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Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis *in vitro*

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ABSTRACT

The inflammatory response to pathogen-associated molecular patterns such as lipopolysaccharide (LPS) in sepsis is mediated *via* Toll-like receptors (TLRs). Since TLRs also trigger various immune functions, including phagocytosis, their modulation is a promising strategy in the treatment of sepsis. As antibiotics have immunomodulatory properties, this study examined the effect of commonly used classes of antibiotics on i) the expression of TLRs and cytokines and ii) the phagocytic activity under sepsis-like conditions *in vitro*. This was achieved by incubating THP-1 monocytes and peripheral blood mononuclear cells (PBMCs) obtained from patients after open-heart surgery with the addition of LPS and six key antibiotics (piperacillin, doxycycline, erythromycin, moxifloxacin or gentamicin). After 24 h, mRNA levels of both cytokines (IL-1β, IL-6) and TLRs (1, 2, 4, and 6) were monitored and phagocytosis was determined following coincubation with *Escherichia coli*. Each antibiotic differentially regulated the gene expression of the investigated TLRs and cytokines in monocytes. Erythromycin, moxifloxacin and doxycyclin displayed the strongest effects and changed mRNA-levels of the investigated genes up to 5.6-fold. Consistent with this, antibiotics and, in particular, moxifloxacin, regulated the TLR-and cytokine expression in activated PBMCs obtained from patients after open-heart surgery. Furthermore, piperacillin, doxycyclin and moxifloxacin inhibited the phagocytic activity of monocytes. Our results suggest that antibiotics regulate the immune response by modulating TLR- and cytokine expression as well as phagocytosis under septic conditions. Moxifloxacin, doxycycline and erythromycin were shown to possess the strongest immunomodulatory effects and these antibiotic classes should be considered for future immunomodulatory studies in sepsis.

1. Introduction

Sepsis is one of the leading causes of death in critically ill patients and is associated with a hospital mortality of approximately 30% [1]. The clinical manifestation of sepsis is characterized by the cardinal signs of inflammation including tachycardia, leukocytosis, tachypnea, and pyrexia, which are collectively defined as systemic inflammatory response syndrome (SIRS). This systemic inflammatory response results from excessive stimulation of innate immune cells by exposure to different evolutionarily conserved molecular structures on infectious microorganisms, known as "pathogen-associated molecular patterns" (PAMPs)[2].

The recognition of PAMPs is mediated by pattern recognition receptors (PRR) including the Toll-like receptors (TLRs), the Nod-like receptors (NLRs), the C-type lectin receptors (CLRs) and the RIG-like receptors (RLRs) [3]. TLRs are the most widely studied PRRs and are considered to be the primary sensors of pathogens [4]. Lipopolysaccharide (LPS) released from gram-negative bacteria is recognized by TLR4 and comprise a major class of PAMP that causes sepsis through the induction of pro-inflammatory cytokines such as IL-1β and IL-6 [4,5]. Beside their role in the recognition of invading microbes, TLR-signaling further triggers the clearance of pathogens by phagocytosis, which plays a key role in the immune response during sepsis [6]. Due to the pivotal contribution of TLRs in sepsis, their modulation and signaling pathways became a research focus and it has been suggested that TLRs could be potential target molecules for the treatment of sepsis [7–9].

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Although significant resources have been invested during the last decade in new treatments for sepsis, almost all have failed to improve outcomes and intervention with antibiotics remains one of the most effective strategies. Antibiotics have widely appreciated bacteriostatic or bactericidal properties and are used in the therapy of numerous infections, including those associated with sepsis. Interestingly, some antibiotics also directly modulate the immune system in addition to exerting their antimicrobial properties. Thereby, antibiotics interact with host immune cells and regulate their immune function, which has beneficial effects in various inflammatory conditions [10,11]. Previous studies have shown that antibiotics improve survival in animals challenged with LPS or multidrug-resistant pathogens, suggesting an immunomodulatory and non-antimicrobial effect of antibiotics under septic conditions [12–15]. Furthermore, clinical data showed for the first time that the immunomodulatory properties of macrolide antibiotics improve the outcome of septic patients [16].

Yet, little is known about the molecular and cellular mechanisms by which antibiotics modulate the immune system during sepsis. Thus, the aim of this study was to examine the effect of antibiotics on the expression of TLRs and sepsis-related cytokines under inflammatory conditions that mimic sepsis. We focused on classes of antibiotics that are commonly used in the treatment of sepsis and investigated their effects on LPS-activated human immune cells, including leukocytes isolated from patients with a systemic inflammatory response after open-heart surgery. These research findings clearly indicate that the classes of chinolones (moxifloxacin), tetracyclines (doxycycline) and macrolides (erythromycin) regulate the expression of TLRs and cytokines in septic conditions. Thus, use of these antibiotics can be one useful clinical treatment for septic patients.

2. Material and methods

2.1. Reagents and THP-1 cells

LPS from *Escherichia coli* (serotype 055:B5) was purchased from Sigma-Aldrich (Taufkirchen, Germany) and antibiotics were obtained from the manufacturer: gentamicin (GMC), piperacillin (PIP) and doxycycline (DXC) from Ratiopharm (Ulm, Germany); erythromycin (ERY) from Amdipharm (Dublin, Ireland) and moxifloxacin (MXF) from Bayer (Leverkusen, Germany). Human monocytic THP-1 cells (ATCC TIB-202) [17] were grown in suspension in RPMI1640 (PAA, Coelbe, Germany) containing 10% FCS (Biochrom, Berlin, Germany), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany), 100 mg/mL penicillin, 100 mg/mL streptomycin (both from Invitrogen, Frankfurt, Germany) and were maintained at 37 °C in a humidified incubator containing 5% CO_2. The cells were cultured in antibiotic- and FCS-free suspension for 48 h before experimental use to avoid any confounding stimulation that might result from antibiotics or endotoxins present in the cell culture medium. The viability of monocytes was determined by the tryptan blue staining method. Cells with viability greater than 90% were used for the experiments.

2.2. Patients and healthy volunteers

With the approval of the Ethics Committee of the Rheinische Friedrich-Wilhelms-University Medical School in Bonn (number of approval: 125/08), and following written informed consent, we recruited six healthy, non-smoking male volunteers and eight patients scheduled to undergo cardiopulmonary bypass (CPB) for routine elective coronary artery grafting. We excluded patients with acute or chronic infection, immunocompromised patients, patients with autoimmune disorders, or those taking immunosuppressive or anti-infective drugs in the preceding five days. All patients underwent a routine procedure with median sternotomy and use of CPB. Anesthesia was induced with etomidate, sufentanil and cisatracurium and was maintained using isoflurane and sufentanil. Thereafter, the patients received 1.5 g cefuroxime as perioperative antibiotic prophylaxis. The CPB technique included non-pulsatile flow with moderate hypothermia (32 °C–34 °C) and the CPB pump was primed with a standard electrolyte solution containing 10,000 IU heparin. Additional heparin was given if the activated clotting time failed to reach at least 480 s. Cardioplegia was induced by application of Calafiore warm blood-cardioplegic solution. Weaning from CPB was achieved after rewarming and inotropic support with epinephrine (0.025–0.15 μg/kg/min) in all patients and additional norepinephrine (0.025–0.15 μg/kg/min) as necessary. After CPB, heparin was neutralized with protamine sulfate until the preoperative activated clotting time was achieved. Postoperative fluid management consisted of isotonic-balanced electrolyte solution (30–40 ml/kg/day) for basic replacement and gelatin, fresh frozen plasma, or packed red cells according to hemodynamic status and laboratory parameters. Postoperative antibiotic prophylaxis was performed with 3 × 1.5 g cefuroxime every 8 h after admission on ICU.

2.3. Blood collection and preparation of peripheral blood mononuclear cells

Blood samples obtained from healthy volunteers were collected by peripheral vein puncture. Patient blood samples were taken through an indwelling central venous catheter before induction of anesthesia, 30 min and 24 h after end of surgery. In general, blood collection was performed before routinely planned administration of antibiotic prophylaxis. Monovette blood collecting system (Sarstedt, Nuembrecht, Germany), containing EDTA as anticoagulant, was used. Peripheral blood mononuclear cells (PBMCs) were immediately isolated from blood samples by sucrose gradient (PANcoll, PAN-biotech, Aidenbach, Germany) as described elsewhere [18]. After washing, PBMCs were cultured as described above.

2.4. In vitro stimulation experiments

THP-1 cells and PBMCs at a concentration of 1×10^6 cells/mL in 6-well plates (Cellstar, Greiner bio-one, Frickenhausen, Germany) were separately incubated in the presence of either LPS (10 μg/ml), GMC (5 μg/ml), ERY (2 μg/ml), MXF (20 μg/ml), DXC (8 μg/ml), PIP (100 μg/ml) or a combination of LPS with each antibiotic. The cells were incubated at 37 °C and were removed from culture plates after 24 h for isolation of RNA.

2.5. Isolation of RNA and Real-time PCR

Total RNA was isolated from the THP-1 cells and PBMCs using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was generated from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) as recommended by the manufacturer. Cycling conditions were 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s. Real-time PCR was carried out on the 7900HT Fast Real-Time PCR system from Applied Biosystems using TaqMan Gene Expression Assays primer/probe sets (Applied Biosystems) and the standard thermal-cycling conditions for relative quantification designed by the manufacturer. Quantification of the PCR signals for each sample was performed by comparing the cycle threshold values in duplicate for the gene of interest with the cycle threshold values for the GAPDH housekeeping gene. The mean relative mRNA expression was determined by using the SDS software V2.2 and the $2^{-\Delta\Delta CT}$ method [19].

2.6. Phagocytosis assay

The phagocytic activity of THP-1 cells was examined by measuring the uptake of pHrodo *E. coli* BioParticles (Invitrogen) as described by Wan et al. [20]. To prepare BioParticles, 2 ml of PBS were used for reconstitution of the BioParticles. THP-1 cells (1×10^6 cells/ml) were incubated with antibiotics in the presence or absence of LPS as

described above for 24 h, followed by an additional incubation with pHrodo *E. coli* BioParticles for 2 h. DNA staining for viability measurement was performed just prior to flow cytometry analysis with 2 μg/ml Hoechst 33342 (Invitrogen). Flow cytometry was conducted using a Canto III Flow Cytometer System (BD Biosciences, Heidelberg, Germany) and FlowJo Analysis Software (Treestar, Ashland, OR, USA). Phagocytic activity was expressed as the percentage of cells that performed phagocytosis compared to the total number of viable monocytes.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Mann–Whitney *U* test was used to examine differences in phagocytic assay and in gene expression of both THP-1 cells and PBMCs from healthy volunteers. Data obtained from CPB surgery patients were analyzed by paired *t*-test. Values of $p < 0.05$ were considered significant. All data are expressed as mean ± SEM unless otherwise noted.

3. Results

3.1. Antibiotics differentially modulate the gene expression of TLRs and cytokines in monocytes

To determine a putative immunomodulatory effect of antibiotics in sepsis, we initially incubated human THP-1 monocytes with clinically relevant concentrations of commonly used classes of antibiotics, namely PIP (beta-lactam), DXC (tetracycline), ERY (macrolide), MXF (quinolone), and GMC (aminoglycoside) in the presence or absence of LPS. The expression of genes encoding IL-1β, IL-6, TLR1, TLR2, TLR4, and TLR6 was monitored by RT-PCR after 24 h. Tested antibiotics showed differential regulatory effects on gene expression of the investigated cytokines and TLR-subtypes (Fig. 1). The magnitude of this regulation differed between the various classes of antibiotics. Whereas ERY displayed strong immunomodulatory properties in terms of increasing the gene expression up to 5.6-fold (TLR4; Fig. 1E; $p < 0.05$) in LPS-stimulated cells, PIP only altered the mRNA levels up to 2.3-fold (TLR1; Fig. 1C; $p < 0.05$) compared to LPS-activated controls. In detail, PIP increased the expression of TLR1, TLR2, TLR4 and TLR6 by ≈2-fold when compared to unstimulated controls (Fig. 1C–F; $p < 0.05$). In co-stimulation with LPS, the number of genes regulated by the presence of PIP also included IL-1β and IL-6 (Fig. 1A,B; $p < 0.05$). In contrast, cells incubated with either DXC or GMC alone exhibited significantly reduced mRNA levels of IL-1β (DXC and GMC; Fig. 1A; $p < 0.05$), TLR1 (DXC, Fig. 1C; $p < 0.05$), TLR4 (GMC, Fig. 1E, $p < 0.05$) and TLR6 (DXC and GMC, Fig. 1F; $p < 0.05$). Furthermore, co-incubation of monocytes with LPS led to a significant down-regulation of IL-6 by both DXC and GMC compared to their LPS-stimulated controls (Fig. 1B; $p < 0.05$). The incubation of monocytes with either ERY or MXF resulted in differential regulation of several genes: THP-1 cells cultured in the presence of ERY alone showed significantly higher mRNA levels of IL-1β ($p < 0.05$), whereas ERY in combination with LPS reduced mRNA levels of IL-1β when compared to their respective controls (Fig. 1B; $p < 0.05$). THP-1 monocytes stimulated with ERY ± LPS exhibited significantly increased mRNA levels of TLR1, TLR2, TLR4 and TLR6 (Fig. 1C–F; $p < 0.05$). The activation status of monocytes also influenced the regulation of TLR4 gene expression by MXF. In LPS-activated cells, MXF significantly increased the TLR4 expression 2-fold, while MXF reduced the TLR4 mRNA level by more than 50% in the absence of endotoxin (Fig. 1E; $p < 0.05$). Furthermore, MXF significantly reduced IL-6 and TLR6 expression when cultured in the absence of LPS (Fig. 1B,F; $p < 0.05$) and reduced both IL-1β and IL-6 mRNA levels in LPS-activated cells (Fig. 1A,B; $p < 0.05$).

3.2. Antibiotics differentially modulate the gene expression of TLRs and cytokines in PBMCs

The immunomodulatory effects of antibiotics on a mixed immune cell population were studied in PBMCs obtained from healthy human donors and were stimulated under the same conditions as described above. Consistent with the results from THP-1 monocytes, PIP increased

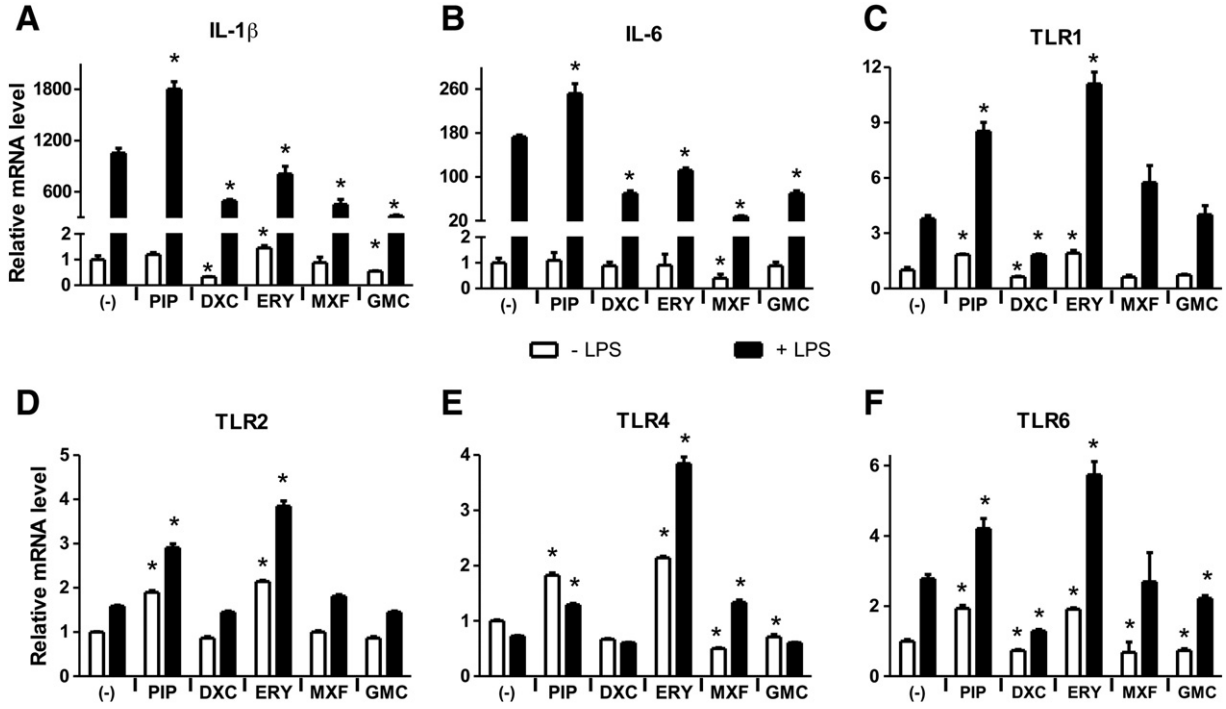

Fig. 1. Effect of antibiotics on cytokines and TLR expression in endotoxin-activated THP-1 monocytes. THP-1 monocytes were incubated with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence or absence of LPS for 24 h. Quantitative Real-Time-PCR (qRT-PCR) analysis was performed to determine the expression levels of genes encoding IL-1β (A), IL-6 (B), TLR1 (C), TLR2 (D), TLR4 (E) and TLR6 (F) after normalization to GAPDH. Each bar represents the fold-change in mRNA expression comparing unstimulated control with antibiotic-incubated cells or LPS-stimulated control with LPS plus antibiotic-stimulated cells, respectively. Data are expressed as mean ± SEM; *, $p < 0.05$.

the TLR2 expression, whereas DXC, MXF and GMC reduced the mRNA levels of TLR4 and/or TLR6 in PBMCs compared to their unstimulated controls (Fig. 2D–E; $p < 0.05$). Furthermore, down-regulation of TLR6 mRNA expression by both DXC and GMC was also found in LPS-activated PBMCs (Fig. 2F; $p < 0.05$). In contrast to the observed immunosuppressive effects of antibiotics on cytokine expression in the THP-1 cell line, PBMC cultured with DXC, MXF or GMC alone and GMC in combination with LPS exhibited significantly higher mRNA levels of IL-1β as compared to the respective controls (Fig. 2A, $p < 0.05$). Furthermore, PBMCs cultured with MXF alone revealed significantly increased mRNA level of IL-6 compared to unstimulated cells (Fig. 2B; $p < 0.05$).

3.3. Antibiotics differentially modulate the gene expression of TLRs and cytokines in PBMCs from patients with a systemic inflammatory response

We next studied the immunomodulatory effects of antibiotics on immune cells from patients with a systemic inflammatory response after open-heart surgery to mimic conditions found in sepsis. Therefore, PBMCs were isolated from 8 patients undergoing CPB-surgery before induction of anesthesia as well as 30 min and 24 h after surgery, followed by incubation with antibiotics and LPS as described above. The operation was uncomplicated in all of the patients and their demographic data as well as clinical profile are presented in Table 1. All patients were broadly immunostimulated, which was represented by a significant increase in the number of leukocytes after surgery (Table 2; $p < 0.05$). Yet, further parameters to diagnose a SIRS after surgery could not be evaluated as these were manipulated by routinely performed treatments such as cardiac pacing and mechanical ventilation. Blood from two patients could not be collected at 24 h after surgery due to their unstable condition. The PBMCs responded to the surgery by down-regulating mRNA levels of TLR2 after 30 min and both IL-1β and IL-6 after 24 h compared to preoperative collected cells (Figs. 3 and 4B; $p < 0.05$). In LPS-activated PBMCs, this effect was extended by up-regulation of IL-1β, IL-6, TLR1, TLR4 and TLR6 in cells obtained after 30 min and down-regulation of TLR2 in cells isolated 24 h after surgery compared to preoperative controls (Figs. 3 and 4; $p < 0.05$). Those effects were partially modulated by antibiotics, suggesting an immunomodulatory effect of antibiotics on either one or both studied time points (Figs. 3 and 4).

Therefore, we analyzed the effect of antibiotics on gene expression within each time point and found that in pre-operatively isolated PBMCs DXC increased the expression both IL-1β and IL-6, while PIP, DXC and MXF significantly increased the mRNA levels of TLR2 and reduced the TLR1 gene expression compared to unstimulated cells (Figs. 3 and 4A,B; $p < 0.05$). Furthermore, PIP significantly increased the

Table 1
Demographic data and perioperative characteristics of patients undergoing CPB surgery.

Patient characteristics (n = 8)	
Age (Years)	72.5 ± 8.3
Weight (kg)	83.0 ± 12.8
Height (cm)	171.9 ± 8.5
Pre-operative ejection fraction (%)	61.0 ± 13.2
Perioperative Data	
Duration of surgery (min)	258.6 ± 75.2
CPB time (min)	66.3 ± 12.4
ACC (min)	16.3 ± 23.5
Intubation time (h)	27.5 ± 9.5
Postoperative blood loss (ml)	1247.5 ± 466.4
Length of stay on ICU (h)	47.1 ± 26.7
Intraoperative red blood cell transfusion (n)	5/8
Postoperative red blood cell transfusion (n)	7/8

Values are presented as mean ± SD except for the number of transfused red blood cells. CPB indicates cardiopulmonary bypass; ACC, aortic cross-clamping; ICU, intensive care unit.

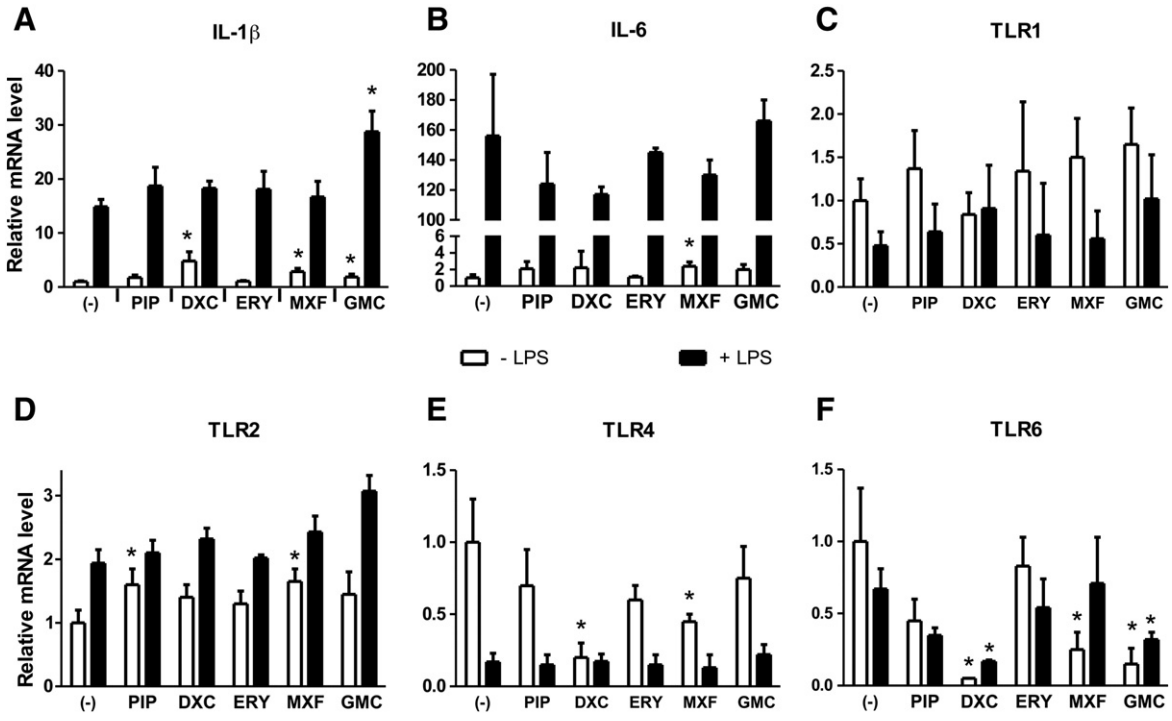

Fig. 2. Effect of antibiotics on cytokine and TLR-expression in endotoxin-activated PBMCs from healthy human donors *ex vivo*. Freshly isolated PBMC from healthy human donors were cultured with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence or absence of LPS for 24 h and mRNA expression of IL-1β (A), IL-6 (B), TLR1 (C), TLR2 (D), TLR4 (E) and TLR6 (F) was measured by quantitative Real-Time PCR. Data represent the fold-change in mRNA expression comparing unstimulated control to antibiotic-incubated cells or LPS-stimulated control to LPS plus antibiotic-stimulated cells, respectively. Data are expressed as mean ± SEM of 6 individual donors in each group; *, $p < 0.05$.

Table 2
Perioperative laboratory data and vital parameters of patients undergoing coronary artery bypass surgery.

Variable	Preoperative	End of CPB (n = 8)	24 h postop (n = 6)
Laboratory data			
Leucocyte count ($\times 10^9$/L)	7.0 ± 1.2	13.6 ± 2.1*	8.0 ± 2.7
Hemoglobin (mg/dL)	13.1 ± 1.2	10.5 ± 0.8	10.5 ± 0.8
Platelet count ($\times 10^9$/L)	224 ± 70	118 ± 65	117 ± 41
Vital Parameters			
Heart rate (/min)	61 ± 18	96 ± 6[a]	95 ± 13[a]
Mean arterial pressure (mmHg)	68 ± 6.7	83 ± 8	
73 ± 13			
Temperature (°C)	36.3 ± 0.3	36.5 ± 0.7	37.8 ± 0.4

Values are presented as mean ± SD.
* $p < 0.05$ *vs.* preoperative data.
[a] Patients received cardiac pacing.

expression of the gene encoding TLR6 compared to unstimulated pre-operatively collected PBMCs (Fig. 4D; $p < 0.05$). In LPS-activated PBMCs, only ERY reduced the mRNA-level of TLR1 whereas GMC increased IL-1β expression (Figs. 3A and 4A; $p < 0.05$). When PBMCs were stimulated *in vivo* by surgery and collected 30 min after surgery, PIP in co-stimulation with LPS significantly reduced their mRNA expression of TLR1 and TLR4 while TLR2 expression was increased compared to LPS-activated controls (Fig. 4; $p < 0.05$). The down-regulation of IL-1β and TLR2 by ERY also required the presence of LPS, whereas MXF increased both IL-1β and TLR2 expression and decreased TLR4 and TLR6 expression in the absence of LPS. The presence of LPS was also important for the regulatory activity of DXC: DXC alone significantly reduced TLR1 while DXC in combination with LPS increased TLR1 expression significantly compared to their respective controls (Fig. 4A; $p < 0.05$). When PBMCs were obtained from patients 24 h after CPB surgery, only MXF significantly reduced the mRNA level of TLR4 while no effect by other antibiotics was observed (Fig. 4C; $p < 0.05$).

3.4. Effect of antibiotics on bacterial phagocytosis

After monitoring an effect of antibiotics on the expression of TLRs, which play a critical role in regulating the phagocytosis of bacteria [6], we examined whether antibiotics may affect the phagocytosis of immune cells. Therefore, THP-1 monocytes were stimulated with antibiotics and LPS as described above, followed by incubation with heat-killed *E. coli*. As determined by flow cytometry, the inclusion of PIP, DXC, or MXF in combination with LPS during culturing decreased the percentage of phagocytizing cells (Fig. 5; $p < 0.05$ and $p < 0.01$, respectively). This effect was LPS-dependent as incubation with antibiotics alone had no effect on the phagocytic activity (data not shown).

4. Discussion

The excessive inflammatory response to microbial pathogens in sepsis is mediated *via* TLRs and their modulation might improve the outcome of septic patients by limiting the pathological inflammation [21]. Increasing evidence suggests that the immunomodulatory properties of antibiotics may have beneficial effects in sepsis [12–15]. Yet, the underlying molecular and cellular mechanisms including the effect of antibiotics on TLR expression remains unclear. The current study showed immunomodulatory properties of various classes of antibiotics on the expression of TLRs and sepsis-associated cytokines. Those effects were particularly found in immune cells that were activated by the sepsis mediator LPS and during a systemic inflammatory response after surgical trauma.

Innate immune cells, such as monocytes, play a central role in the pathogenesis of sepsis and are rapidly stimulated by various PAMPs, in particular LPS, originating from invading pathogens [2]. LPS triggers a

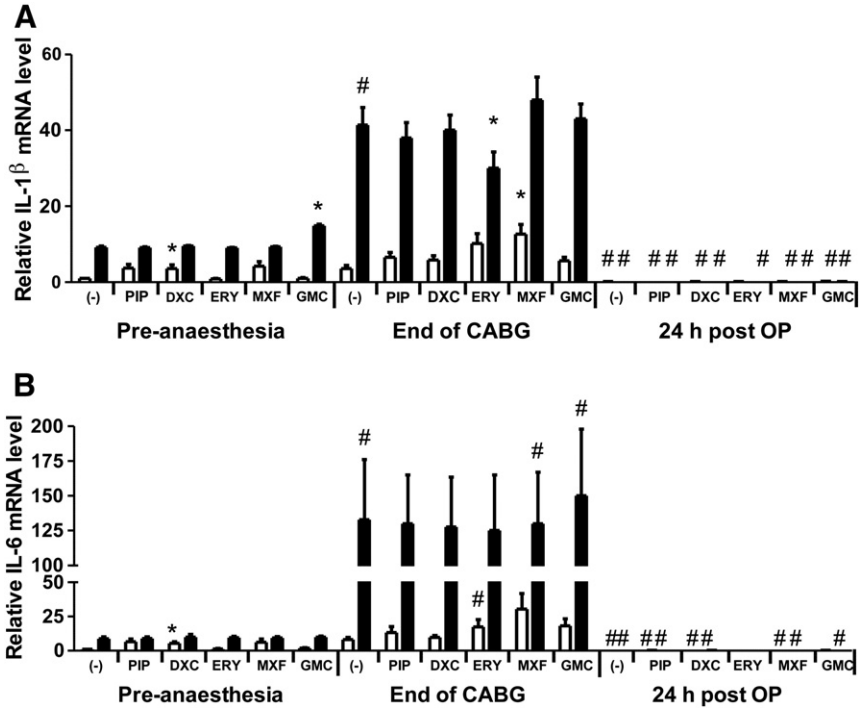

Fig. 3. Effect of antibiotics on cytokine expression in endotoxin-activated PBMCs before, 30 min and 24 h after CPB surgery. PBMCs from 8 patients undergoing CPB surgery were obtained before anesthesia, 30 min (End of CPB) and 24 h after operation (post op). Cells were cultured with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence (solid bar) or absence (open bar) of LPS for 24 h and mRNA expression of IL-1β (A) and IL-6 (B) was measured by quantitative Real-Time PCR. Data are expressed as mean ± SEM and normalized unstimulated cells, which were collected at pre-operative time point. # indicates $p < 0.05$ compared to pre-anesthesia; in intergroup comparisons at the various time points * indicated $p < 0.05$.

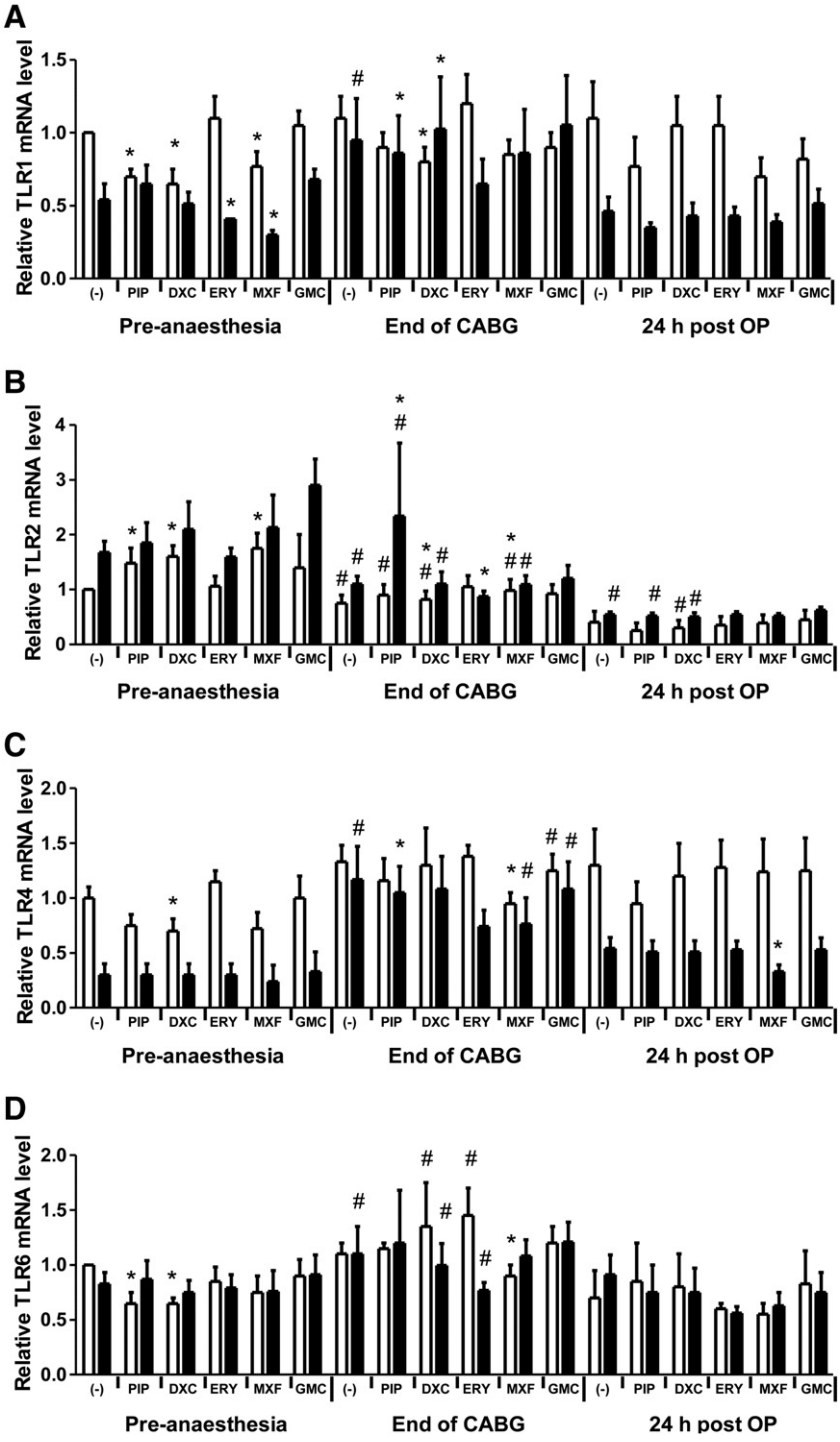

Fig. 4. Effect of antibiotics on TLR expression in endotoxin-activated PBMCs before, 30 min and 24 h after CPB surgery. PBMCs from 8 patients undergoing CPB surgery were obtained before anesthesia, 30 min (End of CPB) and 24 h after operation (post op). Cells were cultured with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence (solid bar) or absence (open bar) of LPS for 24 h and mRNA expression of TLR1 (A), TLR2 (B), TLR4 (C), and TLR6 (D) was measured by quantitative Real-Time PCR. Data are expressed as mean ± SEM and normalized to unstimulated cells, which were collected at pre-operative time point. # indicates $p < 0.05$ compared to pre-anesthesia; in intergroup comparisons at the various time points * indicated $p < 0.05$.

pro-inflammatory response, which is characterized by a production of inflammatory cytokines such as IL-1β and IL-6, *via* TLR4 [4]. Furthermore, LPS-activation changes the expression of TLRs in immune cells and leads to a differential response to subsequent PAMP exposure, which can result in an excessive pro-inflammatory response or immunocompromised status of a septic patient [22]. Consistent with previous reports, we found that tetracyclines (DXC), macrolides (ERY) and fluoroquinolones (MXF) had anti-inflammatory properties, which manifest by inhibiting the expression of inflammatory cytokines in response to LPS [13,23–25]. At the same time, beta-lactams (PIP) showed pro-inflammatory effects by increasing mRNA levels of IL-1β and IL-6 [26]. Furthermore, the aminoglycoside GMC also reduced the mRNA levels of IL-1β and IL-6 in monocytes which is in agreement with the effects of GMC on renal tubule cells [27]. We demonstrated regulatory properties for all tested classes of antibiotics on TLR expression to a various extent. These effects ranged from modest effects found in GMC-

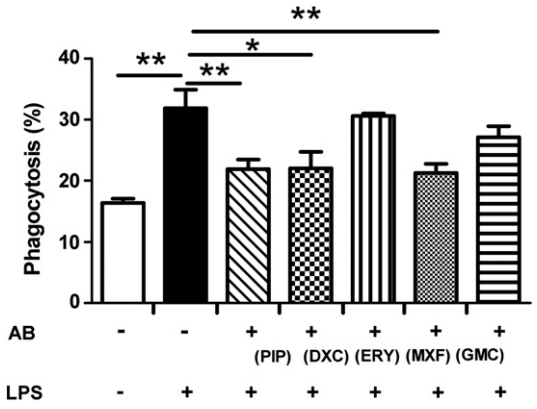

Fig. 5. Effect of antibiotics on phagocytosis of heat-inactivated *E. coli* by THP-1 cells. THP-1 cells were incubated with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence of LPS for 24 h and were incubated thereafter for 2 h with heat-killed rhodamine-conjugated *E. coli*. DNA staining for viability measurement was performed just prior to flow cytometry analysis with Hoechst 33342. Data show percentage of monocytes positive for phagocytosis determined by flow cytometry and are expressed as mean ± SEM; *, $p < 0.05$; **, $p < 0.01$.

incubated cells to a very significant modulation of all investigated TLR-subtypes by ERY. Interestingly, the observed effects of antibiotics on monocytes did not correspond to the results observed in PBMCs. For example, MXF increased the expression of IL-1β and IL-6 in PBMCs whereas the same genes were down-regulated by MXF in monocytes. This observation that antibiotics have different immunomodulatory effects on monocytes and heterogeneous leukocyte populations such as PBMCs, which consist of lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells, has been previously described. Bailly et al. demonstrated that the quinolone ciprofloxacin suppressed the pro-inflammatory cytokine response to endotoxin in monocytes while the treatment of a mixed cell population with ciprofloxacin increased their expression [28,29]. These results suggest that cellular interactions could modify monocytic TLR- and cytokine expression to a modulation by antibiotics.

Although the current data do not provide evidence for the immunomodulation by antibiotics at protein levels, several studies showed that the regulation of the investigated mRNA for TLRs and cytokines is correlated with the change in protein expression [30–32]. Thus, those findings suggest that antibiotics modulate the responsiveness of immune cells to LPS, which might help to understand why antibiotics such as the macrolide clarithromycin have beneficial immunomodulatory effects during sepsis [16].

The molecular mechanisms that underlie the immunomodulatory effects of antibiotics are so far poorly understood. To the authors' knowledge, no previous study has revealed the mechanisms causing the observed effects of PIP and GMC on the immune response. Yet, DXC has been identified to inhibit LPS-induced NO production by immune cells *in vitro* [23]. DXC may also be responsible for the reduction of IL-1β and IL-6 expression. In addition, tetracyclines such as DXC possess strong chelating abilities, which have been associated with immunomodulatory effects by inhibiting protein kinase C (PKC) [33,34]. Since it has been shown that PKC inhibitors reduce LPS-stimulated cytokine secretion [35], the chelating abilities of DXC might also reduce the IL-1β and IL-6 expression through PKC inhibition. The macrolid ERY was found to block the activation of transcription factors NF-κB and IRF3 [24,36], thereby reducing the production of both pro- and anti-inflammatory cytokines. The inhibition of signaling pathways by ERY depends on which PAMP the immune cell is activated by. While IRF3 is inhibited by ERY after LPS stimulation, no effect of ERY on NF-κB, which is critical for the expression of IL1-β and IL-6, has been detected [24]. Thus, the observed effect in the current study might be due to an unknown molecular mechanism. In contrast to ERY, MXF also inhibits the activation of NF-κB by inhibition of I-κB degradation after LPS stimulation [37]. Furthermore, MXF also blocks the activation of mitogen-activated protein kinases, which leads to a suppression of pro-inflammatory cytokines. These inhibitory effects of MXF on the TLR4/LPS-signaling pathway could explain its anti-inflammatory properties including the reduction of the pro-inflammatory cytokines IL-1β and IL-6.

For the first time, we found that the phagocytic activity of LPS-stimulated THP-1 cells toward *E. coli* was inhibited by PIP, DXC and MXF regardless of their effects on TLR expression. The inhibiting properties of MXF on the phagocytic activity have been previously described for the internalization of the gram-positive bacterium *Staphylococcus aureus* [38]. In contrast, another report showed that PIP enhances the phagocytic activity of *E. coli* by murine macrophages [39]. However, the comparison of studies on the phagocytic activity, which focus on murine cells is limited as murine and human immune cells differentially express phagocytic receptors after LPS stimulation [40]. A TLR-independent modulation of these receptors, such as scavenger receptors (SR) or CLRs, might explain inhibitory effects of PIP, DXC and MXF. This constellation was previously described for the effect of the immunomodulatory agent resveratrol on the phagocytosis of bacteria by monocytes [41]. There, resveratrol inhibited phagocytic activity of THP-1 cells by reducing SR and CTRs expression regardless of TLR-signaling. Furthermore, it has been demonstrated that different patterns of cytokines can also modulate the activity of human phagocytes [42]. Since the effect of antibiotics on cytokine production is presumably not limited to IL-1β and IL-6, the generation of a distinct pattern of cytokines by PIP, DXC and MXF could be another reason for the observed effects of antibiotics on phagocytosis.

The examination of direct immunomodulatory effects of antibiotics on immune cells from septic patients is limited due to preexisting combined antibiotic treatment. Given that sepsis is considered a systemic inflammatory response to a suspected or confirmed infection, we examined the effect of antibiotics on immune cells from patients after open-heart surgery. This population exhibits a frequent and robust inflammatory response, which is caused by surgical trauma, CPB, reperfusion injury and endotoxemia [43,44].

As previously reported, the peak of the pro-inflammatory cytokine expression occurred immediately after surgery [45,46]. Furthermore, we observed increased cytokine production after LPS stimulation in immune cells isolated at the end of the operation, whereas no cytokine response to LPS was detectable in cells collected 24 h post-operation. This indicates an early increase in endotoxin sensitivity and late endotoxin tolerance of the collected immune cells. Consistent with this, PBMCs obtained directly after surgery showed an up-regulation of TLR4 expression that is also observed in septic patients [30]. The immunomodulatory effect of antibiotics on PBMCs collected from patients before surgery was in accordance with the effects observed in PBMCs from healthy donors. We further found that all antibiotics (with the exception of GMC) showed to a various extent effects on the expression of cytokines and/or TLRs in immune cells that were *in vivo* activated though CPB surgery. Here, MXF showed the most immunomodulatory effects. Furthermore, the up-regulation of TLR2 expression and the reduction of TLR4 mRNA levels by MXF were also observed in all investigated cell populations including THP-1 monocytes.

The modulation of the immune system *via* TLRs is a promising strategy in the treatment of sepsis [7–9]. Current findings suggest that several classes of antibiotics, which are commonly used in the treatment of sepsis, regulate the immune system in sepsis-like conditions through modulation of the expression of TLRs and pro-inflammatory cytokines as well as the phagocytotic activity. We found that these effects varied in the antibiotics belonging to different classes and thus identified quinolones, tetracycline and macrolides as classes with the highest potential to modulate the TLR expression in septic conditions. Thus, these antibiotics should be considered for future immunomodulatory studies in sepsis.

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Conflict of interest statement

The authors have no conflicts of interest.

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