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Investigating the immunomodulatory activities of omadacycline

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Background: Apart from their antimicrobial activities, some antibiotics have immunomodulatory effects on host cells, particularly monocytes. Because hyperactivation of the pro-inflammatory cytokine response contributes to acute lung injury in patients with bacterial pneumonia and other lung diseases, antimicrobial agents with immunomodulatory activity can reduce cytokine-mediated tissue injury and improve outcomes.

Objectives: Omadacycline has been recently FDA-approved for community-acquired bacterial pneumonia and acute bacterial skin and skin-structure infections. The present study investigated omadacycline's ability to modulate LPS-induced production of pro-inflammatory cytokines (TNF-α, IL-1β), acute-phase reactants (IL-6) and anti-inflammatory cytokines (IL-4, IL-10) by human monocytes *in vitro*.

Methods: Isolated human monocytes from healthy consenting adults were cultured in RPMI with 1% pooled human serum. Cells were pre-exposed to omadacycline (0.5–64 μg/mL), minocycline (25, 50 or 25 μg/mL) or azithromycin (20, 40 or 80 μg/mL) for 2 h, followed by stimulation with *Escherichia coli* LPS for 24 h. Cytokines elaborated in the culture supernatant were quantitated by multiplex immunoassay.

Results: Omadacycline dose-dependently suppressed LPS-induced production of all cytokines tested. Only high-dose minocycline (100 μg/mL) modestly suppressed TNF-α whereas minocycline significantly increased LPS-induced IL-1β production. Lower concentrations of minocycline were also stimulatory for IFN-γ, IL-6 and IL-4. Except for suppression of IL-6, azithromycin was largely without effect.

Conclusions: Omadacycline has unique and broad immunomodulatory properties. Such activity supports its use in settings where hyperactivation of the immune response contributes to tissue injury and poor outcomes, especially at sites where pro-inflammatory M-type 1 macrophages dominate the cellular immune response.

Introduction

Omadacycline is a new aminomethylcycline derivative of tetracycline and has been FDA-approved for community-acquired bacterial pneumonia and acute bacterial skin and skin-structure infections. It is a potent bacterial protein synthesis inhibitor antibiotic with broad-spectrum activity against Gram-positive and some Gram-negative bacteria, including MDR organisms as well as some anaerobic and atypical bacteria.[1,2]

In bacterial pneumonia, a robust inflammatory response is required to kill bacteria and resolve infection; however, excessive and prolonged lung inflammation leads to alveolar epithelial cell injury, pulmonary haemorrhage and death. Thus, modulation of the host cytokine-driven immune response is important to overcome infection while avoiding acute lung injury.

Existing evidence suggests that some pharmacological agents including tetracycline-derived antibiotics, can attenuate cytokine-induced cytotoxicity *in vitro*.[3,4] Similarly, other agents can reduce cytokine and chemokine levels in broncho-alveolar lavage (BAL) fluid in experimental animals and improve lung function without a concomitant reduction in the number of viable organisms in the lung.[5] In particular, high levels of the pleiotropic cytokine TNF-α accumulate rapidly in the lung after acute pulmonary injury in humans and experimental animals, and is considered to initiate early disease pathology.[6]

Thus, as a tetracycline derivative, omadacycline has the potential to mitigate acute lung injury such as in bacterial pneumonia or acute respiratory distress syndrome (ARDS) by modulating monocyte/macrophage production of pro-inflammatory cytokines. Similarly, modulation of TNF-α production could improve

the outcome of more chronic inflammatory pulmonary diseases such as asthma, bronchitis and COPD.[6]

The present study investigated the ability of omadacycline to modulate LPS-induced production of pro-inflammatory cytokines (TNF-α, IL-1β), acute-phase reactants (IL-6) and anti-inflammatory cytokines (IL-4, IL-10) by isolated human monocytes *in vitro*.

Materials and methods

Ethics

This study was approved by Idaho State University's Institutional Review Board (protocol # IRB-FY2021-69) and signed informed consent was obtained from each participant.

Monocyte isolation and culture

The effects of omadacycline on LPS-induced cytokine production by monocytes *in vitro* followed our previously published methods.[7] Three different healthy consenting adult volunteers (one female, two male) were recruited for four independent experiments. PBMCs were isolated from 30 mL of heparinized whole blood by density gradient centrifugation and resuspended at $1–5\times10^6$ cells/mL in RPMI-1640 with phenol red and 2 mM L-glutamine (RPMI; Lonza) but without any antimicrobial agents that are typically used in cell culture (e.g. penicillin, streptomycin or amphotericin).

To ensure good distribution of plated cells, 1 mL of RPMI was added to empty wells of 6-well (35 mm) tissue culture plates (Corning) and the plates equilibrated for 15 min at 37°C in a 5% CO_2, humidity-controlled incubator. Next, prepared PBMCs (1 mL) were added to duplicate wells ($1–5\times10^6$ cells/well) and incubated for 2 h. Wells were washed twice with 1 mL room-temperature Dulbecco's PBS (DPBS) to remove non-adherent cells (largely lymphocytes). The remaining adherent cells (monocytes) were then overlaid with 1.8 mL of RPMI supplemented with 1% pooled human serum (MP Biomedicals) to yield complete RPMI (cRPMI). This method of monocyte preparation has been described previously by us[7] and routinely results in a monocyte population that is >95% pure with >98% viability.

Antibiotics

Stock antibiotics and their suppliers were as follows: (a) omadacycline tosylate (Paratek Pharma) was prepared in cell culture-grade sterile water and adjusted for purity to yield a 64 mg/mL stock; (b) minocycline HCl (Sigma M9511) was prepared as a 25 mg/mL stock solution in cell culture-grade water using gentle heating as directed by the manufacturer; and (c) azithromycin dihydrate (Sigma PZ0007) was prepared as a 20 mg/mL stock solution in anhydrous cell culture grade DMSO. The highest final DMSO concentration in azithromycin-treated wells was 0.4%; pilot studies showed this concentration of DMSO did not influence cytokine production in this system (not shown). Stock antibiotic solutions were aliquoted and frozen at −7°C. Aliquots were freshly defrosted as needed and not refrozen.

Antibiotic treatment and monocyte stimulation

Antibiotics were diluted in RPMI to 10× the final desired concentrations. Prepared 10× antibiotics (0.2 mL) were added to wells of cells containing 1.8 mL of cRPMI to yield the following final concentrations: omadacycline (64–0.5 μg/mL in 2-fold decreasing concentrations); minocycline (100, 50 or 25 μg/mL); and azithromycin (80, 40 or 20 μg/mL). The concentrations of minocycline and azithromycin were based on studies of antibiotic-mediated suppression of LPS-induced cytokine production in human monocyte cell lines (THP-1)[8] and on our studies of azithromycin-mediated suppression of LPS-induced TNF-α production in human mononuclear cells.[9] The concentration range of omadacycline was recommended by the supplier. An equal volume of RPMI alone served as the negative antibiotic treatment control.

After 2 h of antibiotic treatment, cells were stimulated with control standard LPS (*Escherichia coli* O113:H10, Associates of Cape Cod, Woods Hole, MA, USA; 125 ng/10^6 monocytes) or DPBS (negative control). Supernatants were collected at 24 h after LPS treatment, centrifuged to remove cellular debris, and immediately stored at −7°C until assayed for cytokines of interest.

Cells in each well were observed by phase contrast light microscopy before addition of any treatment, again after 2 h of antibiotic pretreatment and lastly after 24 h of LPS exposure to assess cell loss and morphological alterations indicative of cytotoxicity (e.g. blebbing, cell shrinkage). After harvest of culture supernatants, cells in selected wells were stained with trypan blue to assess viability.[10]

Cytokine analysis

Pro-inflammatory cytokines TNF-α and IL-1β, the acute-phase reactant IL-6, the anti-inflammatory mediators IL-4 and IL-10, and the immune modulatory cytokine IFN-γ were measured in duplicate by multiplex bead-based immunoassay (Illumina MAGPIX High Performance Luminex Assay, R&D Systems) following the manufacturer's instructions. Cytokine levels in experimental samples were determined from the standard curves using the instrument's software and logistic 5P-weighted regression analysis.

Statistical analyses

Four independent experiments were performed using monocytes isolated from three different healthy adult donors (one female; two male). Experiments were performed in duplicate; samples from each well were run in duplicate on the multiplex immunoassay. Data are given as the means ± SEM of duplicate experimental samples assayed in duplicate. For each cytokine, a one-way analysis of variance model was fitted within a repeated measures framework separately for each antibiotic. Each dosage was compared with the positive control with a Dunnett's test and considered significantly different from the positive control with adjusted P value <0.05. For cytokines measured after omadacycline treatment, the natural logs of responses were used to stabilize the variance (this was not necessary for minocycline or azithromycin). Analyses were completed using SAS version 9.4 and plots using R version 4.1.0 and Tidyverse (1.3.1).

Results

Compared with untreated control wells, no marked differences in cell numbers/well, viability or morphology were observed after 24 h of treatment with LPS alone, antibiotics alone, or with LPS plus antibiotics at any concentration tested (not shown).

As expected, LPS strongly stimulated production of the classical monocyte-derived pro-inflammatory cytokines TNF-α and IL-1β, as well as the acute-phase reactant IL-6 [Figure 1(a), top row], though individual variations in the quantities of cytokines produced were observed between donors. LPS stimulated only low levels (<10 pg/mL) of IFN-γ and the anti-inflammatory cytokines IL-4 and IL-10 [Figure 1(a) bottom row].

High concentrations of omadacycline (≥32 μg/mL) significantly inhibited these responses [Figure 1(a), top row]. Such inhibition could not be attributed to overt cytotoxicity since no changes in cell numbers, viability or morphology were observed at these concentrations over the 24 h experimental period (not shown). In three of four experiments, mid-range concentrations of omadacycline (4–16 μg/mL) were associated with modestly increased production of TNF-α, IL-1β and IL-6 [Figure 1(b)] compared with LPS alone. However, when data from all donors

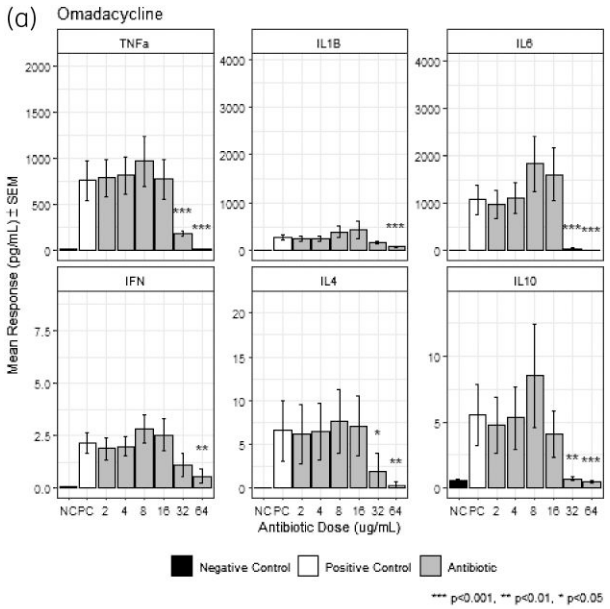

Figure 1. Immunomodulatory effects of omadacycline. PBMCs were prepared and cultured as described in the Materials and methods section. Duplicate wells of cells were pretreated for 2 h with the indicated concentrations of omadacycline followed by 24 h of stimulation with *E. coli* LPS (125 ng/well). Cytokines elaborated into the culture supernatant were quantified in duplicate by commercial multiplex immunoassay. (a) Pooled cytokine data from four independent experiments using three different donors are given as the mean (pg/mL) ± SEM. (b) Cytokine responses of individual donors; note that IL-4 was below the level of detection for all test conditions for donor #E09.20.21. Values that are statistically different from the positive LPS control are indicated as follows: ***$P<0.001$, **$P<0.01$ and *$P<0.05$. Comparator antibiotics minocycline and azithromycin were run in parallel and results are shown in Figures 2 and 3, respectively.

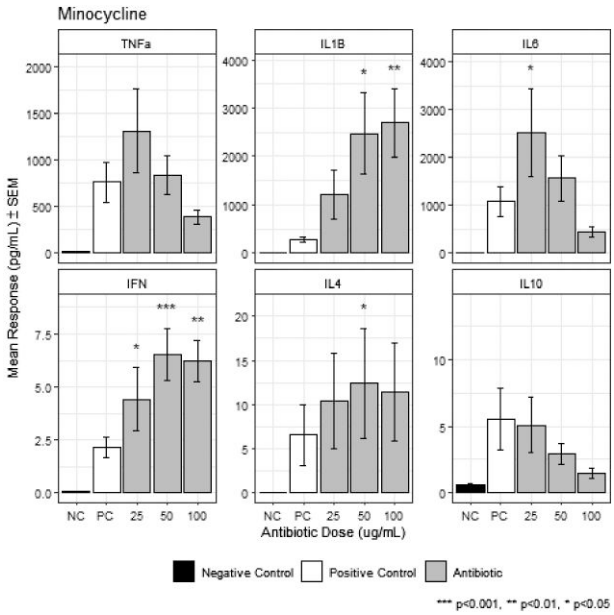

Figure 2. Immunomodulatory effects of minocycline. The effects of minocycline on LPS-induced cytokine production were investigated, analysed and illustrated as described in Figure 1(a).

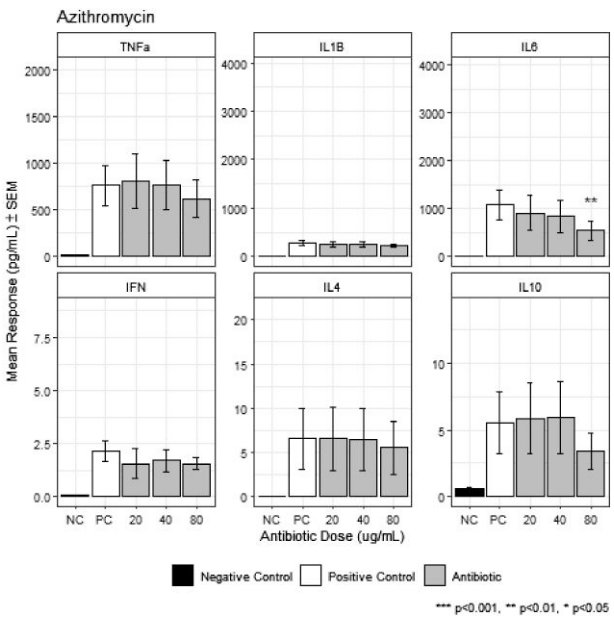

Figure 3. Immunomodulatory effects of azithromycin. Methods to investigate the effects of azithromycin on LPS-induced cytokine production were as described in Figure 1(a).

were analysed collectively, this increase was not statistically significant ($P>0.05$). Lower concentrations of omadacycline (≤4 μg/mL) were largely without effect. As with the other cytokines, omadacycline dose-dependently inhibited production of IFN-γ, IL-4 and IL-10 [Figure 1(a), bottom row].

Minocycline, at all concentrations tested, markedly and significantly stimulated IL-1β production (Figure 2) as much as 10-fold over LPS alone. IL-1β is expressed as a pro-form that requires proteolytic processing for cytokine activity. Typical processing is

mediated by NLRP3 inflammasome-driven caspase activation; however, processing can also occur via other cell type-specific proteases, some of which are calcium dependent (e.g. MMP-12 in macrophages[11]). To better understand the mechanism by which minocycline augmented LPS-induced IL-1β production in our study, one separate experiment included pretreatment of macrophages with either the NLRP3-specific inflammasome inhibitor MCC950 or the intracellular calcium chelator BAPTA-AM. The inflammasome inhibitor significantly suppressed the minocycline-enhanced IL-1β production but had no effect on the TNF-α responses, whereas the calcium chelator suppressed both cytokines (not shown). Minocycline also dose-dependently increased IFN-γ (Figure 2). For TNF-α, IL-6 and IL-4, lower concentrations of minocycline were stimulatory whereas the highest concentration tested, 100 μg/mL, was inhibitory (Figure 2).

The effects of azithromycin were less remarkable than the other antibiotics tested, though at the highest concentration tested (80 μg/mL), it significantly inhibited IL-6 production (Figure 3).

Discussion

The efficacy of some antibiotics is believed to be related, in part, to their non-antimicrobial immunomodulatory effects on host cells, particularly monocytes. Activated monocytes release a broad spectrum of cytokines, which can initiate and sustain a robust pro-inflammatory cytokine cascade to combat infection. However, hyperactivation of these responses—as is common in severe acute infections or during exacerbation of some chronic respiratory diseases—can result in widespread tissue injury, vascular dysfunction and haemodynamic collapse, leading to acute organ failure and death. Thus 'dual-acting' antibiotics, i.e. those that can reduce pathogen load while beneficially modulating the host immune response, may have greater efficacy in these settings.

We and others have previously demonstrated that antibiotics such as tetracycline,[12] azithromycin[8,9,13] and clindamycin[14–16] suppress LPS-induced TNF-α production *in vitro* and *in vivo*. Similarly, the present study utilized an *in vitro* model to assess the immunomodulatory effects of omadacycline on LPS-induced cytokine production in primary human monocytes.

The current study focused on six well-characterized cytokines, namely TNF-α, IL-1β, IL-6, IFN-γ, IL-4 and IL-10. TNF-α and IL-1β are the prototypic pro-inflammatory cytokines produced by classically activated M1 macrophages as part of the innate immune response. Like TNF-α, macrophage production of IL-6 requires activation of the NF-κB signalling pathway and follows temporally the production of TNF-α. In this scenario, IL-6 has largely pro-inflammatory properties and drives the acute-phase response. Interestingly, IL-6 is also produced by skeletal muscle cells after exercise where, as a myokine, its production is independent of TNF-α and NF-κB signalling. In this setting, IL-6 is considered anti-inflammatory, largely due to its ability to suppress TNF-α and IL-1β and to activate IL-1ra and IL-10.

IFN-γ (also known as type II interferon) is a critical mediator of both the innate and adaptive immune responses to microbial pathogens, especially viruses. IFN-γ is produced primarily by T cells, although macrophages, mucosal epithelial cells and other cell types also produce this cytokine. IFN-γ is considered pro-inflammatory in that it enhances killing of intracellular pathogens, activates inducible nitric oxide synthesis and promotes leucocyte adhesion and migration for a successful tissue inflammatory response. It also primes alveolar macrophages against secondary bacterial infection. Non-classically activated M2 macrophages elaborate anti-inflammatory cytokines, including IL-4 and IL-10, which drive the resolution phase of the immune response.

In the present work, high concentrations of omadacycline (≥32 μg/mL) significantly inhibited LPS-induced production of TNF-α, IL-1β and IL-6. Such inhibition could not be attributed to cytotoxicity since omadacycline alone, or in combination with LPS, did not result in overt cell loss or observable cytopathic effects. This result has important physiological relevance, particularly at sites where omadacycline may accumulate. For instance, a recent intrapulmonary pharmacokinetics study in humans showed that 4 h after the last dose, the maximal concentration (C_{max}) of omadacycline in alveolar macrophages was 36 and 18 times higher than in plasma and epithelial lining fluid (ELF), respectively.[17] Mid-range concentrations of omadacycline (4–16 μg/mL) augmented *in vitro* production of TNF-α or IL-1β in some donors, though more studies are necessary to determine whether such increases are significant and reproducible. Lower concentrations of omadacycline (≤4 μg/mL) were largely without effect. Thus, to the best of our knowledge, we demonstrate for the first time the dose-dependent immunomodulatory effects of omadacycline.

Minocycline and azithromycin were used here as comparators. Minocycline is a second-generation tetracycline derivative used to treat a variety of bacterial infections including pneumonia and various skin infections. It is readily absorbed from the upper small intestine and, unlike omadacycline, is highly (70%–75%) bound to plasma proteins. Azithromycin is a macrolide antibiotic used widely to treat many bacterial infections including community-acquired pneumonia.

Minocycline's anti-inflammatory activities, including inhibition of pro-inflammatory cytokine production by monocytic cell lines and reduced generation of reactive oxygen species from polymorphonuclear leucocytes (PMNLs), have been reported.[18] Azithromycin's immunomodulatory activities have also been reported *in vitro*[9,13] and in clinical settings including various respiratory diseases such as asthma, chronic bronchitis and COPD.[19]

Minocycline at 100 μg/mL suppressed TNF-α, IL-6 and IL-10. These findings generally agree with those of Tai *et al.*[8] using the monocytic cell line THP-1, and of Pang *et al.*[20] using circulating human monocytes. However, we show here that minocycline dramatically increased LPS-induced production of IL-1β by more than 10-fold over LPS alone. This unexpected response was consistent across all experiments and occurred at all concentrations of minocycline tested. Pang *et al.*[20] showed that minocycline suppressed LPS-induced transcription of IL-1β mRNA, though the quantity of IL-1β released was not reported despite the fact that they, too, utilized a multiplex immunoassay that included this cytokine.

Unlike other cytokines, mature IL-1β production uniquely depends on activation of an inflammasome complex. In our study, inclusion of an NLRP3-specific inflammasome inhibitor significantly suppressed minocycline-induced IL-1β production but had no effect on TNF-α (not shown), suggesting that minocycline,

but not omadacycline, can directly activate this complex. In agreement with our study, three older reports[21–23] showed that minocycline (and/or tetracycline) significantly increased production of one or more pro-inflammatory cytokines, including IL-1β, in LPS-stimulated monocytes. Our study also showed that minocycline augmented LPS-induced production of IFN-γ and IL-6. Thus, the widely accepted notion that minocycline has broad anti-inflammatory properties requires further clarification.

In contrast to minocycline, omadacycline did not increase IL-1β; in fact, high concentrations significantly inhibited its production. The differences in immunomodulatory effects between these two tetracycline-based antimicrobials may be related to their different chemical structures that provide unique pharmacological characteristics. For instance, unlike other tetracyclines including minocycline, omadacycline demonstrates low (21%) binding to human plasma proteins[24] and good intracellular penetration.[24,25] This latter feature clearly has clinical implications for the treatment of pneumonia caused by intracellular pathogens[24,25] but it may also portend an increased ability to modulate the host cytokine response. Potential differences in their ability to bind and transport calcium into the cell may also contribute.

In contrast to previously published reports, azithromycin had no significant effect on LPS-induced production of TNF-α and IL-1β in the present study. This result was unexpected and was not attributed to pH alterations since, compared with vehicle-treated and other antibiotic-treated wells, no discernible differences in media pH (using phenol red as pH indicator) were observed either upon addition of azithromycin or after prolonged incubation with this drug alone or in combination with LPS. Instead, this variation is likely related to differences in the *in vitro* models used. Features such as the types of cells studied, the LPS source, antibiotic pre-exposure conditions, characteristics of the serum used in cell culture and the presence of other standard antibiotics (streptomycin, amphotericin) in the culture media can all affect responses. For instance, Ikegaya *et al.*[13] showed that azithromycin significantly reduced LPS-induced TNF-α production. These authors used a monocytic cell line (THP-1) cultured with 10% FCS and co-stimulated for 4 h with *Pseudomonas aeruginosa* LPS and azithromycin (0.1 to 50 μg/mL). THP-1 cells are a spontaneously immortalized monocyte-like cell line, derived from a child with monocytic leukaemia. Compared with primary peripheral blood monocytes, THP-1 cells are far less responsive to LPS—a feature that is largely, but not wholly, attributable to the low expression levels of CD14 on their surface.[26] Even when CD14 is forcibly overexpressed in these cells, the LPS-induced TNF-α response is still far less than that of primary monocytes.[26] Thus, in this regard, THP-1 cells are a less than optimal model for LPS-induced monocyte cytokine production. Indeed, the maximal LPS-induced TNF-α level in the Ikegaya study was ∼10 pg/mL and only one concentration of azithromycin (10 μg/mL) significantly reduced this level (to ∼6 pg/mL). In contrast, our studies used primary human monocytes cultured with 1% pooled human serum. Cells were pretreated with antibiotic for 2 h (to allow antibiotic uptake) before stimulation with 125 ng of *E. coli* O113:H10 LPS. Our TNF-α levels at 4 and 24 h were approximately 750 and 1200 pg/mL, respectively; however, none of the azithromycin concentrations tested significantly reduced these levels.

In another example, Khan *et al.*[27] showed that azithromycin reduced TNF-α in human monocytes stimulated for 24 hrs with 100 ng/mL of *E. coli* O26:B6 LPS. Though this model is more comparable to ours, these authors document that the magnitude of the decrease varied widely among the different donors (from 9%–69%; $N=7$) and even among the same individual tested on different days. As in the Ikegaya study, Khan *et al.*[27] cultured their cells in 10% FCS. Adult versus fetal serum can result in marked differences in cell function that are largely attributed to the abundance and nature of growth factors and immune elements in the sera.[28] In general, the contents of the adult material reflects lifelong immune system exposure to numerous and highly varied stimuli whereas the components of fetal serum reflect a more naive immune system. With its abundance of growth factors, proteins and metabolites, plus its low levels of gamma globulin, FBS has been the gold standard to support metabolism, proliferation and differentiation of *pluripotent mesenchymal stem cells* whereas adult (preferably species-matched) serum is preferred for cultivation of *lineage-determined cells* (e.g. monocytes) and tissues. Thus, human monocytes/macrophages are commonly cultured in adult human serum since fetal serum can adversely influence cell phenotype, adherence characteristics, surface antigen gene expression profiles and production of some cytokines (e.g. IL-1β).[29]

Lastly, the origin of the LPS used can significantly affect cellular responses. The structures of both the oligosaccharide and lipid components of LPS vary considerably between bacterial species and can dramatically affect the ability of the host to recognize and respond to this pathogen-associated molecular pattern.[30] LPS moieties can also vary within a single species. For example, the ability of *P. aeruginosa* LPS to interact with human TLR4 depends on the extent to which the lipid A component is acylated.[31] Thus, the *in vitro* model system employed must be carefully considered when drawing conclusions about the ability of a given agent to affect cellular immune responses.

In conclusion, our studies demonstrate that omadacycline has anti-inflammatory immunomodulatory properties. Such activity supports its use in conditions where hyperactivation of the immune response contributes to tissue injury and poor outcomes, especially at sites where pro-inflammatory M-type 1 macrophages dominate the cellular immune response.

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Transparency declarations

The lead author affirms that this manuscript is an honest, accurate and transparent account of the study being reported, that no important aspects of the study have been omitted, and that any discrepancies from the study as planned have been explained. The manuscript was written

by the lead/corresponding author (Bryant) and the authors have no financial conflicts of interest to declare. The funder of this study (Paratek) did not play any decision-making role in the research effort or in the writing of the manuscript.

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