{0\text{-}24}$ (G*h/L)	136.5 ± 41.4	105.2 ± 26.5	0.020[b]
Adverse events			
Episodes of:			
Headache *(n)*	8	3	0.056
Joint pain *(n)*	5	4	0.496
Shivering *(n)*	10	5	0.160
Asystole *(n)*	2	0	0.168
Comedication			
Dispensed paracetamol doses *(n)*	14	4	0.026[b]

Values shown as means ± standard deviations.
[a]Statistically significant after correction for multiple testing according to Bonferroni-Holm. [b]Not statistically significant after correction for multiple testing according to Bonferroni-Holm.

Colistin markedly attenuated the release of IL-6, IL-8, and TNF-α. These cytokines are among the key effectors of the inflammatory reaction in acute sepsis. They act as endogenous pyrogens, upregulate the activation of secondary effector cells, and are involved in a countless number of complex immunological interactions and signaling cascades, only a small part of which

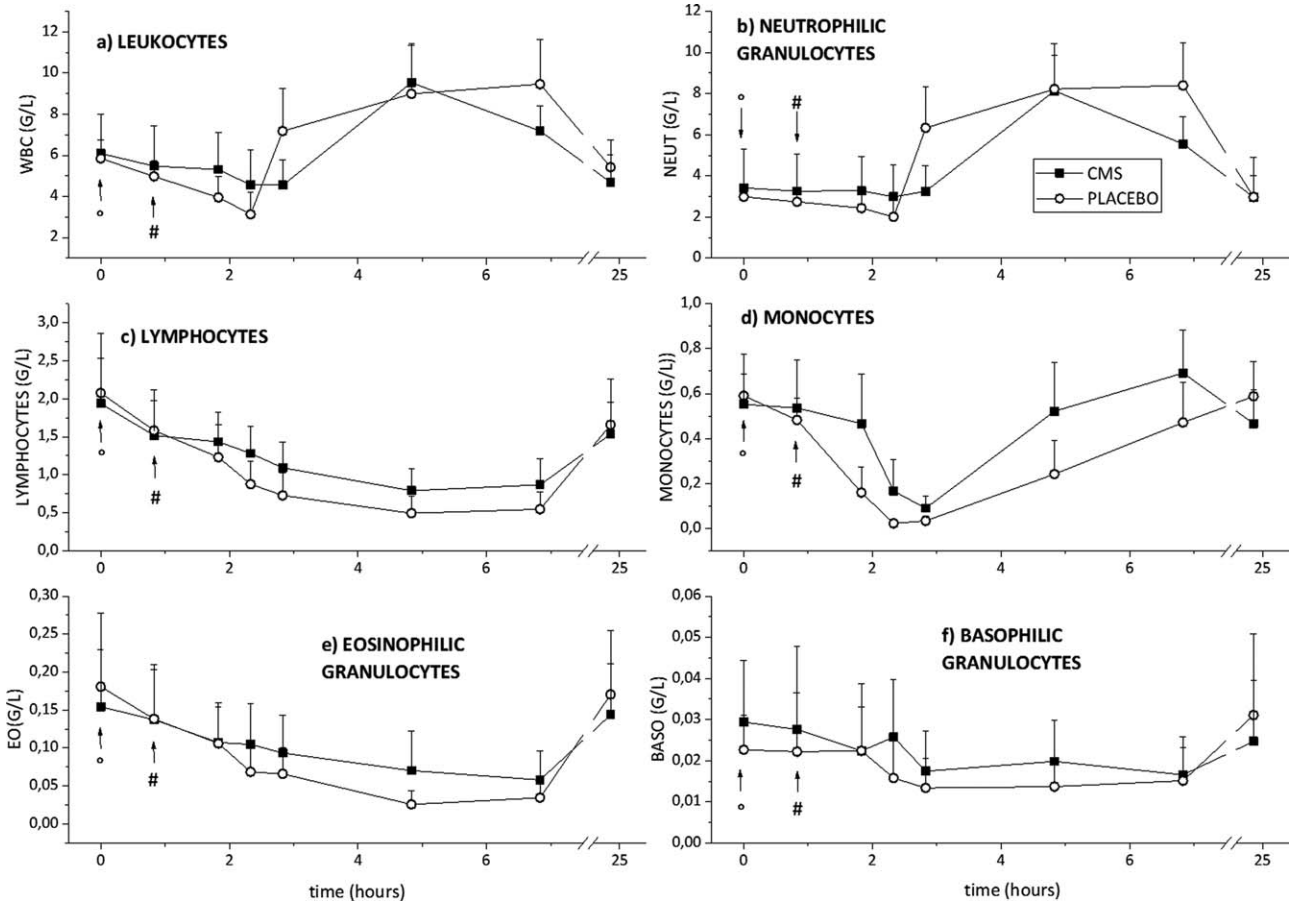

Figure 4 Concentration–time profiles of total leukocyte counts and differential leukocyte counts in blood of LPS-challenged healthy volunteers after treatment with CMS (closed squares) and placebo (open circles). °Timepoint of CMS/placebo infusion. #Timepoint of LPS infusion.

has been fully elucidated.[23] To the best of our knowledge, the inhibition of their release by colistin in humans *in vivo* has not been reported before.

IL-1β, also a major proinflammatory cytokine, showed only minimal deviation from baseline values following LPS infusion. In the *ex vivo* experiments, colistin-driven attenuation of IL-1β reached a noteworthy magnitude only after 4 h. In the *in vivo* study, IL-1β measurements were affected by particularly high intersubject variability (present already at baseline) and no relevant IL-1β attenuation by colistin was detectable at all. However, the lack of impact of LPS administration on IL-1β in blood of healthy volunteers has been documented previously.[24,25] In patients with severe sepsis, IL-1β was shown to persistently increase over several days in association with poor outcome.[26] Thus, the marginal involvement of IL-1β in response to LPS in the present study might also be related to the relatively low dose of LPS administered to healthy volunteers and the transient character of the artificial endotoxemia.

Of note, colistin treatment had almost no effect on total and differential leukocyte counts, which showed no relevant difference between periods. This is in contrast to the colistin-induced reduction of IL-8, one of the major drivers of neutrophil chemotaxis, but agrees with the lack of effect of the IL-8 inhibitor reparixin on neutrophil release during endotoxin-induced systemic inflammation.[27] A potential delayed response of leukocytes to colistin therapy outside the observation window of 24 h cannot be excluded.

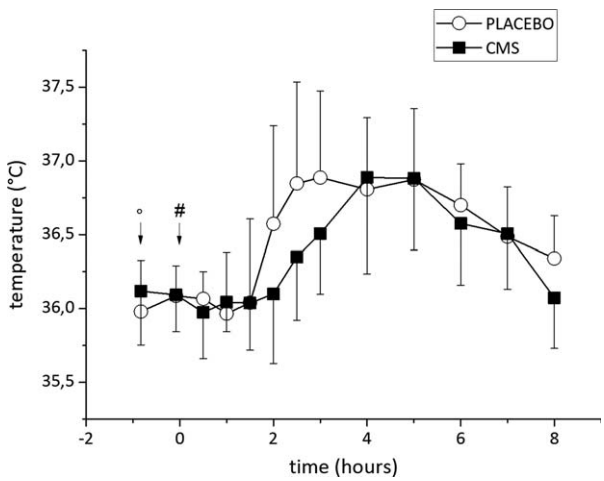

Figure 5 Sublingual temperature-time profiles of LPS-treated healthy volunteers following a single dose of 2.5 million IU CMS or placebo, respectively. °Timepoint of CMS/placebo administration. #Timepoint of LPS administration.

Among the limitations of this study is the fact that it does not provide the mechanism by which colistin reduces effectors of the inflammatory response. Considering the mode of action of the drug, binding to bacterial LPS, and subsequent reduction of circulating endotoxin, with according limitation of its immunogenic potential, seems the most likely explanation. However, the most commonly used method to measure LPS, the limulus amoebocyte lysate (LAL) assay, suffers from a number of methodological flaws and is likely to deliver only semiquantitative results.[28,29] It was therefore not employed in the present study, which could have benefited from showing an association between reduced LPS concentrations and attenuated immune reaction.

Also, this study was conducted in healthy volunteers. Any extrapolation of the data presented here to a clinical context must be done with caution. In a clinical setting, therapy is usually administered only after symptoms have emerged, i.e., treatment with colistin would be started only with a certain delay, when upregulation of the inflammatory cascade is already in place. Hence, the dynamics of the interaction between colistin and the immune system might be different than in the present study, where CMS was administered prior to LPS challenge. However, this was an exploratory study with the aim to characterize the anti-endotoxin effect of colistin in its possibly biggest magnitude, and the design of the study was adapted to this circumstance. Moreover, it should be kept in mind that in specific situations LPS release into systemic circulation might as well continue even after therapy has been initiated, e.g., as intermittent endotoxin bursts from an abscess. Also, an increase of circulating endotoxin levels due to bacterial cell death might also be induced by antibiotic therapy itself.[30] These scenarios are better depicted by the experimental design of the present study rather than by LPS challenge followed by CMS infusion. Nevertheless, future trials might consider CMS administration after or at the time of LPS infusion in order to better reflect specific clinical situations.

With respect to clinical relevance, a crucial question is whether the colistin concentrations achieved in the present study are representative of levels attained in patients. In current clinical practice, therapy with CMS is typically started with a loading dose of 9 million IU and pursued with a maintenance dose of either 4.5 million IU every 12 h or 3 million IU every 8 h.[31,32] In a recently published study, the mean peak concentration of formed colistin in patients treated with a loading dose of 9 million IU CMS was reported to be 2.65 mg/L (range 0.9–5.1 mg/L),[31] and the median average steady-state concentration of formed colistin under maintenance therapy observed by another group was 2.36 mg/L (range 0.48–9.38 mg/L).[33] In comparison hereto, the present project used colistin sulfate concentrations of 0.25, 1 and 4 mg/L for the *ex vivo* experiments. Healthy subjects in our *in vivo* study received a single dose of 2.5 million IU of CMS, which was shown to produce a mean colistin peak plasma concentration of 1.4 mg/L.[34] These numbers show not only that colistin concentrations attained in this study do reflect values observed in patients treated according to current dosing recommendations. Even more importantly, the abovementioned concentration ranges suggest that a consistent portion of CMS-treated patients will have circulating colistin levels well above the values attained in this study, and that the true magnitude of colistin's anti-endotoxin effects in these patients might be even bigger.

In conclusion, this study has shown that colistin, in addition to its direct antibacterial activity, has potent immunomodulatory properties that might be beneficial for patients with cytokine dysregulation, e.g., during sepsis. Despite high variability of some of the investigated parameters (most notably, IL-1β), modulation of key inflammatory cytokines IL-6, IL-8, and TNF-α in blood of LPS-challenged subjects was highly significant. A profound, specifically immunological interpretation of the impact of colistin on individual cytokines is hardly feasible based on the evidence presented here and is beyond the scope of this article. However, future investigations on the topic are highly desirable and might include a larger number of both pro- and antiinflammatory cytokines as well as other immunologically relevant biomarkers in order to provide further insights into the mechanisms of immune modulation by colistin. Ultimately, with increasing knowledge of the interaction between colistin and the effectors of the immune system, it might be possible to define specific subgroups of patients for whom treatment with colistin could be particularly beneficial.

METHODS

Substances and equipment

CMS was purchased from Grünenthal (Brunn am Gebirge, Austria). Colistin sulfate was purchased from Sigma-Aldrich (Vienna, Austria). LPS (*E. coli* 0113 Reference Endotoxin, CC-RE Lot 3) was obtained from the National Institutes of Health (NIH, Bethesda, MD). Quantikine colorimetric sandwich enzyme-linked immunosorbent assay (ELISA kits (R&D Systems) were purchased from Biomedica Medizinprodukte (Vienna, Austria).

Ethical aspects

This trial was conducted at the Department of Clinical Pharmacology at the Medical University of Vienna, Austria, in compliance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) guidelines and the Declaration of Helsinki. The study was registered under EudraCT number 2014-002857-20 and received the approval of the Ethics Committee of the Medical University of Vienna and the Austrian Agency for Health and Food Safety. Written informed consent was obtained from all subjects before inclusion in either the *in vitro* or *in vivo* part of the study. Also, all subjects underwent a screening visit which included physical examination, blood sampling for hematology, clinical chemistry, virology (hepatitis B and C, HIV serology), and coagulation tests as well as ECG tracing and noninvasive arterial blood pressure measurement.

In vitro study

Key inclusion criteria were: male, age between 19 and 40 years, laboratory parameters within the given reference range. Key exclusion criteria were findings indicative of a clinically relevant illness within 3 weeks from the study day, recent blood donation or recent participation in other clinical studies involving treatment with investigational drugs and/or LPS, regular use of medication, or alcohol or drug abuse. No medication at all was allowed within 1 week from the study day. Seventeen healthy males underwent a screening visit for the *in vitro* part of the study. One subject had to be excluded due to a common cold shortly before the scheduled study day. Sixteen male healthy volunteers (mean ± SD age, 27.3 ± 4.9 years) were included in the *in vitro* part of the study. At scheduled appointments, ~50 mL of venous blood per subject were drawn into EDTA-precoated VACUETTE blood collection tubes (Greiner Bio-One, Kremsmünster, Austria). Then blood was spiked with LPS to give a final concentration of 50 pg/mL and with colistin sulfate to give final concentrations of 0.25, 1, and 4 mg/L. From previous experience with CMS administration to healthy volunteers, peak plasma

concentrations of formed colistin after a single intravenous dose of 2.5 million IU of CMS were expected to be around 1.4 mg/L.[34] However, single doses and corresponding peak concentrations reached in plasma of critically ill patients are higher than in this study,[31,33,35] and colistin susceptibility breakpoints for relevant pathogens reach up to 4 mg/L.[36] Hence, the above-mentioned colistin concentrations were chosen to reflect a range of values representative both for the present study in healthy volunteers as well as for the clinical setting in patients. The above-mentioned LPS concentration of 50 pg/mL is representative of values reached in plasma of septic patients.[37,38] Previous *in vitro* models of endotoxemia have employed higher concentrations which, however, are not, or very rarely, attained *in vivo* in the clinical setting.[38] In previous *in vitro* experiments at our institution, incubation of healthy volunteers' blood with LPS at a concentration of 50 pg/mL yielded a sufficiently high increment in inflammatory cytokines for experimental purposes.[38,39] Positive (blood + LPS only) and negative (blood + 0.9% saline solution only) controls were included. After 2 and 4 h of incubation at 37°C, blood was centrifuged and the resulting plasma stored at −80°C until analysis. Eventually, IL-1β, IL-6, IL-8, and TNF-α levels were determined by means of a commercially available cytokine ELISA method (Quantikine, R&D Systems).

In vivo study

Key inclusion criteria for the *in vivo* part of the study were: male, age between 19 and 40 years, laboratory parameters within the given reference range, normal medical history, and physical examination. Key exclusion criteria were: allergy to the trial product or related products (polymyxin B), history of severe allergic or anaphylactic reactions, recent blood donation or recent participation in other trials involving treatment with investigational drugs and/or LPS, blood donation for the *in vitro* part of the study, known coagulation disorders, liver or kidney dysfunction, intake of any medication within 1 week prior to study days, clinically relevant illness within 3 weeks prior to study days, weight $>$ 95 kg or $\leq$ 60 kg.

Eighteen healthy volunteers were screened for the *in vivo* part of the study. Two subjects were excluded due to poor peripheral vein situation and withdrawal of informed consent, respectively. Sixteen healthy male volunteers were enrolled in the *in vivo* study, which was designed as a prospective, investigator- and participant-blinded, single-center, randomized two-way crossover study. Subjects were randomly allocated to receive 2.5 million IU of CMS or placebo (250 mL of 0.9% saline solution) as intravenous infusion over 60 min followed by 2 ng/kg body weight of LPS as intravenous bolus infusion over 1–2 min. No premedication was administered prior to LPS infusion. After a washout period of at least 6 weeks, subjects underwent the alternate treatment in a crossover fashion.

CMS/placebo infusion was administered 50 min prior to LPS infusion. In a previous study in healthy volunteers, maximum plasma concentrations of formed colistin were reached ∼2.4 h after the start of CMS infusion.[34] On the other hand, it is known that the peak levels of most LPS-induced inflammatory cytokines in plasma can be expected within a time frame ranging from 1.5–3 h after LPS infusion.[14,40] Therefore, the above-mentioned time schedule was chosen to allow both agents to reach their maximum effect in plasma simultaneously, i.e., ∼1.5 h after challenge with LPS.

During the 8 h following LPS challenge, all subjects received a continuous infusion of 0.9% saline solution at a flow rate of 100 mL/h. Noninvasive arterial blood pressure, heart rate, and sublingual body temperature were closely monitored. If needed to alleviate LPS-triggered flu-like symptoms, the intake of paracetamol at single oral doses of 500 mg or single intravenous doses of 1,000 mg was allowed with a maximum total dose of 3 × 1,000 mg. Paracetamol does not affect cytokine release in this model.[41] One standardized meal was served not earlier than 4 h after LPS administration.

At defined timepoints before and up to 24 h after CMS/placebo infusion, venous blood was drawn from an indwelling venous cannula placed into a suitable vein of the contralateral arm to the one used for LPS infusion.

Differential blood counts were performed by means of an automated Sysmex 7100 analyzer. In analogy to the *in vitro* part of the study, IL-1β, IL-6, IL-8, and TNF-α were determined in plasma by means of a commercially available cytokine ELISA kit. Furthermore, levels of CRP in blood of healthy volunteers were determined at baseline and 24 h after LPS challenge.

Randomization and blinding

Block randomization with block sizes of eight was performed using an open access randomization generator (www.randomization.com). Two sets (one main set, one backup set) of sealed envelopes with the randomization number containing information about the sequence of treatment allocation (sequence AB or sequence BA) were prepared for each individual subject and kept throughout the study. In order to prevent potential influence on study outcomes (e.g., expectation of less pronounced LPS-induced symptoms in the CMS group), investigators and study subjects were not aware of treatment allocation. CMS and/or placebo infusions were prepared by an unblinded study nurse under supervision of an unblinded pharmacist. These unblinded members of study staff were not involved in other study-related procedures.

Pharmacokinetic analysis

Concentrations of inflammatory cytokines and differential leukocyte counts in plasma of healthy volunteers were elaborated using noncompartmental analysis (NCA) by means of a commercially available computer program (Kinetica 3.0, Innaphase, USA). Maximum plasma concentration (c_{max}), time to maximum plasma concentration (t_{max}), and area under the concentration–time curve (AUC) from 0 to n h ($AUC_{0\text{-}n}$) were calculated from nonfitted data by employing the trapezoidal rule.

Sample size considerations

Sample size considerations were based on previous experience with crossover trials involving endotoxin infusion. Since the present study tested the impact of colistin on LPS-triggered inflammation for the first time in humans, the effect size of colistin on LPS and cytokine levels could only be estimated relying on preclinical data. Among the outcome parameters investigated in this study, inflammatory cytokines were chosen as the basis for sample size calculations, and IL-6 selected as the most representative one. In two recent publications in this regard, colistin achieved effect sizes in terms of IL-6 reduction of approximately 50%.[2,3] Assuming 0.5 standard deviations and a two-tailed α error of 0.05, a sample size of $n = 16$ subjects was estimated to be appropriate to detect a 50% difference in means between the study groups with a power of 80%.[42]

Statistical analysis

Statistical analysis was performed using a commercially available computer program (SPSS, IBM, Armonk, NY). Occurrence of normal distribution was assessed by the Shapiro–Wilk test. Normally distributed parameters are given as mean and standard deviation, nonnormally distributed parameters were natural log transformed. A linear mixed model with treatment, period, and sequence as fixed effects and subject ID as random effect was used to assess the impact of colistin on the dependent outcome variables as well as to exclude relevant sequence and period effects. Statistical significance was defined as $P < 0.05$. The Bonferroni–Holm correction method was applied to correct for errors related to multiple testing. Throughout the manuscript, the term "significant" is used only for p values which retained statistical significance after correction for multiple testing. **Table 1** shows P values prior to multiplicity adjustment. For each P value <0.05, statistical significance after correction is evincible from identification with a specific symbol and the corresponding legend.

ACKNOWLEDGMENTS

This trial was supported in part through the Special Research Program SFB-54 by the Austrian Science Fund.

CONFLICT OF INTEREST/DISCLOSURE

No conflicts to declare.

AUTHOR CONTRIBUTIONS

P.M. wrote the article; M.Z., P.M., C.S., and B.J. designed the research; S.S., C.D., K.P., and E.L. performed the research; M.Z., P.M., C.S., and B.J. analyzed the data.

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