TLR2 and TLR4 activity in monocytes and macrophages after exposure to amoxicillin, ciprofloxacin, doxycycline and erythromycin

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Background: Antibiotics are used to treat bacterial infections but also impact immunity. This is usually attributed to antibiotic-induced dysbiosis of the microbiota, but antibiotics may have a direct effect on immune cells and immunity-associated receptors, such as Toll-like receptors (TLRs).

Objectives: To investigate whether antibiotics alter TLR2/1, TLR2/6 and TLR4 activity in immune cells.

Methods: We evaluated the effects of amoxicillin, ciprofloxacin, doxycycline and erythromycin on TLR2/1-, TLR2/6- and TLR4-induced NF-κB activation in THP1-XBlue™-MD2-CD14 cells. Furthermore, we studied TNF-α and IL-6 levels in THP-1-derived macrophages after exposure to these antibiotics and TLR ligands.

Results: Amoxicillin had no effect on any of the TLRs studied. However, ciprofloxacin reduced TLR2/1, TLR2/6 and TLR4 activity in THP1-XBlue™-MD2-CD14 cells and decreased TLR2/1-induced TNF-α and IL-6 in macrophages. Doxycycline reduced TLR2/6 and TLR4 activity in THP1-XBlue™-MD2-CD14 cells and TNF-α and IL-6 levels in response to TLR2/6 stimulation in macrophages. Erythromycin decreased TLR2/1 and TLR4 activity in THP1-XBlue™-MD2-CD14 cells without changes in TNF-α and IL-6 levels in macrophages. In addition, ciprofloxacin decreased the expression of TLR2 mRNA.

Conclusions: These results suggest that some antibiotics may attenuate TLR-dependent monocyte/macrophage responses and likely reduce bacterial clearance. The latter is particularly important in infections with AMR bacteria, where misprescribed antibiotics not only fail in control of AMR infections but might also weaken host defense mechanisms by limiting innate immune responses. Our data suggest that efforts should be made to prevent the deterioration of the immune response during and after antibiotic treatment.

Introduction

Antibiotics are drugs that limit the spread or directly kill microorganisms by targeting bacterial structures that are crucial for proper reproduction or vital bacterial cellular processes.1–3 Discovery of antibiotics is one of the most crucial findings of mankind. It allowed us to cure infections and prolong life expectancy.4 However, aberrant or too frequent use of antibiotics, as well as environmental contamination with antibiotics, led to the ever-growing problem of enhanced frequency of antimicrobial-resistant (AMR) organisms.5–8 Estimates suggest that by 2050, AMR microorganisms will kill as many people as cancer does today.9

Although antibiotics have high affinity for conserved bacterial structures, such as amoxicillin for cell wall-forming PBPs10 or ciprofloxacin for bacterial DNA topoisomerase,11 studies have shown that some antibiotics may impact eukaryotic cells in the host, inducing changes in the immune system, for example.12,13 These changes are usually attributed to antibiotic-induced dysbiosis of intestinal microbiota,14,15 resulting in a weaker immune response and higher susceptibility to infections.16,17 A lower immune response may give the pathogen a higher chance to spread, which is highly undesired. As antibiotics recognize conserved structures, such as PBPs,17 which are also recognized by, for example, Toll-like receptors (TLRs),18,19 we questioned whether antibiotics could influence immunity by impacting TLRs on immune cells. There is, however, minor knowledge on the potential direct effects of certain antibiotics on immune cells.

TLRs are a family of pattern-recognition receptors specialized in recognizing specific microbial- or damage-associated
molecular patterns (MAMPs and DAMPs, respectively). Among the 10 TLRs described in humans, TLR2, including TLR2/1 and TLR2/6 heterodimers, and TLR4 are crucial for nuclear factor kappa B (NF-κB) pathway activation, and for coordinating cytokine release, phagocytosis and infection clearance in response to Gram-positive and Gram-negative bacteria. Many antibiotics, such as ciprofloxacin, seem to directly impact the host cell metabolism, reducing antibiotic efficacy, and interact with human immune cells influencing phagocytic functions and cytokine production, as well as lymphocyte proliferation. We evaluated whether antibiotics influence immune cell function via TLRs. To this end, we investigated TLR2-1-, TLR2-6- and TLR4-induced NF-κB activation in the THP1 reporter cell line after exposure to amoxicillin, ciprofloxacin, doxycycline or erythromycin. Also, we studied the impact of these antibiotics on cytokine release of THP-1-derived macrophages.

**Materials and methods**

**Antibiotics and TLR2/1, TLR2/6 and TLR4 agonists**

All the antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amoxicillin (product number A8523), ciprofloxacin (product number 17850), doxycycline (product number D9891) and erythromycin (product number E6376) were dissolved in 0.1% acetic acid. Antibiotic stock solutions were freshly prepared for each experiment at concentrations of 1 and 0.1 mg/mL. The different antibiotics were chosen due to their broad spectrum of use or increasing resistance. The antibiotic doses used are in line with the different MICs reported by EUCAST for the tested antibiotics in limiting the growth of different pathogenic and opportunistic bacteria, and also based on in vitro studies of several antibiotics.

Specific TLR ligands, Pam3CSK4 (Pam3CysSerLys4), FSL-1 (Pam2CysGlyPHKSF) and LPS (LPS-EK, Ultrapure) were purchased from InvivoGen (Toulouse, France). Details about the TLR ligands used are shown in Table 1.

**Human THP-1 monocytic cell line**

The human THP-1 monocytic cell line was obtained from ATCC (Manassas, USA). THP-1 cells were cultured in antibiotic-free RPMI 1640 medium (Lonza, Bornem, Belgium) with 10% FBS (Sigma-Aldrich, MO, USA), 2 mM L-glutamine (Lonza, Belgium), 1 mM sodium pyruvate (Lonza, Belgium) and 0.05 mM 2-mercaptoethanol (Scharlau, Barcelona, Spain). Cells were cultured at 37°C and 5% CO2.

**THP-1 differentiation to macrophages**

Differentiation of THP-1 cells to macrophages was performed according to a previously described method with minor modifications. For details, see Supplementary Methods, available as Supplementary data at JAC Online.

**TLR reporter cell assays**

Antibiotic-dependent inhibition of TLR2/1-, TLR2/6- and TLR4-NF-κB/AP-1 signalling pathways was evaluated using THP1-XBlue™-MD2-CD14 expressing all TLRs and an NF-κB/AP-1-sensitive reporter gene for SEAP. TLR inhibition assays were performed in the presence of amoxicillin, ciprofloxacin, doxycycline or erythromycin (at 1 or 10 mg/L). THP1-XBlue™-MD2-CD14 were seeded in 96-well plates (~10000 cells/well) and were incubated with amoxicillin, ciprofloxacin, doxycycline or erythromycin (at 1 or 10 mg/L). After 24 h, specific TLR2/1, TLR2/6 or TLR4 ligands (Pam3CSK4, FSL-1 and LPS, respectively) were added to the cells previously treated with antibiotics, at a concentration of 10 ng/mL. After 24 h, cell supernatant was collected for SEAP activity quantification.

**SEAP activity quantification**

After TLR reporter assays, THP1-XBlue™-MD2-CD14 supernatant was mixed with QUANTI-Blue (InvivoGen) (1:10 ratio) and incubated (37°C and 5% CO2) for 1 h. NF-κB/AP-1 activation was quantified at 650 nm in a Versa Max ELISA plate reader (Molecular devices, Sunnyvale, CA, USA). Data were represented as fold-change compared with the positive control (Pam3CSK4, FSL-1 or LPS).

**Antibiotic and TLR ligand treatments in THP-1-derived macrophages**

Phorbol-12-myristate-13-acetate (PMA)-induced THP-1-derived macrophages were incubated (24 h) with 1 and 10 mg/L amoxicillin, ciprofloxacin, doxycycline and erythromycin in fresh culture medium. Acetic acid (0.001% final concentration) was used as vehicle. Incubation steps were performed at 37°C and 5% CO2. After 24 h of incubation with antibiotics, the cell culture media were refreshed and Pam3CSK4 (10 ng/mL), FSL-1 (10 ng/mL) or LPS (10 ng/mL) (ligands of TLR2/1, TLR2/6 and TLR4, respectively) were added in combination with amoxicillin, ciprofloxacin, doxycycline or erythromycin (at 1 or 10 mg/L). After 24 h, cell supernatants were collected for cytokine quantification using ELISA.

**ELISA**

Tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in the supernatant of THP-1-derived macrophages were determined after incubation with antibiotics and specific TLRs ligands (as described above). Sandwich ELISA kits (R&D Systems Inc., Minneapolis, USA) were used and performed according to the protocol delivered by the manufacturer.

**Table 1. Specific TLR ligands and dosage**

| Ligand name | Origin | Target | Concentration |
|-------------|--------|--------|---------------|
| Pam3CSK4 | Synthetic tri-acetylated lipoprotein | TLR2/1 | 10 ng/mL |
| FSL-1 | Synthetic di-acetylated lipoprotein | TLR2/6 | 10 ng/mL |
| LPS | Escherichia coli K12 | TLR4 | 10 ng/mL |
Table 2. RT–qPCR primer sequences

| Target gene                  | Symbol | Forward primer sequence (5′→3′) | Reverse primer sequence (5′→3′) |
|------------------------------|--------|---------------------------------|---------------------------------|
| β-2-Microglobulin            | B2M    | GGTTTCATCCATCCGACATT            | ACGGCGAGCATAACTCATCTT           |
| β-Actin                      | ACTB   | AGAAAATCTGGCACAACC              | TAGCAGCGCCCTGGATAGCAA           |
| Toll-like receptor 1         | TLR1   | CAGTGTCTGGTACACGATAGTT         | TTTCAAAAACCGTGTCTTAAAGAGA       |
| Toll-like receptor 2         | TLR2   | GCCTCTCAAAGGAAGATCC            | TCCGTGTGTTGACAGGTC             |
| Toll-like receptor 4         | TLR4   | AAACCGAAAGGTTGATTGTT            | CTGACGAGGTTCTCCAC             |
| Toll-like receptor 6         | TLR6   | GGAGGTGCCCTCATTATCCT            | TAACCTACCGCCTAGCTCA           |

Figure 1. Amoxicillin, ciprofloxacin, doxycycline and erythromycin effect on TLR2/1-induced NF-κB activation in THP1-XBlue™-MD2-CD14. SEAP activity determined by QUANTI-Blue in THP1-XBlue™-MD2-CD14 supernatant. Cells were incubated with (a) amoxicillin (AMX), (b) ciprofloxacin (CIP), (c) doxycycline (DOX) or (d) erythromycin (ERY). TLR2/1 activation was then induced by Pam3CSK4. Data are normalized to Pam3CSK4 response. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01 versus Pam3CSK4 (n = 7). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
RNA isolation and cDNA synthesis

After treatment with antibiotics and specific TLR ligands, THP-1-derived macrophages were washed two times in cold Dulbecco’s PBS (DPBS) (Gibco, Gaithersburg, MD, USA). TRIzol™ (Invitrogen) was then used for RNA isolation as described by the manufacturer. RNA concentration and purity were measured with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 200 ng of total RNA were used for reverse transcriptase cDNA synthesis.

The reverse transcription (RT) reaction was performed in total RNA (DNase I treated) following a standard protocol for Superscript III Reverse Transcriptase (Thermo Fisher Scientific Inc., Landsmeer, The Netherlands). For detailed explanation, see the Supplementary Methods.

Quantitative PCR (qPCR)

qPCR for TLR1, TLR2, TLR4 and TLR6 was performed with FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions (for detailed primer sequences, see Table 2). β-2-Microglobulin (B2M) and β-actin (ACTB) were used as housekeeping genes. Changes in expression were analysed using 2^(-ΔΔCt). For detailed methods, see the Supplementary Methods.

Statistical analysis

Statistical testing was performed using GraphPad Prism version 8.0 (San Diego, CA, USA). The Shapiro-Wilk normality test was used to evaluate normality of data distribution. Comparisons between two groups were performed by paired Student’s t-test for normally distributed data or...
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Wilcoxon test for non-parametrically distributed data. TLR ligand-dependent TNF-α production in THP-1-derived macrophages was tested with one-tailed Wilcoxon test versus the unstimulated cells. The TLR ligand-dependent IL-6 production was tested against a hypothetical value of zero using one-tailed Wilcoxon test versus the unstimulated cells. The differences between more than two groups were tested with the Friedman test. When ANOVA demonstrated a significant effect between variables, multiple comparisons with Dunn’s post hoc test were performed. \( P < 0.05 \) was considered as statistically significant; \( * P < 0.05, ** P < 0.01 \) versus LPS (n = 7). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Figure 3. Amoxicillin, ciprofloxacin, doxycycline and erythromycin effect on TLR4-induced NF-κB activation in THP1-XBlue™-MD2-CD14. SEAP activity determined by QUANTI-Blue in THP1-XBlue™-MD2-CD14 supernatant. Cells were incubated with (a) amoxicillin (AMX), (b) ciprofloxacin (CIP), (c) doxycycline (DOX) or (d) erythromycin (ERY). TLR4 activation was then induced by LPS. Data are normalized to LPS response. Data are shown as mean ± SEM. \( * P < 0.05, ** P < 0.01 \) versus LPS (n = 7). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Results
To determine whether antibiotics affect the regulation of NF-κB/AP-1 induced by TLRs in immune cells, we first tested the effect of antibiotics on the activation of TLR2/1, TLR2/6 or TLR4 in THP1-XBlue™-MD2-CD14 cells.

TLR2/1-dependent NF-κB/AP-1 activation attenuated by ciprofloxacin and erythromycin but not by amoxicillin or doxycycline
THP1-XBlue™-MD2-CD14 cells were incubated with 1 or 10 mg/L amoxicillin, ciprofloxacin, doxycycline and erythromycin for 24 h, then TLR2/1 was activated by Pam3CSK4. Ciprofloxacin and erythromycin reduced Pam3CSK4-induced TLR2/1 activation at higher 10 mg/L concentrations. We observed no effect of the lower concentration at 1 mg/L for either amoxicillin, ciprofloxacin, doxycycline or erythromycin. We found that
Figure 4. TLR2/1-, TLR2/6- and TLR4-induced production of TNF-α and IL-6 in THP-1-derived macrophages. (a) TNF-α and (b) IL-6 levels in THP-1-derived macrophage supernatants. Cells were incubated with Pam3CSK4, FSL-1 or LPS. Data are shown as mean ± SEM. u.d., under limit of detection. * P < 0.05, ** P < 0.01, *** P < 0.001 versus unstimulated cells. ^P < 0.05 versus FSL-1-stimulated cells (n = 6). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Figure 5. Antibiotics and TLR2/1-dependent TNF-α and IL-6 production in macrophages. TNF-α and IL-6 levels in THP-1-derived macrophage supernatants. Cells were treated with amoxicillin (AMX; a and e), ciprofloxacin (CIP; b and f), doxycycline (DOX; c and g) or erythromycin (ERY; d and h). TLR2/1 activation was then induced by Pam3CSK4. Data are normalized to Pam3CSK4 response. Data are shown as mean ± SEM. * P < 0.05, ** P < 0.01 versus Pam3CSK4 (n = 6). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
amoxicillin had no effect on TLR2/1-dependent NF-κB/AP-1 (Figure 1a). However, ciprofloxacin at a concentration of 10 mg/L reduced TLR2/1-dependent NF-κB/AP-1 activation by 14% (P < 0.01; Figure 1b). Doxycycline at a concentration of 10 mg/L also did not reduce TLR2/1-induced NF-κB/AP-1 activation (Figure 1c). However, erythromycin at 10 mg/L caused a small but significant (7%; P < 0.05) reduction in NF-κB/AP-1 (Figure 1d).

**TLR2/6-dependent NF-κB/AP-1 activation attenuated by ciprofloxacin and doxycycline but not by amoxicillin or erythromycin**

Similar to the previous experiment, we examined the effect of antibiotics on TLR2/6-dependent NF-κB/AP-1 activation induced by FSL-1 in THP1-XBlue™-MD2-CD14 cells.

FSL-1-induced TLR2/6 activation was reduced by ciprofloxacin (26%; P < 0.05; Figure 2b) and doxycycline (7%; P < 0.05; Figure 2c) only at the higher 10 mg/L concentration. We observed no effects of amoxicillin (Figure 2d) or erythromycin (Figure 2e) on FSL-1-induced NF-κB/AP-1 activation.

**TLR4-dependent NF-κB/AP-1 activation reduced by ciprofloxacin, doxycycline and erythromycin but not by amoxicillin**

We also evaluated the effect of antibiotics on TLR4-dependent NF-κB/AP-1 activation induced by LPS in THP1-XBlue™-MD2-CD14 cells.

Only amoxicillin had no effect on TLR4-dependent NF-κB/AP-1 activation (Figure 3a). None of the antibiotics had an effect at 1 mg/L. Three of the studied antibiotics attenuated TLR4-dependent NF-κB/AP-1 activation at 10 mg/L. Ciprofloxacin reduced NF-κB/AP-1 activation by 14% (P < 0.01; Figure 3b) and doxycycline by 34% (P < 0.01) (Figure 3c). Erythromycin had a minor but significant (9%; P < 0.05) reduction in NF-κB/AP-1 activity (Figure 3d).

**TLR2 and TLR4 ligands activated TNF-α and IL-6 production in macrophages**

Since antibiotics attenuated TLR2/1, TLR2/6 and TLR4 activity in THP1-XBlue™-MD2-CD14 reporter cells, we next wanted to test the effects of these antibiotics on cytokine production by TLR ligand-stimulated THP-1-derived macrophages. We first tested the effects of TLR stimulation on THP-1-derived macrophages as such.
We determined TNF-α and IL-6 production after stimulation of macrophages with the specific TLR ligands (Figure 4). Stimulation of TLR2/1 with Pam3CSK4 resulted in a 43-fold increase in TNF-α production compared with unstimulated cells (basal) (7659 versus 169 pg/mL) (P<0.05). Similarly, TLR2/6 stimulation with FSL-1 resulted in a 56-fold increase in TNF-α (9462 pg/mL) (P<0.05; Figure 4a). In addition, TLR4 stimulation with LPS increased TNF-α levels to 1038 pg/mL, a 6-fold increase compared with unstimulated cells (P<0.05; Figure 4a). Although the average TNF-α concentration was different after TLR2/1, TLR2/6, and TLR4 stimulation compared with unstimulated cells, no significant differences were found when comparing TNF-α production induced by the three different agonists. The results were different for IL-6 (Figure 4b). This cytokine was not detectable in unstimulated cells. Stimulation with TLR2/1 ligand increased IL-6 to 1038 pg/mL, a 6-fold increase compared with unstimulated cells (P<0.05; Figure 4b). In cells stimulated with FSL-1, a specific TLR2/6 ligand, we found a significant increase in IL-6 to 318.54 pg/mL (P<0.001; Figure 4b). Similarly, stimulation of TLR4 with LPS increased IL-6 levels to an average concentration of 34.2 pg/mL (P<0.05; Figure 4b). Moreover, no significant differences in IL-6 after TLR2/1 and TLR2/6 stimulation were found. However, we found that FSL-1 elicited higher IL-6 release compared with TLR4 stimulation with LPS (P<0.05; Figure 4b).

Ciprofloxacin and doxycycline but not amoxicillin or erythromycin lowered TLR2/1-dependent cytokine production in macrophages. THP-1-derived macrophages were incubated with the specific TLR2/1 ligand Pam3CSK4 and 1 or 10 mg/L amoxicillin, ciprofloxacin, doxycycline or erythromycin for 24 h, then TNF-α and IL-6 were measured in the supernatant of the cultures.

The effects were dependent on the antibiotics and antibiotic concentration, as amoxicillin, ciprofloxacin, doxycycline or erythromycin had no effect on TNF-α or IL-6 production by macrophages at 1 mg/L, but strong effects were observed with 10 mg/L ciprofloxacin and doxycycline. Similar to in THP1-XBlue™-MD2-CD14, amoxicillin had no effect on TLR2/1-dependent TNF-α (Figure 5a) or IL-6 levels in macrophages (Figure 5e). Ciprofloxacin at a concentration of 10 mg/L reduced TLR2/1-dependent TNF-α release by 21% (P<0.05; Figure 5b) and IL-6 release by 27% (P<0.01; Figure 5f). Doxycycline also reduced cytokine release. Doxycycline at a concentration of 10 mg/L reduced TLR2/1-induced TNF-α release by 31% (P<0.05) and IL-6 by 46% (P<0.01) (Figure 5c and 5g, respectively). In contrast, erythromycin had no regulatory...
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effects, as both TNF-α (Figure 5d) and IL-6 levels (Figure 5h) remained unchanged in macrophage supernatants.

**Doxycycline but not amoxicillin, ciprofloxacin or erythromycin reduced TLR2/6-dependent cytokine production**

Next, the effects of amoxicillin, ciprofloxacin, doxycycline and erythromycin on TLR2/6-dependent release of TNF-α and IL-6 were determined. Again, the effects were antibiotic- and concentration-dependent, as no changes were observed at the lower concentration of 1 mg/L, but profound changes were observed after 10 mg/L doxycycline treatment. The effects on TLR2/6 were also antibiotic-dependent. We found that amoxicillin did not affect TLR2/6-dependent release of TNF-α (Figure 6a) or IL-6 (Figure 6e). Similarly, we did not detect any effect of ciprofloxacin on TNF-α (Figure 6b) or IL-6 (Figure 6f) levels induced by TLR2/6 stimulation. However, we found that doxycycline at a concentration of 10 mg/L significantly prevented TNF-α (P < 0.01) and IL-6 (P < 0.01) production. The production of TNF-α (Figure 6c) and IL-6 (Figure 6g) was on average 40% and 54% lower, respectively, in cells treated with 10 mg/L doxycycline. Similar to amoxicillin, no effect of ciprofloxacin and erythromycin on TLR2/6-induced release of TNF-α (Figure 6d) or IL-6 (Figure 6h) was seen in THP-1-derived macrophages.

**Amoxicillin, ciprofloxacin, doxycycline and erythromycin did not significantly impact the TLR4-dependent cytokine production in macrophages**

The effect of amoxicillin, ciprofloxacin, doxycycline and erythromycin was studied on TLR4-dependent release of TNF-α (Figure 7a–d) and IL-6 (Figure 7e–h). In contrast to TLR2 dimers, we found here that none of the antibiotics tested had a significant effect on the regulation of either cytokine at either of the concentrations tested (1 or 10 mg/L).

**TLR expression changes in macrophages might explain the immunomodulatory effects of antibiotics**

Since the above data show that antibiotics have an effect on TLR2- and TLR4-dependent NF-κB/AP-1 activation and cytokine release in response to TLR2 heterodimers, we investigated whether the expression of TLR1, TLR2, TLR4 or TLR6 in macrophages is affected by antibiotics. To this end, we examined the mRNA levels of these TLRs in THP-1-derived macrophages treated (24 h) with amoxicillin, ciprofloxacin, doxycycline or erythromycin at concentrations of 1 and 10 mg/L. We found that TLR1 mRNA levels were not significantly altered by treatment with amoxicillin, ciprofloxacin, doxycycline or erythromycin at

![Figure 8. TLR mRNA levels in THP-1-derived macrophages under antibiotic conditions. (a) TLR1, (b) TLR2, (c) TLR4 and (d) TLR6 mRNA level in THP-1-derived macrophages after 24 h incubation with vehicle (Veh), amoxicillin (AMX), ciprofloxacin (CIP), doxycycline (DOX) or erythromycin (ERY). Data are normalized to vehicle. Data are shown as mean ± SEM. *P < 0.05 versus vehicle (n = 5). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.](https://academic.oup.com/jac/article/77/11/2972/6650899)
Antibiotic effect in TLR2 and TLR4

concentrations of 1 and 10 mg/L (Figure 8a). TLR2 mRNA levels were also not significantly altered by treatment with 1 or 10 mg/L amoxicillin, doxycycline or erythromycin (Figure 8b), but ciprofloxacin at a concentration of 10 mg/L reduced TLR2 mRNA levels by 53% compared with cells treated with vehicle \((P < 0.05)\) (Figure 8b). Similarly, TLR4 (Figure 8c) and TLR6 (Figure 8d) mRNA was not affected by amoxicillin, ciprofloxacin, doxycycline or erythromycin at either dose tested.

**Discussion**

Here we show that some antibiotics may reduce NF-κB/AP-1 activation by directly interacting and attenuating extracellular TLR2/1, TLR2/6 and TLR4. By that means, antibiotics may contribute to lowering the innate immune response and the fight against infections in users of antibiotics.\(^\text{14,31}\) This was corroborated by the observation that some of the antibiotics tested, i.e. ciprofloxacin and doxycycline, reduced the cytokine production in macrophages in response to TLR1 dimers. The effects were, however, very antibiotic-dependent as amoxicillin had no effect on the studied TLR ligand-induced cellular responses. This was different with ciprofloxacin, which reduced TLR2/1, TLR2/6 and TLR4 activity in THP-1-XBlue\(^\text{™}-MD2-CD14 cells and attenuated the TLR2/1-induced TNF-α and IL-6 levels in THP-1-derived macrophages. Doxycycline down-regulated TLR2/6 and TLR4 activity in THP-1-XBlue\(^\text{™}-MD2-CD14 cells, as well as the TNF-α and IL-6 levels in response to TLR2/6 stimulation in macrophages. Interestingly, erythromycin down-regulated TLR2/1 and TLR4 activity in THP-1-XBlue\(^\text{™}-MD2-CD14 but did not reduce the levels of TNF-α and IL-6 in macrophages, in contrast to the other antibiotics that impacted TLR signalling. Hence, we also evaluated the impact of antibiotics on regulation of TLR expression. This study suggests that down-regulation of TLR2 mRNA induced by ciprofloxacin may explain the effect of this antibiotic on the lower cytokine release by macrophages.

The foregoing findings suggest that some but not all antibiotics can attenuate immune cell responses by reducing TLR2 or TLR4 signalling. Furthermore, different immune cell types may be differently affected by antibiotics. For instance, our findings show that the studied antibiotics affect THP-1 cells (monocytes) or THP-1-derived macrophages differently. This is of particular importance during infections caused by AMR bacteria, in which incorrectly prescribed antibiotics will be ineffective in treating the AMR infections but will also weaken the host defence mechanisms by limiting the innate immune response. This likely increases the consequences of infections. This could explain why during the use of ciprofloxacin, alterations in host metabolites are produced and decreased antibiotic efficacy is observed.\(^\text{13}\) It might even contribute to enhanced chances of opportunistic pathogen infections, for instance with *Clostridioides difficile*, a well-known side-effect of antibiotics such as ciprofloxacin.\(^\text{32}\)

The potential attenuation of TLR2 and TLR4 signalling by ciprofloxacin, doxycycline and erythromycin needs some further consideration as these TLRs are the major receptors for products from Gram-positive and Gram-negative bacteria.\(^\text{33}\) Notably, these...
boosting the host’s defence mechanisms and TLRs has been proposed as a way of reducing AMR infections. Human studies have shown that lowered TLR signalling increases the risk of infection and infection-associated mortality. For instance, Kim et al. have shown that down-regulation of TLR2 and IL-6-production implies a higher mortality risk by infection with Staphylococcus aureus bacteraemia. Based on our findings, antibiotics may be detrimental to the outcome of infections, especially those caused by AMR bacteria, as ciprofloxacin, doxycycline and erythromycin lowered TLR2/1, TLR2/6 or TLR4 signalling, and ciprofloxacin and doxycycline down-regulated TNF-α and IL-6 in macrophages. Therefore, it cannot be excluded that these antibiotics, while restricting pathogenic growth and spreading, at the same time attenuate the host’s ability to remove pathogens by its own immune response.

The studied TLRs are also crucial for differentiation of circulating monocytes to macrophages and dendritic cells. By interfering with the TLR signalling, ciprofloxacin, doxycycline and erythromycin may contribute to lowering of bacterial phagocytosis and monocyte-to-macrophage differentiation. Moreover, IL-6, a well-recognized orchestrator for developing adaptive immune responses, was lowered by ciprofloxacin and doxycycline, suggesting that these antibiotics might have a profound impact on altering not only innate but also adaptive immune responses. This suggestion is supported by several animal studies that have shown that TLR2 or TLR4 down-regulation increases the risk of suffering infections or more severe consequences.

Our findings regarding the immunomodulatory effect of ciprofloxacin and doxycycline are corroborated by the findings of others. Bode et al. showed that antibiotics such as doxycycline, moxifloxacin (same family as ciprofloxacin) and erythromycin altered the function of TLRs in a cell- and antibiotic-dependent manner. Interestingly, in our study, ciprofloxacin had no impact on TLR4-activated macrophages, while ciprofloxacin had a strong effect on TLR4 signalling in the reporter THP-1 cell line. Previous research shows that ciprofloxacin reduces LPS-induced proinflammatory cytokines in THP-1 macrophages and attenuates TLR4/NF-κB-induced inflammatory responses in rat microglia. However, these studies used higher concentrations (i.e. ≥50 mg/L) of ciprofloxacin than us. This might imply that ciprofloxacin and probably other antibiotics at different doses induce different cellular effects in monocytes/macrophages and attenuate macrophage function.

In summary, our data indicate that antibiotics can exert direct immunomodulatory effects in monocytes and macrophages, but effects are very antibiotic-dependent. Though amoxicillin had no effects, ciprofloxacin, doxycycline and erythromycin may attenuate innate immunity-associated receptors in monocytes or macrophages, such as TLR2/1, TLR2/6 and TLR4. In this manner, antibiotics might weaken the host defence mechanisms by limiting the innate immune response and likely increase the risk and consequences of infections (Figure 9). This incentivizes us to further study other antibiotics and other potential mechanisms of regulation that can be a target for antibiotics. Further research is needed to find means to prevent or accelerate the recovery of TLR function during and after antibiotic treatment to boost the immune system and the physiological defences, and reduce antibiotic usage.

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Transparency declarations
None to declare.

Supplementary data
Supplementary Methods are available as Supplementary data at JAC Online.

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