The glycopeptide vancomycin does not enhance toll-like receptor 2 (TLR2) activation by *Streptococcus pneumoniae*

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**Objectives:** The exposure of *Streptococcus pneumoniae* to cell-wall-active antibiotics in vivo and in vitro results in the release of bacterial components that can induce proinflammatory activation of human cells via toll-like receptor 2 (TLR2). The aim of this study was to compare the activation of human TLR2 pathways after exposure of *S. pneumoniae* to faropenem, cefotaxime and vancomycin.

**Materials and methods:** *Streptococcus pneumoniae* D39 was exposed to cefotaxime, faropenem or vancomycin for 6 h during lag or early log phase growth. IL-8 promoter activity of HeLa cells was measured using a dual luciferase reporter plasmid system. HeLa cells were transfected with an expression vector containing TLR2/CD14, or empty vector/CD14 and IL-8 promoter activity was measured using luminescence. Cells were stimulated with antibiotic-treated bacteria, untreated bacteria or medium-only controls.

**Results:** Lag phase *S. pneumoniae* treated at sub-MIC (1/8 MIC) cefotaxime or faropenem induced 11-fold and 8-fold increases, respectively, in TLR2-mediated IL-8 promoter activity when compared with untreated bacteria. Early log MIC cefotaxime or faropenem-treated bacteria also enhanced TLR2 activation by 3-fold and 4-fold, respectively, when compared with untreated bacteria. Vancomycin treatment had no effect on TLR2 induction at any growth stage or MIC ratio tested.

**Conclusions:** β-Lactam antibiotics induce surface changes and release of cell wall structures from bacteria that are proinflammatory via TLR2, but the glycopeptide vancomycin does not.

**Keywords:** faropenem, cefotaxime, vancomycin, inflammation

**Introduction**

Community-acquired pneumonia and meningitis caused by *Streptococcus pneumoniae* is treated with single or combined antibiotic therapy. Common agents include β-lactam antibiotics (e.g. penicillin, cefotaxime), vancomycin, macrolides (e.g. erythromycin) or a fluoroquinolone such as levofloxacin or moxifloxacin.1

*S. pneumoniae* and its cell wall components are recognized by cells of the human innate immune system via a number of pattern recognition receptors including toll-like receptor 2 (TLR2).2,3 Penicillin treatment of *S. pneumoniae* is associated with enhanced activation of TLR2-mediated IL-8 promoter activity by transfected HeLa cells.3 Lag phase pneumococci release more TLR2-activating molecules during exposure to sub-MIC penicillin. In contrast early log phase pneumococci release more TLR2-activating molecules when exposed to the MIC.3 Antibiotics that do not interact with the cell wall (e.g. erythromycin, moxifloxacin) do not enhance TLR2 activation by pneumococci in this system.5 These events have clinical significance. In animal models of meningitis, treatment with β-lactam antibiotics is associated with increased leucocytosis into the subarachnoid space and exacerbation of brain oedema. This is due to enhanced release of proinflammatory cell wall structures such as lipoteichoic acid (LTA) and peptidoglycan (PGN), which are known to be activators of the TLR2 signal cascade.3,5 Altered bacterial morphology due to β-lactam antibiotics is also inflammatory.6,7 The aims of the current study were to test the effect of three important cell wall synthesis inhibitors, cefotaxime, faropenem and vancomycin, on TLR2 induction by *S. pneumoniae*.

**Materials and methods**

**Bacteria and antibiotics**

D39, a type 2 pneumococcus, was used in these experiments. Mid-log stocks of bacteria were prepared by culture in brain–heart
infusion (BHI) broth supplemented with 10% heat-inactivated fetal calf serum (HIFCS) in 5% CO2 at 36°C.

Cefotaxime and vancomycin were purchased from Sigma-Aldrich. Faropenem was a gift from Bayer Corp., Germany. Cefotaxime and faropenem were used as representatives of antibiotics that inhibit the penicillin binding proteins (PBPs). Vancomycin was used as a representative of a non-PBP cell-wall-inhibiting antibiotic. The MIC of each antibiotic was determined using the macrobroth method according to NCCLS guidelines, 1997. 

Lag phase and early log phase bacteria were exposed to one-eighth MIC or MIC of antibiotic as described previously. Briefly, freshly inoculated bacterial cultures at lag phase growth (5 × 108 cfu/mL, optical density at 620 nm, 0.0044), or early log growth (OD 620 nm, 0.099, adjusted to 5 × 108 cfu/mL) were treated with antibiotic for 6 h before inoculation onto transfected HeLa cells. Bacterial counts were checked spectrophotometrically and by viable count analysis. Controls included untreated bacteria, and broth plus antibiotic.

**Results**

**Cefotaxime and faropenem enhance TLR2 activation by *S. pneumoniae***

The known TLR2 agonist, PGN, at a concentration of 1 μg/mL was shown to induce a strong response in this system (relative IL-8 luciferase promoter activity, 130 ± 15, data not shown). Treatment of lag phase *S. pneumoniae* in cefotaxime at 1/8 MIC elicited an 11-fold increase in TLR2-mediated activity when compared with untreated controls (Figure 1a) (P < 0.005, paired t test). Early log treatment with MIC cefotaxime enhanced TLR2 proinflammatory activity 3-fold when compared with untreated controls (Figure 1b) (P < 0.005, paired t test).

Faropenem exhibited TLR2-inducing effects that were very similar to those noted for cefotaxime. Treatment of *S. pneumoniae* in 1/8 MIC faropenem induced an 8-fold increase of TLR2 activity when compared with untreated controls (Figure 1a) (P < 0.005, paired t test). Treatment of *S. pneumoniae* with faropenem at the MIC during early log growth induced a 4-fold increase in TLR2-mediated proinflammatory activity when compared with untreated cells (Figure 1b) (P < 0.005, paired t test).

**Vancomycin does not affect *S. pneumoniae* TLR2 inducibility at any MIC ratio or bacterial growth phase**

Vancomycin at 1/8 MIC or the MIC did not induce TLR2 proinflammatory changes by either lag or early log phase

**Statistics**

All data were tested for normal distribution using the Anderson–Darling normality test. For the comparison of treated versus untreated normally distributed data were analysed by the t test.

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**Figure 1.** TLR2-mediated IL-8 promoter activity in response to *S. pneumoniae* treated with various cell-wall-active antibiotics for 6 h during (a) lag or (b) early log growth. Untreated, hatched bars; 1/8 MIC, black bars; MIC, grey bars; negative, white bars. Values are for firefly luciferase normalized with Renilla luciferase and vector control. Negative control is BHI and 10% HIFCS plus antibiotic at the MIC. Data are expressed as mean ± SEM for four separate experiments. The paired t test was used to compare values for antibiotic-treated versus untreated live bacteria, **P < 0.005** versus untreated controls.
S. pneumoniae when compared with untreated bacteria (Figure 1a and b).

Discussion

These data have shown that exposure of S. pneumoniae to the glycopeptide vancomycin does not result in release of bacterial components that have the potential to activate human cells via TLR2. In contrast, cefotaxime and faropenem, β-lactam antibiotics that kill bacteria by non-lytic inhibition of the PBPs, do release TLR2-activating moieties. Inhibition of the PBPs produces a cell that is osmotically unstable and morphologically abnormal. The glycopeptide vancomycin interacts with D-alanyl-D-alanine peptide intermediates, but this antibiotic differs from the β-lactams as the PBPs are not involved.

In rabbit models of pneumococcal pneumonia and meningitis, β-lactam therapy is associated with increased leucocytosis, brain oedema and mortality. Such adverse effects correlate with increased release of bacterial cell wall structures such as LTA and PGN. PGN and LTA signal proinflammatory activity via TLR2 and this is associated with an increase in the production of proinflammatory cytokines such as TNF-α. Increases in free LTA and PGN occur during treatment with the lytic penicillins and this is due to interaction with pneumococcal PBP2b and activation of autolysin enzymes. Cefotaxime and faropenem treatments may impose major morphological changes in the pneumococcus that optimize the interaction of surface LTA and/or PGN with TLR2.

These data also confirm that the antibiotic MIC ratio and the target bacterial growth phase have a major influence on TLR2 activation after antibiotic exposure. Sub-MIC (1/8 MIC) cefotaxime or faropenem enhanced TLR2 activation only when applied to lag phase bacteria, but MIC concentrations of these antibiotics enhanced TLR2 activation when applied to early log phase cells. In the clinical context sub-MIC or near-MIC concentrations of antibiotic may occur during the dosing interval. The growth phase of bacteria during the course of natural bacterial infection is largely unknown. These data suggest that transition of bacteria to different points in the growth cycle (lag to early log in these experiments) alters the cell wall dehiscence caused by β-lactam antibiotics and the subsequent proinflammatory effects. This relationship with the growth cycle may be related to differences in PBP expression during the different phases.

Vancomycin is routinely used in the treatment of pneumococcal meningitis in many countries. In contrast to β-lactam antibiotics, vancomycin does not enhance the bacterial-triggered release of proinflammatory cytokines from ex vivo human monocytes. β-Lactam antibiotics are associated with increased meningeval inflammation in animal models of meningitis but limited data exist as to the specific inflammatory effect of vancomycin on the course of bacterial meningitis. The data presented here suggest that vancomycin is unlikely to enhance inflammatory effects during treatment of pneumococcal bacterial meningitis, as TLR2 is the major pattern recognition receptor for pneumococcal cell wall components. The key factors promoting enhanced inflammation during β-lactam therapy are the release of bacterial muramyl dipeptides and the generation of abnormal morphologies; vancomycin does not induce these changes. In summary, our data show that vancomycin does not release bacterial components from the cell wall that are active via TLR2.

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References